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Effects of Acute and Chronic Hypoxia Exposure on the Contractile Properties of Isolated Compact and Spongy Ventricular Myocardium From Rainbow Trout (Oncorhynchus mykiss)

Roberts, Jordan Cyril

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Effects of Acute and Chronic Hypoxia Exposure on the Contractile Properties of Isolated Compact and Spongy Ventricular Myocardium From Rainbow Trout (Oncorhynchus mykiss)

by

Jordan Cyril Roberts

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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Abstract

The ventricle of salmonids is composed of an outer compact layer, routinely supplied with well-oxygenated arterial blood, and inner spongy myocardium receiving low oxygen venous blood. During hypoxia oxygen exposures for both these tissue layers declines. As well, hypoxia results in an increase to circulating epinephrine for trout, which may affect the contractile response during hypoxia. This thesis presents an investigation into effects of high epinephrine, as well, acute and chronic hypoxia exposure on the contractile performance of these ventricular tissues from rainbow trout (*Oncorhynchus mykiss*). Acute hypoxia resulted in a similar decline to work output in compact and spongy myocardium. High epinephrine however, improved contractile performance of spongy myocardium during hypoxia. Hypoxia acclimation resulted in a decline to work output from spongy myocardium across all oxygen exposures. It appears hypoxia causes a decline in ventricular performance; however, elevated epinephrine exposure may be important to maintain higher performance during hypoxia.
Acknowledgements

I would like to acknowledge the following people for their roles in helping me complete this thesis. My supervisor, Dr. Doug Syme for giving me so many great opportunities, and teaching me what it means to be a good scientist. My committee members, Dr. Sean Rogers and Dr. Mat Vijayan for your guidance and suggestions at the supervisory committee meetings. My lab mate, Scott Seamone, I would like to thank you for being a supportive friend while I stumbled through graduate school. Dr. Kurt Gamperl for hosting us in his lab and making sure our trip to St. John’s was productive. Kimberly Stewart for her help conducting experiments. Kyle Wilson and Brandon Allen for sharing their knowledge of statistics. Madison Bartlett, for being a supportive partner. Finally, family and friends who have kept me level throughout this experience.
Dedication

This thesis is dedicated to Madison Bartlett
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## List of Symbols, Abbreviations and Nomenclature

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<th>Definition</th>
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<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AR</td>
<td>Adrenergic receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BPM</td>
<td>Beats per minute</td>
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<td>CV</td>
<td>Coefficient of variance</td>
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<tr>
<td>HIF-1</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>P_{a}O_{2}</td>
<td>Partial pressure of oxygen in arterial blood</td>
</tr>
<tr>
<td>PO_{2}</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>ppt</td>
<td>Parts per trillion</td>
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<td>P_{v}O_{2}</td>
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<td>SE</td>
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Chapter One: Introduction

1.1 Overview:

The thesis presents an investigation into the contractile performance of ventricular myocardium from rainbow trout (*Oncorhynchus mykiss*) during acute hypoxia, following acclimation to normoxia or hypoxia, and when exposed to epinephrine. The ventricle of the salmonid heart is composed of an outer compact layer supplied with well oxygenated arterial blood and an inner spongy layer supplied with poorly oxygenated venous blood. During hypoxia, the oxygen in venous and arterial blood decline differentially. The aim of this thesis was to explore the contractile performance of these tissue layers following acute and chronic hypoxia. It was hypothesized the different routine oxygen exposures for these two tissue layers would affect the relationship of contractile performance to subsequently changing oxygen levels. Comparisons of contractile performance were made between the two myocardial tissues in acute exposure to hypoxia and following acclimation to normoxia or hypoxia. In addition to the changes in oxygen exposure for the myocardium, severe hypoxia can elicit a stress response in trout. In acute hypoxia this stress response involves increases to circulating catecholamines. Elevated catecholamines were hypothesized to improve contractile function in the myocardium layers dependent on the density of adrenergic receptors present on each. The contractile performances were measured in the compact and spongy myocardium in tonic and high levels of epinephrine, during normoxia and hypoxia, to assess the impact of epinephrine on contractile performance. The following chapter provides background on hypoxia as an environmental stressor and current understanding of the cardiac performance for fish exposed to this stressor.
1.2 Background:

1.2.1 Hypoxia as an environmental state and physiological stressor

There is a lower content of oxygen in water than in air. As such, organisms that rely on gas exchange with water must be adapted to survive in this relatively low oxygen environment. Additionally, the partial pressure of oxygen (PO$_2$) in a body of water may be subject to flux through numerous environmental factors. This can be stressful or even lethal to aquatic organisms if the dissolved oxygen drops below organism-specific critical thresholds (Farrell and Richards 2009; Greenbank 1945; Kangur et al. 2005).

Hypoxia can occur under many conditions in both fresh water and marine environments. In shallow lakes of Canada subject to seasonal freezing, prolonged ice cover can limit primary production in the water column and gas exchange with the atmosphere (Barcia and Mathias 1979). Hypoxia resulting from these conditions can lead to major die-offs of fish species, impacting both the local ecology and the recreational fishery, in events known as winter kills (Barcia and Mathias 1979; Greenbank 1945). From the perspective of fisheries management, there is a considerable cost associated with restocking recreational fisheries from winter kills; the government of Alberta spent 7.3 million dollars in 2006 stocking 3.7 million trout into Alberta lakes (Patterson and Sullivan 2013). In 2013, it was estimated 86 lakes in Alberta were at “medium risk” or higher for winter fish-kills, with 53 lakes being stocked (ESRD 2013). Additionally, hypoxia can occur in eutrophic and hypereutrophic waters (waters with high nutrient levels) due to mass phytoplankton and macrophyte die-offs (Almazan and Boyd 1978; Barica 1974; Kangur et al. 2005). Increases in temperature can also cause a decline in PO$_2$
(Loperfido et al. 2009). Even within a body of water, stratification can cause major differences in PO$_2$ between water layers (Rahel and Nutzman 1994).

The extent and duration of hypoxia can vary substantially depending on environmental conditions. This can include seasonal ice cover resulting in chronic oxygen depletion over the course of a winter (Barcia and Mathias 1979). More acutely, fluctuation in PO$_2$ can occur on a diel cycle, with several hours of hypoxic conditions occurring daily (Tyler et al. 2009) due to temperature fluctuations or photosynthetic activity. As well, a fish can even encounter hypoxia by moving through the water column (Pihl et al. 1991), from deep unmixed layers toward well-mixed surface layers, or through the thermocline.

Hypoxia becomes challenging for a fish when there is difficulty obtaining sufficient oxygen to meet their aerobic needs. However, since fish can be particularly prone to experiencing hypoxic stress in their environment, they commonly possess well-developed responses to hypoxia (Farrell and Richards 2009). The responses to both acute and chronic hypoxia can manifest as changes to behaviour down to the level of gene expression (Kramer 1987; Pihl et al. 1991; Soitamo et al. 2001; Ton et al. 2003). The following sections will summarize hypoxic responses relevant to cardiovascular function in fishes, as context for the questions posed in my thesis, which will follow.

1.2.2 Hypoxia in fish hearts

Many physiological responses to hypoxia are noted for their effects on gas exchange and delivery. The heart generates the pressure needed to pump blood through the gills and deliver blood to the tissues, therefore, cardiac performance will affect both gas exchange and delivery. As the heart is composed of highly aerobic muscle tissue itself, cardiac performance is affected,
in turn, by hypoxia. Thus, hypoxia impacts not only the availability of oxygen, but the function of the organ that is key to ensuring adequate oxygen exchange and delivery. Hence, understanding how oxygen availability affects cardiac function is critical to understanding the physiological response of fishes to hypoxia.

In some of the more active groups of fish the ventricle of the heart is composed of two layers of myocardium: the compact outer layer and spongy inner layer (Farrell and Jones 1992; Pieperhoff et al. 2009) (Figure 1.1). The compact layer is vascularized with a coronary circuit, supplying well oxygenated blood to this tissue from a branch of the dorsal aorta. The spongy myocardium, however, is composed of numerous trabeculae and in many species, including in rainbow trout, this layer lacks any vascularization from the coronary circuit (Davie and Farrell 1991). The spongy myocardium in trout is supplied oxygen by the poorly oxygenated venous blood returning to the heart. The trabecular structure of this layer reduces diffusion distances by allowing the luminal venous blood to reach most regions of this tissue layer, perhaps partially compensating for the lack of vascularization.

In addition to differences in the PO$_2$ experienced by the compact and spongy myocardium during even normoxic conditions, the PO$_2$ of arterial and venous blood perfusing the myocardium is subject to change with environmental PO$_2$ (Gamperl et al. 1994a; Thomas et al. 1994). During air-saturated conditions, the PO$_2$ of water (P$_w$O$_2$) is about 20 kPa (100% air saturation) (see Table 1.1 for conversion to kPa), the arterial PO$_2$ (P$_a$O$_2$) in rainbow trout is 13 kPa, and the venous PO$_2$ (P$_v$O$_2$) is lower at about 5 kPa (Gamperl et al. 1994a). When exposed to acute environmental hypoxia of 12 kPa (about 60% air saturation), both the P$_a$O$_2$ and P$_v$O$_2$ decline; however, while P$_a$O$_2$ perfusing the compact myocardium (4.7-6 kPa) still remains higher than P$_v$O$_2$ (2-3.3kPa) that perfuses the spongy layer (Gamperl et al. 1994a), the decline in P$_a$O$_2$ is
greater in magnitude than the decline in P\textsubscript{v}O\textsubscript{2}. Hence, the compact layer will experience a greater decline in PO\textsubscript{2} during hypoxia than the spongy layer, and this may have implications for not only overall cardiac performance, but also the relative performance of each layer and how they contribute to a functional heart.

1.2.3 Cardiovascular response to acute hypoxia

The cardiovascular response to acute hypoxia has been well characterized in several species of fish in terms of changes to cardiac output, heart rate and stroke volume. One of the more apparent and consistent responses to acute hypoxia observed in fish is a slowing of the heart rate (bradycardia) (Farrell 2007). This response is at least partially attributed to increases in vagal tone (Fritsche and Nilsson 1989). Hypoxic bradycardia is mediated through branchial oxygen sensors and cardio-inhibitory vagal innervation (Farrell 2007). Despite the slowing of heart rate during acute hypoxia, cardiac output in rainbow trout and several other species (e.g. Atlantic cod (\textit{Gadus morhua}) and hagfish (\textit{Myxine glutinosa}) remains constant or slightly increases (Axelsson and Farrell 1990; Gamperl et al. 1994a; Peterson and Gamperl 2010a). This maintenance of cardiac output during acute hypoxia is a result of increased stroke volume (Gamperl et al. 1994a). The increased stroke volume during hypoxia is proposed to be associated with increased venous pressure and increased ventricular filling times (Gamperl et al. 1994a; Farrell 2007).

\textit{In vivo}, increased adrenergic tone has also been observed during hypoxic conditions (Perry and Reid 1992). Epinephrine has been shown to be critical for the maintenance of cardiac function during acute hypoxia through the modulation of contractile function and vascular
resistance (Hanson et al. 2006). In support of this, epinephrine results in increased contractile force in ventricular isolates of rainbow trout (Overgaard and Gesser 2004).

Despite the maintenance of cardiac output during hypoxia in vivo, acute hypoxia exposure results in a loss of contractile performance of isolated ventricular myocardium (Overgaard and Gesser 2004; Syme et al. 2013). For example, a decline in oxygen from 100% air saturation (above physiological) to 40% air saturation (8kPa, near normoxic P\textsubscript{O}\textsubscript{2}) resulted in a 60% decline in net work output from spongy ventricular myocardium of Atlantic cod (Syme et al. 2013). Given the differences between the compact and spongy myocardium in the PO\textsubscript{2} they are routinely exposed to, it might be expected that these tissues differ in their contributions to cardiac output and responses to hypoxia. It has been shown that coho salmon (Oncorhynchus kisutch) can survive with a ligated coronary circulation, but at the expense of pressure generation capabilities (Steffensen and Farrell 1998). When swum in progressively more hypoxic water, the water PO\textsubscript{2} at which the ligated fish began to fatigue was similar to that for sham-operated fish, however, the cardiac workload in ligated fish was also decreased with an associated reduction in arterial resistance, which may have helped compensate for the lack of a coronary supply. As well, coronary perfusion in isolated trout hearts results in increased work production compared to hearts that are not perfused (Agnisola et al. 2003), and in hypoxic waters, rainbow trout will increase the coronary blood flow, perhaps to meet the oxygen demands of the compact myocardium (Gamperl et al. 1994a). These results indicate that the contractile performance of compact myocardium is dependent on adequate PO\textsubscript{2} exposure, and hence that this tissue has an important role for generating pressure in acute hypoxia.
1.2.4 Cardiac responses to chronic hypoxia

As discussed above, hypoxia can occur over longer time scales. Chronic hypoxia may require additional physiological compensation to occur as the need to maintain oxygen delivery shifts from short-term to long-term. For Atlantic cod, chronic hypoxia results in a loss in the ability to raise cardiac output during swimming challenges (Peterson and Gamperl 2010a). Similarly, there is an inability to raise cardiac output in steelhead trout (anadromous rainbow trout) following chronic hypoxia (Motyka et al. 2016). However, the loss in the ability to raise cardiac output is accompanied by an increase in tissue oxygen extraction for steelhead trout and Atlantic cod (Motyka et al. 2016; Peterson and Gamperl 2010b). Regardless, these results indicate there may be some loss in the contractile performance of myocardium following chronic exposure to hypoxia.

Exposure to chronic hypoxia does not result in major morphological changes (ventricular dimensions, relative ventricular mass, or proportion of compact) to the ventricle of steelhead trout (Motyka et al. 2016). However, chronic, pharmacologically induced hypoxemia (low blood oxygen) increases the vascularity of compact myocardium by 2.17x when compared to control trout (Simonot and Farrell 2009). As well, coronary ablation, which would decrease the PO\textsubscript{2} exposure of the compact myocardium, resulted in rapid growth of coronary vasculature (Farrell et al. 1990). These changes may not impact the contractile performance of the compact myocardium following chronic hypoxia directly, however, they indicate that long-term changes in PO\textsubscript{2} exposure result in activation of mechanisms for the maintenance of oxygen delivery in the heart. Thus, this in turn suggests that hypoxia sensitive mechanisms are activated in the compact myocardium that may affect contractile performance.
1.2.5 Adrenergic response to hypoxia and affect on myocardial function

As mentioned above, there is an increase in circulating epinephrine during hypoxia, which appears to be important for regulating cardiac function (Perry and Reid 1992). Following 30 minutes of exposure to a $P_{w}O_{2}$ of 4.5kPa, epinephrine levels in rainbow trout increase to ~350nM from a tonic level of near 5nM (Perry and Reid 1992). Epinephrine exposure results in an increased contractile force and work output when applied directly to ventricular isolates (containing both spongy and compact myocardium) of rainbow trout (Overgaard and Gesser 2004; Shiels et al. 1998). It is thought the positive effect of epinephrine on contractile performance of the myocardium in fish is partially associated with an increase in sarcolemmal Ca$^{2+}$ influx (Vornanen 1990). Additionally, administering a high dose of epinephrine resulted in a lower blood PO$_2$ threshold for cardiac collapse (no epinephrine 5kPa, 500nM 1.3kPa) during intense exercise conditions (Hanson et al. 2006).

It is not currently known if increases in circulating epinephrine affect the contractile performance of compact and spongy myocardium to a different extent. The spongy myocardium of Coho salmon has a 14% higher density of surface $\beta$-adrenergic receptors than in the compact myocardium (Gamperl et al. 1998). If this difference in receptor density is consistent across species, it may suggest spongy myocardium of rainbow trout is more sensitive to increases in circulating epinephrine and has a greater contractile response than the compact myocardium.

1.2.6 Evaluating the contractile performance of isolated myocardium

Most of the current literature exploring hypoxia and fish cardiac physiology is based on $in vivo$ or $in situ$ approaches. During $in vivo$ measurements, minimally instrumented fish are exposed to hypoxia and cardiovascular parameters are monitored (e.g.: Burleson et al. 2002).
With the *in situ* approach, the heart is instrumented to allow for control of venous input pressure and ventricular output pressure with the integrity of the semi-rigid pericardium maintained, but other elements can be excluded, such as neural feedback (e.g.: Petersen and Gamperl 2010a; Overgaard 2004). These approaches have the benefit of including aspects of organ and whole organism integration in the response, but preclude a clear interpretation of direct affects on the tissue or different tissue layers.

For my studies, I employed isolated tissues to investigate effects at the tissue level and potential differences between the compact and spongy myocardium, where strips of myocardium can be used to directly measure the contractile performance. This approach allows comparisons of contractile performance with changes in the perfusate, which can be controlled directly, and is independent of neural and humoral effects in the fish. The myocardial preparations can be held at a static length and stimulated to determine the isometric twitch force as a measure of performance (e.g.: Aho and Vormanen 1999; Shiels and Farrell 1997). Alternatively, one end of the muscle can be attached to a servomotor and the length of the preparation can be changed inducing strain on the muscle to simulate the cardiac cycle, known as the work-loop method (e.g.: Harwood et al. 1998; Harwood et al. 2002; Shiels et al. 1998; Syme and Josephson 1995; Syme et al. 2013). In this approach, the amount of work produced when the muscle shortens (shortening work) and the amount of work required to lengthen the muscle (lengthening work) can be quantified (Syme and Josephson 1995), analogous to the stroke work and filling work, respectively, during a cardiac cycle. The sum of these two measurements (when lengthening work is expressed as a negative quantity) will yield the net work output, a measure of the net mechanical energy produced by the muscle during a cardiac cycle. The name work loop arises from the practice of plotting the force exerted by the preparation against the muscle length during
a contraction cycle, resulting in a graphical construct in the form of a loop. The integral of the plot (i.e. the area in the resultant loop) being the net work done by the muscle during a complete cycle of shortening and lengthening (i.e. analogous to one heart beat) (Figure 1.2). The power output (the rate of doing work) can be determined by multiplying work by the frequency of contraction (analogous to the heart rate). The work-loop method is intended to emulate the movement the muscle would experience \textit{in vivo} and allows observation of how both shortening work and lengthening work can change while simulating an environmental stressor such as hypoxia (Syme et al. 2013). Therefore, the work-loop method serves as an informative procedure in comparing the mechanical performance of the two myocardial tissues of interest under different levels of hypoxia.

\textbf{1.2.7 Study Species}

Rainbow trout were chosen as a model species to explore the contractile performance of the compact and spongy myocardium following acute and chronic hypoxia in my thesis. The size of the ventricle (0.1g-0.5g) and relatively high proportion of compact myocardium (~30% ventricular mass) (Davie and Farrell 1991) was thought to allow for more consistent dissection of living isolates, relative to smaller species or those with a lower proportion of compact myocardium. Additionally, rainbow trout have no coronary vascularization in the spongy myocardium, which increases the difference in PO$_2$ exposure between the tissues. Rainbow trout are often considered relatively hypoxia sensitive (Gamperl et al. 2001). This may manifest in a more sensitive contractile response to hypoxia. Finally as discussed above in the context of winter-kills, hypoxia can be a frequent threat for rainbow trout in some regions.
From a practical perspective, rainbow trout were readily obtainable and husbandry for rainbow trout had already been established at the university. Of note, the experiments in chapter 2 were conducted on seawater acclimated steelhead trout (anadromous rainbow trout) as this experiment was conducted as a collaborative effort at the Ocean Science Centre of Memorial University and only sea water was available for fish husbandry. This collaboration allowed access to a tank system designed for hypoxia acclimation. It is unclear how use of these different life histories may have affected the results. However, intra-specific variability in the hypoxia tolerance of rainbow trout has been demonstrated (Faust et al. 2004).

1.3 Hypotheses:

Chapter 2 will address the effects of acute and chronic exposure to hypoxia on the contractile properties of both the compact and the spongy myocardial tissue layers. The objective is to understand if the two tissue layers differ in their response to hypoxia (given their routine exposure to different PO₂), whether acclimation to hypoxia results in improved performance of the tissues in subsequent hypoxia, and whether differences exist in the acclimation response of the two tissue layers. Measurements of work and isometric twitch force were taken from isolated segments of compact and spongy myocardium from rainbow trout acclimated to hypoxia and normoxia over a series of declining PO₂. It was hypothesized: 2.1) the contractile performance of the spongy myocardium, given its routine exposure to low PO₂, would be better under oxygen limiting conditions relative to the compact layer; and 2.2) compact myocardium from trout acclimated to hypoxia (40% air saturation) would show an improvement in performance during subsequent hypoxia exposure, however, the spongy myocardium would not. This latter expectation is based on the observation that the compact myocardium experiences a relatively
large decline PO$_2$ during hypoxia while the spongy does not, and that the spongy myocardium routinely experiences relatively low PO$_2$ even during normoxia.

**Chapter 3** will determine if the different PO$_2$ experienced by the spongy and compact myocardium when fish are exposed to environmental normoxia and acute, mild environmental hypoxia have a different magnitude of effect on the work output and maximum contraction rate produced by the two myocardial tissue layers. The objective is to understand if, at the PO$_2$s actually experienced by these tissues during environmental hypoxia, they respond differently to the drop in oxygen availability (given that the change in PO$_2$ is quite different between the two tissue layers), and what the implications might be for mechanical function of a heart that relies on contributions from both tissue layers. Work output was measured from tissue isolates exposed to the blood PO$_2$ the tissues would experience during routine environmental normoxia and hypoxia. It was hypothesized 3.1) that the larger drop in PO$_2$ experienced by the compact myocardium during hypoxia would result in a greater loss in work output relative to spongy, and 3.2) exposure to lower PO$_2$ would result in a reduction in maximum sustainable heart rate in both ventricular tissues.

**Chapter 4** will assess the role that adrenergic stimulation (epinephrine) might play in protecting the myocardium from hypoxic insult. Due to the 14% higher density of β-adrenergic receptors present in the spongy vs. compact myocardium of the related coho salmon, it was expected high levels of circulating epinephrine may result in a different intensity of contractile response between the tissue types. For this experiment, work output was measured at high and low PO$_2$ from compact and spongy myocardium exposed to low (5nM) and high (500nM) epinephrine. The objective was to understand if high adrenaline, as might occur in fish exposed to hypoxic stress, might mitigate some of the depressive effects of hypoxia on contractile
performance of the myocardium, and if the spongy and compact myocardium might respond differently to epinephrine, again given the significant difference in the PO$_2$ they routinely experience and the differences in the changes experienced during hypoxia. It was hypothesized 4.1) that a high dose of epinephrine would have a positive effect on the work output of compact and spongy myocardium compared to tonic epinephrine levels, in both normoxia and hypoxia. 4.2) Spongy myocardium exposed to high levels of epinephrine would exhibit a larger increase in work output than the compact myocardium, in both air saturation and in hypoxia, due to potential tissue type differences in β-AR density. 4.3) It was also hypothesized that high epinephrine exposure would result in an increase in the maximum sustainable contraction rate for both myocardium tissue layers.

**Chapter 5** will provide a brief synthesis of the results, limitations and conclusions.
1.4 Tables

**Table 1.1:** Conversions between % air saturation and kPa for PO$_2$ exposures used in experiments throughout thesis.

<table>
<thead>
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<td>65%</td>
<td>13.1</td>
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<tr>
<td>25%</td>
<td>5.1</td>
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<tr>
<td>10%</td>
<td>2</td>
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</tbody>
</table>
1.5 Figures

Figure 1.1 Salmonid ventricle
Salmonid ventricle highlighting the compact and spongy myocardial layers. A) Two scanning electron micrographs of sockeye salmon (*Oncorhynchus nerka*) ventricle showing a clear morphological distinction between the compact (designated by square brackets in both panels) and spongy (designated by T - trabeculae in both panels) myocardium (adapted from Pieperhoff et al. 2009, used with kind permission of John Wiley and Sons). B) Sirius red staining showing the compact (upper) and spongy (lower) myocardial layers in cross section (adapted from Pieperhoff et al. 2009, used with kind permission of John Wiley and Sons). Note the lacunae and trabecular structure of the spongy layer, allowing perfusion of the muscle with blood in the chamber of the ventricle, while the compact layer is dense and must be supplied with a coronary circulation. C) Rainbow trout ventricle in cross section showing the trabecular spongy myocardium in the lumen of the ventricle and the compact myocardium around the ventricular wall. D) Surface view of a rainbow trout ventricle showing the coronary vasculature (arrow). Scale bars A) 200 µm B) 0.4mm C) 0.5cm and D) 0.5cm.
Figure 1.2 Graphical representation of “work loop”

A) Length and force traces from a spongy myocardium preparation from rainbow trout undergoing a cycle of work. The preparation was subjected to 10% peak-to-peak strain (length change) in a sinusoidal trajectory, and electrically stimulated at approximately 0.2s on the time scale, resulting in contraction (a rise then fall in force) during the shortening portion (blue) of the strain cycle. B) The resultant “work-loop” generated from force vs. length of a single strain cycle. Colours correspond to length changes in panel A and arrows indicate the direction of the trace.
Chapter Two: Effects of hypoxia acclimation on the contractile properties of spongy and compact ventricular myocardium isolated from rainbow trout (*Oncorhynchus mykiss*)
2.1 Abstract

Trout ventricles have an outer compact layer supplied with well-oxygenated arterial blood from the coronary circulation and an inner spongy myocardium supplied with low oxygen venous blood that percolates through it. Based on these differences, it was hypothesized: 2.1) the contractile performance of the spongy myocardium, given its routine exposure to low PO$_2$, would be better under acute exposure to hypoxia relative to that of the compact layer; 2.2) in trout acclimated to hypoxia (40% air saturation), compact myocardium would show an improvement in performance relative to myocardium from fish acclimated to normoxia (100% air saturation) during acute hypoxia exposure, however, spongy myocardium, being routinely exposed to low PO$_2$, would not improve from hypoxia acclimation. Work output was measured in ventricular strips from rainbow trout (Oncorhynchus mykiss) (n=19) acclimated to either normoxia or hypoxia when exposed to a series of decreasing PO$_2$ levels (100% air saturation, 65% air saturation, 25% air saturation, 10% air saturation) and when returned to 100% air saturation. Work output of the spongy and compact myocardium from normoxia acclimated fish decreased to a similar extent (55% and 60%, respectively) when PO$_2$ was lowered from 100% air saturation to 10% air saturation, and recovery of contractile performances were comparable. In contrast to expectations, hypoxia acclimation resulted in a reduction of the net (about 45%) and shortening (36%) work output of spongy, but not of compact myocardium. Hypoxia acclimation did not improve the depression of work under acute hypoxia in either tissue. Thus, spongy myocardium does not appear better suited than compact to function under low PO$_2$ conditions, and its performance declines following chronic hypoxia while that of compact appears reversed, but marginally insignificant. These results may indicate a higher relative contractile contribution of the compact myocardium to cardiac output in rainbow trout acclimated to hypoxia.
2.2 Introduction:

For some fish, in both fresh water and marine habitats, environmental hypoxia can be a common stressor. This limitation in oxygen availability can have major implications to the physiology of these fish. Hypoxia as a stressor can occur over extended periods of time (weeks-months) or acutely with relatively rapid changes to environmental partial pressure of oxygen (PO$_2$) (Barica 1974; Kangur et al. 2005; La and Cooke 2011). Major daily fluxes in dissolved oxygen can occur in hypereutrophic waters and result in large die-offs in fish (Kangur et al. 2005; Mulholland et al. 2005). Depletion of oxygen can also occur over prolonged periods during winter ice cover, and in regions of the ocean (Barica 1974; Diaz 2001). Even movement through the water column can subject a fish to rapid changes in environmental PO$_2$ as different strata in the water can have highly different PO$_2$ (Rahel and Nutzman 1994). Chronic and acute hypoxia differ in the time for which the stressor is occurring, and to a fish this may dictate different aspects of the physiological response.

Both acute and prolonged exposure to hypoxia can be stressful and potentially deadly for fish. However, as hypoxia can be a highly common occurrence in aquatic habitats, many fish appear to possess mechanisms making them capable of living over a range of PO$_2$ that may occur regularly in their habitats (Farrell and Richards 2009). As oxygen becomes limiting, physiological changes occur in fish to allow survival under these conditions (Farrell and Richards 2009). In the case of cardiovascular function, there is a need to maintain cardiac performance (cardiac output) during hypoxia to an extent which allows for adequate oxygen transfer to the tissues (Farrell and Jones 1992). Yet the heart itself is composed of highly aerobic tissue and so the contractile properties of myocardium are negatively affected by exposure to acute hypoxia.
Isolated myocardium shows a rapid reduction in inotropy following exposure to acute hypoxia (Syme et al. 2013). This rapid change is thought to be the result of cellular oxygen sensing mechanisms (Syme et al. 2013). Several oxygen sensitive mechanisms which could have such a rapid affect on the contractile properties of the myocardium have previously been identified (Fago et al. 2012; Scaringi et al. 2013).

It is also expected that fish might acclimate to the local oxygen environment, via changes in cardiac performance or sensitivity to reduced oxygen availability. Yet, evidence suggests such acclimation in cardiac performance is either absent or detrimental. Atlantic cod (Gadus morhua), following hypoxia acclimation, have a lower cardiac output when reintroduced to normoxia conditions and during subsequent hypoxic insult (Petersen and Gamperl 2010a). For Atlantic cod, the loss in cardiac output following hypoxia acclimation seems to result from a loss in stroke volume. This is further demonstrated through the reduced ability of the hypoxia acclimated Atlantic cod to raise stroke volume during progressive swimming challenges (Petersen and Gamperl 2010a). A similar reduction in the ability to raise cardiac output is observed from steelhead-rainbow trout (Oncorhynchus mykiss) exposed to chronic hypoxia (Motyka et al. 2016). These results seem to imply hypoxia acclimation results in a loss in inotropy of the ventricular myocardium, and is thus detrimental rather than beneficial.

The results above are based on measures from working hearts, but we know little about the basis of these responses, specifically how the cardiac muscle itself responds to acute hypoxia and hypoxia acclimation. Further, we know little about how different regions of the heart may respond. The ventricle of most fish species is composed of spongy myocardium; this tissue lacks any coronary vasculature, and only receives oxygen from the venous blood percolating through the ventricle (Farrell and Jones 1992). The ventricle of salmonids has an additional layer in the
myocardium, an outer layer referred to as the compact myocardium. The compact myocardium receives well-oxygenated blood from a coronary artery (Farrell and Jones 1992). This configuration creates microenvironments where the two layers are exposed to different PO$_2$ (Gamperl et al. 1994a; Thomas et al. 1994). This difference persists following exposure to hypoxia (Gamperl et al. 1994a; Thomas et al. 1994), where the arterial PO$_2$ ($P_a$O$_2$) remains greater than the venous PO$_2$ ($P_v$O$_2$), but the magnitude of $P_a$O$_2$ change is greater than that for $P_v$O$_2$. Therefore, during hypoxia the compact myocardium is still exposed to a higher blood PO$_2$ than in the spongy myocardium, however, the compact layer experiences a larger overall drop in PO$_2$. We thus might expect differences in the responses of the contractile properties of ventricular myocardium to hypoxia acclimation based on the prolonged exposure of the two tissue layers to these different $P_v$O$_2$ and $P_a$O$_2$. While both acute and chronic time scales of hypoxia have been examined for their effects on cardiac function, it is unclear if hypoxia acclimation has an effect on the contractile performance of myocardium in subsequent acute exposure to hypoxia.

The prolonged tissue-specific reductions in PO$_2$ for compact and spongy myocardium caused by hypoxia acclimation may result in changes, perhaps differential, to their contractile performance. It was hypothesized 2.1) the contractile performance of the spongy myocardium, given its routine exposure to low PO$_2$, would be higher under oxygen limiting conditions relative to the compact layer. It was also hypothesized 2.2) following hypoxia acclimation, the compact and spongy myocardium would differ in their performance in subsequent hypoxic exposure. It was predicted that compact myocardium from hypoxia acclimated rainbow trout, which experience a large drop in PO$_2$ during environmental hypoxia, will be better able to maintain performance during subsequent hypoxic insult than compact myocardium from a normoxia
acclimated trout. In contrast, as suggested by the loss in cardiac function for Atlantic cod (that possess a ventricle with only spongy myocardium) following hypoxia acclimation, and based on the relatively small drop in PO$_2$ experienced by spongy myocardium during hypoxia, it was expected the contractile performance of spongy myocardium would not improve following hypoxic acclimation in subsequent hypoxic insult. To test these hypotheses, the work output and twitch force of the two tissue types were measured in samples dissected from the ventricles of steelhead rainbow trout (*Oncorhynchus mykiss*) acclimated to normoxia (20.2kPa) or hypoxia (8.1kPa) over a range of PO$_2$ exposures.

2.3 Methods:

2.3.1 Animal care and tissue handling

All animal handling procedures were approved by animal care committees from both University of Calgary and Memorial University of Newfoundland. Experiments were conducted on 19 female juvenile seawater acclimated rainbow trout obtained from Cold Water Fisheries (Coldwater, Ontario). During the acclimation period fish were housed at the Dr. Joe Brown Aquaculture Research building at the Ocean Science Centre of Memorial University. All fish were exposed to a 12h:12h light:dark photo period, and held in water at 14°C, with a salinity of 32ppt. Nine trout were held in 100% air saturation (20.2 kPa) water and the other 10 were held in an adjacent tank maintained at 40% air saturation (8.1kPa) via a nitrogen valve regulator controlled by a PO$_2$ monitoring system. Each tank had a volume of 1200l. Fish were held in these conditions for at least 8 weeks prior to experimentation. Hypoxic fish were fed commercially available trout pellets (Skretting 500 EP) to satiation over the course of 5 minutes.
(maximum 1.2% body mass/day) and an equivalent mass of food (per fish) was given to the trout in normoxic conditions.

Fish were removed from the tank and euthanized with a sharp percussion to the cranium followed by pithing the brain and spinal cord. Immediately following euthanasia hearts were extracted and placed in chilled saline: in mM NaCl 132; KCl 2.6; CaCl$_2$ 2.7; MgSO$_4$ 1.0; NaH$_2$PO$_4$ 1.0; glucose 10; HEPES buffer 10, pH=7.8 (adapted from Altringham & Johnston 1990). From each heart, strips of compact and spongy myocardium were removed from the ventricle. Preparations were dissected out attempting to maintain similar dimensions between experiments (see Table 2.1 for dimensions). To reduce the confounding effects of diffusion limitations in the tissues, the diameter of all preparations were maintained near or below 1mm. For preparations of spongy myocardium, whole trabeculae were selected based on a columnar shape with minimal apparent branching of the muscle fibres. As muscle fibre alignment is not superficially apparent for compact myocardium, preparations were selected based on the apparent alignment of fibres as observed during spontaneous contractions. Both tissue preparations were extracted in immediate succession, and experiments were conducted on both preparations from the same heart simultaneously, with simultaneous exposure to the same series of PO$_2$.

Following the dissections, ties of 6-0 silk suture were secured on either end of the preparations and preparations were attached via these sutures to the arm of a servo motor (model 350, Cambridge Technology Inc., Bedford, MA) and a force transducer (model 400a, Aurora Scientific, Aurora, ON, Canada). Mounted preparations were bathed in saline held at 14°C throughout the experiment.
2.3.2 Determination of contractile properties from isolated myocardium

Data were collected and experimental parameters were controlled with custom-written LabView software (ver 6.1, National Instruments, Austin, TX) and a 12-bit analog/digital converter card (PCI MIO 16E-4, National Instruments, Austin, TX). Muscle preparations were stimulated to elicit contractions with a stimulator (Isostim A320, WPI, FL, USA) that allowed control of voltage and stimulus pulse duration. The stimulator was connected to two platinum plates on either side of the submerged muscle preparation. Voltage was set approximately 50% above that needed for maximum force production; stimulus pulse duration was set to 1ms. Measurements of work were obtained from spongy and compact myocardium from hypoxia acclimated fish (n=10 and 9, respectively), and from spongy and compact myocardium from normoxia acclimated fish (n=9 and 9, respectively). The work-loop method was used in order to measure the ability of the preparations to do work, with the repeated cycles of lengthening and shortening (strain) simulating contractions of a beating heart (Syme and Josephson 1995; Harwood et al. 1998; Syme et al. 2013). The shortening of the preparation simulates strain during systole, while the lengthening strain emulates ventricular filling during diastole. The frequency of the strain cycles and electrical stimulation allowed simulation of different heart rates. The servomotor applied strain in a sinusoidal pattern with an amplitude of 10% peak-to-peak of the muscle’s resting length in both the spongy and compact preparations.

The resting length of each preparation was optimized to maximize work output before experiments began. To do so, preparations were subjected to a series of 5 cycles of strain at a cycling frequency of 30 beats per minute (BPM), with the net work output from the final strain cycle used for comparison. Optimal length was found by increasing the preparation length in
increments of 0.1mm between series of work measurements until work output no longer increased following an increase in preparation length. This optimum length was then used for all subsequent measures from the muscle.

In some cases, a symmetrical strain pattern, where the period of muscle lengthening and shortening were the same, did not maximize work output. This was due to a mismatch between the duration of contraction of the myocardium and the period of shortening from the applied strain. Thus, at each cycle frequency used, the proportion of the imposed strain cycle that comprised muscle shortening was adjusted until work was maximized, with the remainder of the cycle then comprising muscle lengthening. For example, for some preparations at a lower cycling frequency of 30BPM, a strain cycle where 30% of the period was shortening and 70% was lengthening resulted in maximal work output. Stimulation was set to occur at a point in each strain cycle to maximize the net work done by the muscle at each frequency, generally so that the muscle was mostly relaxed while being lengthened and active/contracting while shortening, as would occur in a beating heart.

To measure the relationship between work output of the muscle and the cycle frequency (i.e. heart rate), and how this might differ between the hypoxia and normoxia acclimated experimental groups, each preparation was subjected to a set of 30 strain cycles at 30BPM, 50BPM, 70BPM and 90BPM at 100% air saturation. Work measured from the last strain cycle was used for analysis, as work between successive cycles was the most consistent at that point (e.g. Figure 2.1).

Two measures of twitch kinetics were compared between the experimental groups as well, to assess potential impacts of acclimation on muscle contraction. The measures were time taken for force to rise from 10-90% of maximal during contraction, and the time taken for force
to fall from 90-10% during relaxation. These values were measured from the last twitch in a set of 30 isometric twitches at a frequency of 50BPM in 100% air saturation.

The effects of acute hypoxia exposure on the work output and isometric twitch force of spongy and compact preparations from both acclimation groups were then measured. Preparations were subjected to a set of 30 strain cycles at a frequency of 50BPM. 50BPM was chosen as it is close to the in vivo heart rate for rainbow trout at temperatures similar to those used in the present study (Stevens and Randall 1967). Each preparation was also subjected to 30 consecutive stimuli at 50BPM at a fixed length to measure isometric force. The work output and twitch force were measured at 100% air saturation, 65% air saturation (13.1kPa), 25% air saturation (5.1kPa), and 10% air saturation (2.0kPa). The PO$_2$s below 100% air saturation were chosen based on the similarity to in vivo P$_a$O$_2$ and P$_v$O$_2$ during normoxia and hypoxia (Gamperl et al. 1994a); 65% air saturation and 25% air saturation are near the P$_a$O$_2$ and P$_v$O$_2$, respectively, during environmental normoxia, while 10% air saturation is close to P$_v$O$_2$ during hypoxia. The PO$_2$ was briefly (about 10 minutes) returned to 100% air saturation between the measures taken at 25% and 10% air saturation allowing a brief recovery from exposure to severe hypoxia to help avoid deterioration of the preparations. PO$_2$ was controlled by bubbling the saline with different proportions of N$_2$ and air; mixtures of the gas were controlled via a gas mixing pump (Wosthoff M200, Bochum, Germany). PO$_2$ was monitored throughout the experiment using an optical oxygen sensor (Presens Fibox 3 LCD, Espoo, Finland).

Following the exposure to graded hypoxia, the PO$_2$ was returned to 100% air saturation and work output was measured again at 50BPM with a set of 30 strain cycles. This final measure of work output was compared to the initial measure of work output at 100% air saturation to assess recovery of the preparations from hypoxia.
2.3.3 Analysis:

Following the completion of an experiment, preparations were removed from the apparatus and viewed under a microscope; tissue that was clearly non-viable was dissected away from the preparation. The preparation was then blotted on filter paper to remove surface moisture, and weighed (Mettler-Toledo MT5, Highston NJ, USA). Masses of preparations and optimal lengths are presented in Table 2.1.

Measures of trout size (mass (g), fork length (cm), and standard length(cm)) and ventricular size (ventricular mass (g) and relative ventricular mass (% of body mass)) were compared between hypoxia and normoxia acclimated groups using a 2-sample T-test.

The net work output was calculated as the difference between the shortening work and lengthening work done during a complete cycle. Shortening work was the work produced during shortening. The lengthening work was the work required to lengthen the preparations. When net work differed by more than 10% between the last two cycles (29 and 30) of a set (i.e. work tended to oscillate between successive cycles), that particular measure was excluded from analysis as such instability was considered not representative of normal function (i.e. the membrane action potential is likely becoming refractory), and is difficult to interpret. This occurred most commonly at the higher heart rates (70BPM and 90BPM), particularly in compact preparations, and during hypoxia. For example, in compact myocardium from normoxia acclimated fish at a heart rate of 50 BPM, out of 9 preparations studied this occurred once at 100% air saturation, once at 65% air saturation, twice at 25% air saturation and three times at 10% air saturation, but did not occur in any of the other experimental groups (compact from hypoxia acclimated fish or any spongy preparations).
Values of work unadjusted for the mass of each preparation were used for statistical analysis, instead of mass specific work, due to the possibility of bias introduced during dissection of the preparations, where the amount of non-viable tissue left attached to each preparation varied between individual dissections and between spongy and compact preparations. Inclusion of such bias in the analyses would make the biological relevance of differences between tissues and the PO$_2$-tissue type interactions difficult to interpret. The unadjusted values for work in the analyses avoids potential dissection bias and allows greater confidence in comparing treatment effects, but limits the ability to make comparisons of absolute performance between tissue types. There did not appear to be any dissection bias between preparations from hypoxia and normoxia acclimated fishes, as two-sample T-tests confirm the preparation masses were not significantly different between acclimation groups within a tissue type (spongy $P=0.483$; compact $P=0.745$).

For the analysis of developed isometric twitch force (the difference between resting and maximum force during an isometric twitch), force was expressed relative to muscle cross-sectional area in kN/m$^2$ assuming a muscle density of 1.05g/cm$^3$.

The contractile properties (net, lengthening, shortening work, and developed isometric twitch force) were analyzed using a mixed-effects model with the following fully-crossed fixed effects structure: PO$_2$/ tissue-type/ acclimation, where the effect of a three way interaction would indicate acclimation affects the work output in subsequent exposure to declining PO$_2$ differently between compact and spongy myocardium. A random intercept term was included in the model for individual preparations to control for preparation specific performance (i.e. the non-independence of repeated measures on tissues, and also controls for size differences among tissue samples). In these analyses PO$_2$ was included as a continuous covariate. To improve the fit for the assumption of normally distributed residuals, the net work, shortening work, and lengthening
work were transformed with log(x+1). Developed twitch force was log(x) transformed. Normality of residuals and other assumptions of the mixed effects models were visually assessed with histogram, QQ-norm and Pearson residual vs. fitted plots. For the analysis of lengthening work, a single value was removed from the compact, normoxia-acclimated group from the measurement taken at 100% air saturation, as this value was considerably higher than all other values of lengthening work (however, inclusion or removal of this value did not affect the outcomes of the analysis). For analysis of recovery of preparations from hypoxia, on return to 100% air saturation, the mixed effects model had the structure: pre/post hypoxia / exposure /tissue type / acclimation, again with a random intercept estimated for each preparation.

Work performance and developed twitch force (kN/m²) were compared at 30, 50, 70, and 90BPM. Power output, calculated from the product of work per cycle (J) and heart rate (Hz), was also analyzed. Separate analyses were conducted for each experimental group (i.e. separate analysis for both tissue types at each acclimation level). These analyses were conducted as a mixed effects model with the fixed effect being the heart rate (as an ordinal variable) and a random intercept included at the level of each preparation on log(x) transformed values of work and power.

Analyses of the measures of contraction and relaxation twitch kinetics were conducted using two-way ANOVAs with the main effects of acclimation and tissue, with an interaction term included.

P-values of the fixed effects in mixed effects models were estimated using Satterthwaite estimation of denominator degrees of freedom. Degrees of freedom using this method are estimated from one moment and two moment approximation (Fai and Cornelius 1996; Kuznetsova et al. 2013). All analysis were conducted using R software (R-core team 2015).
Mixed effects models were assessed using the lme4 package, and lmertest (Bates et al. 2005; R-core team 2015; Kuznetsova et al. 2013). Where significant differences were found, Tukey’s correction factors were applied to post-hoc contrasts.

2.4 Results

For a summary of preparation dimensions used in the present study see Table 2.1. A summary of trout size and ventricular size is presented in Table 2.2. None of the measures of trout and ventricle size differed significantly between acclimation groups.

2.4.1 Effects of acclimation on contractile performance during acute hypoxia exposure

At a contraction rate of 50BPM, there was a statistically significant effect of PO$_2$ on net work output (P<0.0001), where net work declined with reduced PO$_2$ to a similar extent in both spongy and compact myocardium (no statistical interaction between tissue type and PO$_2$, P=0.545), and declined similarly in tissue from both normoxia and hypoxia acclimated fish (no statistical interaction between acclimation and PO$_2$, P=0.272) (Figure 2.2). There was a significantly higher net work produced by spongy than compact myocardium (P=0.029). The interaction between tissue type and acclimation was also significant (p=0.011), where hypoxia acclimated spongy produced less net work than normoxia acclimated spongy tissue (P=0.032) (Figure 2.2). The difference between net work output in normoxia and hypoxia acclimated compact myocardium was marginally not significant (P=0.066). Acclimation did not have a significant effect on the relationship between PO$_2$ and net work output (P=0.96), where work declined with PO2 similarly regardless of normoxia or hypoxia acclimation (Figure 2.2). The three-way interaction was not significant (P=0.38).
There were also effects of PO$_2$ and acclimation on shortening work. Low PO$_2$ resulted in a reduction of shortening work in both tissue types (P<0.00001) (Figure 2.3), and the effect was similar in both compact and spongy myocardium, although only marginally (P=0.065). There was no significant difference in shortening work between compact and spongy myocardium (P=0.35), and likewise acclimation alone did not have a significant effect on shortening work (P=0.88). However, there was a significant interaction between tissue type and acclimation (P=0.045), where as observed with the net work, hypoxia acclimation resulted in a decline in shortening work output for spongy myocardium (P=0.050) but no significant effect on compact myocardium (P=0.24). Additionally, the interaction between PO$_2$ and acclimation had a significant effect on the response of shortening work to declining PO$_2$, with hypoxia acclimation resulting in a smaller decline in shortening work with declining PO$_2$ (P=0.036). The three-way interaction between PO$_2$-tissue type-acclimation was not significant (P=0.85).

The analysis of lengthening work indicated that declining PO$_2$ resulted in a reduction in lengthening work (P<0.00001) (Figure 2.4). As well, compact myocardium produced significantly more lengthening work than spongy (P=0.00016). Acclimation did not affect lengthening work from either compact or spongy myocardium (P=0.62). None of the interactions were significant for lengthening work (tissue type-PO$_2$ P=0.32, acclimation-PO$_2$ P=0.42, tissue type-acclimation P=0.28, tissue type-acclimation-PO$_2$ P=0.82).

Developed isometric twitch force declined with reduced PO$_2$ in both tissue types (P<0.00001) (Figure 2.5). Spongy myocardium produced significantly higher developed twitch force than compact (P=0.0024). However, there was no effect of acclimation on force in either spongy or compact myocardium (P=0.59), and there were no significant interactions between
PO\textsubscript{2}-tissue type (P=0.320), PO\textsubscript{2}-acclimation (P=0.11), tissue type-acclimation (P=0.52), or PO\textsubscript{2}-tissue type-Acclimation (P=0.80).

2.4.2 Recovery of work output following hypoxic insult

Net work output was significantly lower following recovery from acute hypoxic insult than preceding (P=0.0059) (Figure 2.6). There were no significant interactions between exposure (i.e. pre-hypoxia, post-hypoxia) and tissue type (P=0.92), acclimation group (P=0.81), or exposure-acclimation-tissue type (P=0.48).

2.4.3 Effects of cycle/contraction frequency on developed twitch force, work and power production

There was a significant negative effect of increased contraction rate (i.e. heart rate or cycle frequency) on the amount of net work produced per cycle, in all experimental groups: spongy normoxia acclimated (P<0.0001), spongy hypoxia acclimated (P<0.0001), compact normoxia acclimated (P=0.028), and compact hypoxia acclimated (P<0.0001) (Figure 2.7). In contrast, there was a significant but mostly positive effect of contraction rate on net power output in spongy normoxia acclimated (P<0.0001), spongy hypoxia acclimated (P<0.0001), and compact hypoxia acclimated (P<0.0001) (Figure 2.8), but no effect in compact myocardium from normoxia acclimated fish (P=0.97).

2.4.4 Twitch kinetics in the compact and spongy myocardium

The measures of isometric twitch contraction kinetics (time for force to rise from 10-90\%) and relaxation kinetics (time for force to drop from 90-10\%) did not differ significantly
between the acclimations groups (contraction P=0.925, relaxation P=0.866) (Figure 2.9). There was, however, a significant difference in these measures between the two tissue types, where both contraction and relaxation took longer in the spongy myocardium versus compact (contraction P=0.0022, relaxation P=0.0046). There was no significant interaction between acclimation and tissue type in either measures of twitch kinetics (contraction P=0.59, relaxation P=0.18).

2.5 Discussion

2.5.1 Tissue level differences in work output

The significantly higher net work output observed in the spongy vs. compact myocardium may have biological significance, however, it is difficult to have confidence in the difference between tissues due to potential dissection bias. From the analysis of shortening and lengthening work, the compact myocardium had a higher lengthening work than spongy (Figure 2.4) but similar shortening work (Figure 2.3), resulting in lower net work output of compact vs. spongy (Figure 2.2). Whether this reflects dissection bias or a real difference in inherent work capacity, is unclear. Since the direction of muscle fibres in spongy myocardium was superficially apparent, it was possible to isolate preparations that likely contained a high proportion of viable tissue, while the reduced ability to observe fibre orientation in compact myocardium likely resulted in more of the preparation being non-viable. Thus, as outlined in the methods, results of work were not adjusted for the mass of the preparation in statistical analyses to avoid this potential dissection bias. Further, the values of work will be dependent on the inherent capacities of the muscle but also on the size of the preparation, which was not rigorously controlled, hence, the main effect of tissue type is likely difficult to interpret in the context of biological significance.
If there is truly a significant difference in the work capacity between compact and spongy myocardium the present results would indicate a higher contractile contribution of spongy myocardium to cardiac output than would be expected based on the proportion of the ventricle composed of spongy myocardium.

2.5.2 Effects of acute PO\textsubscript{2} change on contractile performance

Acute reduction of PO\textsubscript{2} resulted in a decline in the amount of net work produced by both tissue types (Figure 2.2). Further, the slopes of the PO\textsubscript{2}- net work responses were similar between compact and spongy myocardium. For example, the average proportion of net work output lost when PO\textsubscript{2} was reduced from 100% air saturation to 10% air saturation was about 61% in compact myocardium and 55% in spongy myocardium. A loss in net work during exposures to reduced PO\textsubscript{2} was also observed in isolated spongy myocardium from Atlantic cod, where the proportion of work output was ~55% following a PO\textsubscript{2} change from 110% air saturation to 40% air saturation (Syme et al. 2013). The present results indicate work output is reduced to a similar extent for both tissue types in acute hypoxia.

The decrease in net work output at lower PO\textsubscript{2} has previously been associated solely with a decrease in shortening work (inotropy), but not to a change in lengthening work (lusitropy) (Syme et al. 2013). Here it appears that low PO\textsubscript{2} resulted not only in a decline in shortening work (Figure 2.3), but also to a decrease in lengthening work (i.e. a reduction in resistance to lengthening) (Figure 2.4). As PO\textsubscript{2} was reduced from 100% air saturation to 10% air saturation, lengthening work decreased ~16% in spongy and ~20% in compact myocardium. The decrease in lengthening work with reduced PO\textsubscript{2} (which tends to increase net work) was smaller than the decrease in shortening work (which tends to reduce net work), and therefore net work output was
still reduced in lower PO$_2$. For example, when PO$_2$ was reduced from 100% air saturation to 10% air saturation, the ratio of the average change in shortening work to average change in lengthening work was 12.3 in the spongy myocardium, and 4.4 in compact myocardium.

A decrease in the amount of work required to extend the myocardium (i.e. lengthening work) during acute hypoxia exposure would likely result in less resistance to ventricular filling. When hypoxic bradycardia occurs, there is an accompanying increase in stroke volume (Gamperl et al. 1994a). The decline in lengthening work during acute hypoxia would promote ventricular filling, without requiring changes to atrial contractility and vascular resistance. As such, the reduced lengthening work in hypoxia might be considered a mechanism to facilitate the hypoxic cardiovascular response.

The mechanism responsible for the reduced work output in the face of reduced PO$_2$ is not known. The similarity between tissue types in the relationships between PO$_2$ and net work may suggest that the same mechanisms are acting on these tissues to limit the work output during rapidly declining PO$_2$ exposure. It has been suggested these changes are the result of oxygen sensing mechanism at the cellular level, rather than just a reduction of work output throughfatiguing of the myocardium in reduced oxygen (Syme et al. 2013). Several rapidly acting cellular mechanisms that could be involved in cellular oxygen sensing have been identified. For example, nitric oxide (NO) levels in a cell are related to the oxygen availability, as NO is rapidly oxidized by the free radicals produced by aerobic metabolism (Fago et al. 2012; Hill et al. 2010). NO is known to supress oxidative phosphorylation by competitively binding to complexes in the electron transport chain (Fago et al. 2012). A suppression in the amount of ATP produced could potentially result in a reduction in cellular metabolism, including the amount of work muscle is capable of producing, as a mechanism to match cell metabolism to oxygen availability. As well,
changes to ion currents important to the action potential in myocardium could result in rapid PO$_2$ sensitive changes to inotropy. O$_2$ sensitive Ca$^{2+}$ channels found in rat papillary muscle are known to alter ion conductance within seconds upon changes to PO$_2$ (Scaringi et al. 2013). As the relationship between Ca$^{2+}$ current and muscle force production is very well established, this mechanism could plausibly regulate the inotropy of the myocardium (Brutsaert et al. 1973). While currently the mechanisms responsible for the cellular regulation of muscle contraction associated with PO$_2$ remain unknown in fish, the presence of a rapid and fine scale regulation of work output in response to PO$_2$ in myocardium is reinforced by the present study.

2.5.3 Effects of hypoxia acclimation on contractile properties

Hypoxia acclimation did not have the hypothesized effect of reducing the impact of acute hypoxia exposure on net work output in a tissue specific manner. Rather, it appears that hypoxia acclimation resulted in a decrease to net work output in spongy myocardium at all PO$_2$ exposures, not just hypoxia. This indicates that hypoxia acclimation does not improve the function of spongy myocardium in further hypoxic insult. Thus changes to the work output of myocardium associated with hypoxia acclimation are likely different from those mechanisms affecting the contractile properties during acute hypoxia.

The net work output of spongy myocardium decreased as a result of hypoxia acclimation (Figure 2.2). Yet for compact myocardium there was a trend toward higher net work output following hypoxia acclimation, although this effect in compact was not significant by a small margin. The negative effects of hypoxia acclimation in the spongy myocardium may be associated with this tissue type also being exposed to the lowest PO$_2$. PO$_2$ exposure of the spongy myocardium during hypoxia acclimation may have reached a threshold level where cellular changes
(discussed below) occurred resulting in a reduction of work output, whereas the compact myocardium, being exposed to higher PO$_2$ via its coronary supply, either did not reach this threshold or respond differently to cellular hypoxia. The changes in shortening work following chronic hypoxia acclimation largely reflect changes in the net work output described above.

Following hypoxia acclimation the decline of shortening work (at all PO$_2$) in spongy myocardium but no increase to lengthening work indicates the reduction in net work output in spongy myocardium occurred largely through a loss in shortening work. Thus, the overall changes to net work output following chronic hypoxia acclimation were mostly mediated through changes to inotropy (shortening work) rather than to lusitropy (lengthening work) of myocardium. The significant PO$_2$-acclimation interaction in the shortening work might indicate hypoxia acclimation improved the ability to maintain shortening work output in subsequent acute hypoxia. This response appears to occur to a similar extent in both tissues types (indicated by the lack of significant 3-way interaction). However, the effect of hypoxia acclimation on the ability to maintain net work output in subsequent acute hypoxia was not significant, indicating that this effect was not important when considering overall changes in net work output.

An effect of hypoxia acclimation on spongy and compact myocardium appears to be absent based on the analysis of isometric twitch force (Figure 2.5). Isometric twitch force is thought to be most representative of myocardial contractile capacity during the isovolumetric portion of the cardiac cycle (Syme 1993). This lack of an effect of acclimation on twitch performance may indicate that the effects of hypoxia acclimation that appear to influence shortening work affect mostly dynamic aspects of function (i.e. shortening/lengthening), and less-so the ability to generate force.
It would also appear hypoxia acclimation does not have an effect on the twitch kinetics (Figure 2.9). Any changes that occurred during hypoxia acclimation did not affect rates of contraction and relaxation in either of the myocardium tissue layers. The between tissue type differences in twitch kinetics may be explained partially by the differences in developed force production between tissues. The developed force/cross sectional area was higher in the spongy preparations than in the compact (although as discussed with the work output this may be reflective of quality of dissections rather than actual tissue differences) and therefore the longer contraction and relaxation of spongy muscle may simply be a result of the higher force production.

The underlying mechanisms that result in tissue specific inotropic changes following hypoxia acclimation are currently unknown. Cellular changes resulting from prolonged exposure to hypoxia could contribute to changes in inotropy. For example, HIF-1 (hypoxia inducible factor) is a transcription factor referred to as the master regulator of the hypoxic response; the HIF-1α subunit of this protein heterodimer is stabilized during hypoxic conditions (Semenza 2000). Genes identified to be under the transcriptional regulation of HIF-1 are often associated with the hypoxia response (Semenza 2000; Ton et al. 2003). Changes in the expression profile of the myocardium due to increased activity of this transcription factor during hypoxia acclimation could potentially result in changes to the contractile properties.

It has been hypothesized that during normoxic conditions, venous blood of rainbow trout perfusing spongy myocardium has a sufficiently low PO$_2$ such that HIF-1α proteins are not degraded (Soitamo et al. 2001). In hypoxia, venous PO$_2$ drops even further (Gamperl et al. 1994a; Thomas et al. 1994). This may indicate higher levels of HIF-1α are stabilized during hypoxia. It has recently been shown that the loss in cardiac function of steelhead trout following
chronic hypoxia exposure is accompanied by an increase in tissue oxygen uptake (Motyka et al. 2016). Thus, while there is an apparent loss in cardiac function in hypoxia, adjustments in tissue oxygen extraction show compensation to the low oxygen conditions. This associated increase in oxygen uptake may suggest the loss in inotropy for spongy myocardium could serve as a protective response. Lower inotropy could allow better survival for spongy myocardium in the lower PO$_2$ venous blood during chronic hypoxia by reducing aerobic demand. Zebrafish (Danio rerio) embryos exposed to hypoxia show large changes in gene expression related to metabolism, which indicate a shift to lower aerobic energy consumption and a change in the dependence of energy substrates (Ton et al. 2003). In the present study, as only glucose was provided as an exogenous energy source, the loss in inotropy could also partially result from an increased metabolic reliance on other substrates. Future research could explore these possibilities by investigating the metabolism of spongy myocardium exposed to chronic hypoxia.

As net work from compact myocardium output increased following hypoxia acclimation, it seems that any hypoxia induced changes in gene expression which were speculated on above did not have the same affect on compact myocardium. During chronic hypoxia acclimation, the PO$_2$ of blood perfusing compact myocardium would have been greater than the PO$_2$ in the spongy myocardium (Thomas et al. 1994; Gamperl et al. 1994a). This indicates that HIF-1α may not be stabilized in the compact myocardium to the same extent speculated for spongy during chronic hypoxia (i.e. HIF-1α is less active in compact), and thus there may have been less impact of HIF-1α on gene expression and its downstream cellular effects in compact muscle, resulting in no reduction in the shortening or net work of the compact myocardium. However, it is unclear how an increase to net work output would have occurred on a cellular level in the compact myocardium following hypoxia acclimation.
In contrast to compact myocardium, spongy experienced the lowest PO$_2$ and thus perhaps the highest HIF-1α activity during hypoxia acclimation, which may have resulted in cellular changes that reduced work output. This could be further supported by exploring the expression profiles of key genes in compact and spongy myocardium from hypoxia acclimated trout, and through comparing HIF-1α protein abundance.

In terms of the function of the heart, the results found here suggest that spongy myocardium reduces its relative contribution to cardiac output following hypoxia acclimation, while compact does not. As discussed above, there appears to be some level of compensation following chronic hypoxia through an increased oxygen extraction at the tissues in steelhead trout (Motyka et al. 2016). From the apparent maintenance of inotropy in compact myocardium following exposure to chronic hypoxia, the need for compensation via increase tissue oxygen extraction may be lessened when compared to hearts of species entirely lacking a compact tissue layer. Further, the difference between the two ventricular tissues in work output following hypoxia acclimation might indicate a change in pumping mechanics for the ventricle. The present data suggest that the compact myocardium is increasing its relative mechanical contribution to cardiac output following hypoxia acclimation, and there is a decline in work output from spongy myocardium. Use of echocardiography or other imagining techniques on trout hearts following hypoxia and normoxia acclimation may elucidate to what extent changes in pumping mechanics are occurring.

### 2.5.4 Recovery of work output following exposure to acute hypoxia

While the relative extent of recovery was similar between the different experiment groups, there was some loss in work output on re-exposure to air-saturation compared to pre-
hypoxic performance, in all groups (Figure 2.6). The loss of some work output may be the result of tissue death through the course of the experiment as a result of trauma from the dissection, or slipping of the preparation on the mounting pins following repeated contractions. However, the similarity in the extent of recovery suggests all tissues are able to rapidly restore a high proportion of their initial work output on return to high \( \text{PO}_2 \) conditions (Spongy normoxia acclimation 95± 9.7%, spongy hypoxia acclimation 82±6.3%, compact normoxia acclimation 82±17%, compact hypoxia acclimation 92±7.7%). While neither tissue type would be exposed to 20.2kPa (air saturated) conditions \textit{in vivo}, rapid increases in blood \( \text{PO}_2 \) (to a lesser extent) could occur through movement in the water column through areas of high water \( \text{PO}_2 \). Thus, the ability of the myocardium to rapidly and near-completely recover function on return to high \( \text{PO}_2 \) from low could provide a physiological benefit to cardiac function and blood supply.

\subsection{2.5.5 \textit{Relationship between work, power and contraction rate}}

In all of the experimental groups, increased cycling frequency (i.e. heart rate) resulted in reduced net work output (Figure 2.7), over at least part of the range of frequencies studied, and never an increase. This matches previous observations in the myocardium of fish (Harwood et al. 1998; Syme et al. 2013). Conversely, power output tended to increase with cycle frequency (Figure 2.8) and was maximal between 70-90BPM, the exception being for normoxia acclimated compact myocardium, where power was not significantly different between cycling frequencies. This latter observation, of power not changing with cycle frequency, is inconsistent with previous work where power output from the compact myocardium of rainbow trout increased to an optimum cycle frequency of 70BPM (at 15°C) (Harwood et al. 1998). As noted previously, several values of work and power were excluded in the present analysis, as many of the
preparations went refractory at the highest stimulus frequencies (7 of 9 for 90BPM). The reduced number of observations at 90BPM will skew the averages and thus patterns of change with cycle frequency, making the results for 90BPM less reliable. However, at the lower heart rates this did not occur often (only 1 of 9 refractory observation at 70BPM), thus, preparations becoming refractory cannot fully explain why power output of the compact myocardium from normoxia acclimated trout was not affected by cycling frequency. Regardless, the present results suggest that acclimation did not have a notable effect on the relationship between contraction rate and work or power output, other than reducing the absolute power output of the tissues.

There were fewer refractory work measurements from hypoxia acclimated compact tissue at 90BPM (3 of 9) than in normoxia acclimated compact (7 of 9). Additionally, only 2 of 9 normoxia and 1 of 10 hypoxia acclimated spongy myocardium preparations were refractory at 90BPM. This may indicate an increase occurs to the maximum sustainable contraction rate in myocardium following exposure to chronically lowPO2.

2.6 Conclusions

Both spongy and compact myocardium had a similar reduction in contractile performance when exposed to acutely reduced PO2. This suggests a rapid drop in environmental PO2 would result in a rapid reduction of contractile performance for the myocardium. In order to maintain cardiac function in acute hypoxia, rainbow trout exposed to these conditions may need to modulate other factors (e.g. endocrine) that would improve cardiac output. Alternatively, the power output of the myocardium may be sufficient to maintain adequate cardiac function even in the face of reduced performance in hypoxia, and the drop in performance may actually be a result of cellular oxygen sensing mechanisms, as a means to safeguard cardiac metabolism during
hypoxia. The similarity between spongy and compact myocardium in the extent of reductions in work in reduced PO$_2$ suggest that the contractile mechanics of cardiac pumping are not impacted by hypoxia.

Additionally, chronic hypoxia acclimation did not have an effect on the pattern of the decline in net work output in subsequent acute exposure to hypoxia. This indicates that the change to contractile properties of myocardium following chronic hypoxia is largely independent of the rapid change in work output occurring in acute hypoxia. Compact and spongy myocardium differ in response to chronic hypoxia acclimation. It appears chronic hypoxia does not result in a large intropic changes in the compact layer but a loss of inotropy occurs in the spongy myocardium layer. The loss in work output that occurred in the spongy myocardium may be the result of PO$_2$ exposure reaching a threshold where cellular changes occur. This reduction in work output from the spongy myocardium following hypoxia acclimation suggests changes to the pumping mechanics of the ventricle may occur in hypoxia acclimation. As the ventricle of rainbow trout is mostly composed of spongy myocardium, these results indicate the decline in cardiac function observed in vivo, following chronic hypoxia acclimation, is mediated largely through a loss in contractile ability in the spongy ventricular layer.
2.7 Figures

Table 2.1 Ventricular preparation size

Comparisons of ventricular preparation dimensions used in the present study. Data are mean±SE.

<table>
<thead>
<tr>
<th>Ventricular Tissue</th>
<th>Acclimation</th>
<th>mass (mg)</th>
<th>Length (mm)</th>
<th>Cross sectional area (mm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spongy</td>
<td>Normoxia</td>
<td>2.80±0.46</td>
<td>6.10±0.62</td>
<td>0.39±0.068</td>
</tr>
<tr>
<td></td>
<td>Hypoxia</td>
<td>2.36±0.43</td>
<td>5.79±0.26</td>
<td>0.40±0.066</td>
</tr>
<tr>
<td>Compact</td>
<td>Normoxia</td>
<td>5.36±1.05</td>
<td>7.02±0.41</td>
<td>0.68±0.081</td>
</tr>
<tr>
<td></td>
<td>Hypoxia</td>
<td>4.96±0.56</td>
<td>8.33±0.44</td>
<td>0.60±0.099</td>
</tr>
</tbody>
</table>
Table 2.2 Trout size

Trout size and ventricular measures of the 19 rainbow trout used in the present study. Two-sample T-test show none of the measurements were different between acclimation groups.

<table>
<thead>
<tr>
<th></th>
<th>Fork length (cm)</th>
<th>Total length (cm)</th>
<th>Mass (g)</th>
<th>Relative ventricular mass(%)</th>
<th>Ventricular mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>31.5 ± 0.48</td>
<td>34.0 ±0.54</td>
<td>600±31</td>
<td>0.087 ±0.0055%</td>
<td>0.52 ±0.036</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>30.7 ±0.46</td>
<td>33.1 ±0.53</td>
<td>510±34</td>
<td>0.090 ±0.0076%</td>
<td>0.46g ±0.052</td>
</tr>
</tbody>
</table>
Figure 2.1 Difference in net work between subsequent strain cycles

The % difference between values of net work output of subsequent strain cycles across a set of 30 contractions from a single preparation of compact myocardium.
Figure 2.2 Net work of spongy and compact myocardium across PO$_2$ exposures

The net work output (unadjusted for muscle mass) of spongy and compact myocardium from trout acclimated to normoxia or hypoxia, when exposed to decreasing PO$_2$. Data are means ±SE. Standard errors were calculated from group means (tissue type and acclimation) at each PO$_2$. Asterisk indicates a significant difference (P<0.05).
Figure 2.3 Shortening work of spongy and compact myocardium across PO$_2$ exposures

The shortening work output (absolute work) of spongy and compact myocardium tissue preparations from trout exposed to normoxic conditions or acclimated to hypoxia, exposed to decreasing PO$_2$. Data are displayed as means ±SE. Standard errors were calculated from group means (tissue type and acclimation) for a PO$_2$. Asterisk indicates a significant difference ($P<0.05$).
The lengthening work output (absolute work) of spongy and compact myocardium tissue preparations from trout exposed to normoxic conditions or acclimated to hypoxia, exposed to decreasing PO$_2$. Data are displayed as means ±SE. Standard errors were calculated from group means (tissue type and acclimation) for a PO$_2$. 

**Figure 2.4 Lengthening work of spongy and compact myocardium across PO$_2$ exposures**
Figure 2.5 Isometric twitch force of spongy and compact myocardium across PO$_2$ exposures

The developed isometric twitch force (kN/m$^2$) of spongy and compact myocardium tissue preparations from trout exposed to normoxic conditions or acclimated to hypoxia, exposed to decreasing PO$_2$. Data are displayed as means ±SE. Standard errors were calculated from group means (tissue type and acclimation) for a PO$_2$. 
Figure 2.6 Net work prior to and following exposure to hypoxia

The net work output of spongy and compact myocardium from trout acclimated to normoxia or hypoxia, on initial exposure to 100% air saturation (pre-hypoxia) and upon re-exposure to 100% air saturation following acute exposure to hypoxia (post-hypoxia). Data are means ±SE. Standard errors were calculated from group means (tissue type and acclimation) at that exposure.
Figure 2.7 Net work of spongy and compact myocardium at increasing cycling frequencies

The net work output (unadjusted for muscle mass) of spongy and compact myocardium from trout acclimated to normoxia or hypoxia, subjected to different rates of contraction (cycling frequency or heart rate) (30, 50, 70, and 90BPM). Data are means ±SE. Standard errors were calculated from group means (tissue type and acclimation) at that frequency.
The net power output (unadjusted for muscle mass) of spongy and compact myocardium tissue preparations from trout exposed to normoxic conditions or acclimated to hypoxia, subjected to increasing cycling frequencies (30, 50, 70, and 90BPM). Data are displayed as means ±SE.

**Figure 2.8 Net power of spongy and compact myocardium at increasing cycling frequencies**
Isometric twitch kinetics during contraction (time for force to rise from 10 to 90% of maximal) and relaxation (time for force to drop from 90 to 10% of maximal) from spongy and compact myocardium from trout acclimated to normoxia or hypoxia. The contraction frequency was 50BPM. None of the measures of twitch kinetics were significantly different between normoxia and hypoxia acclimation for either tissue type.
Chapter Three: Effects of normoxic and hypoxic PO$_2$ on the contractile properties of compact myocardium and spongy myocardium isolated from rainbow trout (*Oncorhynchus mykiss*)
3.1 Abstract

The hearts of trout have two ventricular tissue layers, an outer compact layer normally supplied with well-oxygenated arterial blood, and an inner spongy myocardium receiving low oxygen venous blood percolating through the ventricle. However, during exposure to environmental hypoxia, the PO$_2$ drop experienced by compact myocardium is much greater than the PO$_2$ change in the spongy layer. It was hypothesized that: 3.1) the larger drop in PO$_2$ experienced by the compact myocardium during hypoxia would result in a greater loss in work output relative to spongy; 3.2) exposure to lower PO$_2$ would result in a reduction in maximum sustainable heart rate in both ventricular tissues. Measures of work output and maximum sustainable contraction rate were obtained from isolated segments of ventricular compact and spongy myocardium of 11 rainbow trout at the PO$_2$ that is physiologically relevant to each tissue during normoxia and hypoxia. A decline in the PO$_2$ from normoxia to hypoxia was associated with a drop in the maximum sustainable contraction rate in compact myocardium, by about 10%. The difference in maximum contraction rates, however, was not significant between 100% air saturation (above physiological) and hypoxia exposure. Both tissues exhibited a significant decline (P=0.0015) in net work output with the physiologically relevant drop in hypoxic PO$_2$, and the loss in work output from compact myocardium and spongy myocardium was similar. This may suggest that while cardiac performance is impaired by hypoxia, pumping mechanics are largely maintained, whereby each tissue layer experiences a similar change in performance.

3.2 Introduction

Some groups of fish possess an outer ventricular tissue layer in their heart known as compact myocardium (Farrell and Jones 1992). This tissue layer is supplied with well-
oxygenated arterial blood by a coronary artery branching off of the dorsal aorta (Davie and Farrell 1991). The inner layer of the ventricle is known as spongy myocardium, and in species with a “Type II” cardiac morphology, such as rainbow trout (*Oncorhynchus mykiss*), the spongy myocardium lacks a coronary vascularization and as a result is perfused only with poorly-oxygenated venous blood in the lumen of the ventricle (Farrell and Jones 1992). This arrangement leads to consistently different partial pressures of oxygen (PO$_2$) exposure between the two tissues, even when the fish is breathing well-oxygenated water. It is currently unknown how these PO$_2$ exposures may influence the contractile performance of these ventricular tissues.

In addition to differences in PO$_2$ experienced by these tissue layers during normoxia, many fishes, including trout, can often experience changes in environmental PO$_2$ (Farrell and Richards 2009). As such, the PO$_2$ of arterial and venous blood perfusing the myocardium of the trout heart is subject to change with environmental conditions (Gamperl et al. 1994a; Thomas et al. 1994). During 100% air saturation, the PO$_2$ of water (P$_w$O$_2$) is about 20 kPa, the arterial PO$_2$ (P$_a$O$_2$) in rainbow trout is 13 kPa and the venous PO$_2$ (P$_v$O$_2$) is much lower at about 5 kPa (Gamperl et al. 1994a). When exposed to acute environmental hypoxia of 12 kPa, (about 60% air saturation) both the P$_a$O$_2$ and P$_v$O$_2$ decline; however, while P$_a$O$_2$ perfusing the compact myocardium (4.7-6 kPa) still remains higher than P$_v$O$_2$ (2-3.3kPa) that perfuses the spongy layer (Gamperl et al. 1994a), the decline in P$_a$O$_2$ is much greater in magnitude than the decline in P$_v$O$_2$. Hence, the compact layer will experience a much greater decline in PO$_2$ during hypoxia than the spongy layer, and this may have implications for not only overall cardiac performance, but also the relative performance of each layer and how they contribute to a functional heart.

Rainbow trout exposed to progressive hypoxia are largely able to maintain cardiac output (Gamperl et al. 1994a). Coronary ligation, however, results in a loss of pressure generating
ability of the heart by approximately 28% in normoxic conditions for exercising rainbow trout (Steffensen and Farrell 1998). This indicates restricting oxygen supply to the compact myocardium results in a loss of function (Steffensen and Farrell 1998). Additionally, coronary perfusion of isolated trout heart results in an increase to the stroke work of the heart, compared to isolated hearts with no coronary perfusion (Agnisola et al. 2003). Thus, altering oxygen availability to the compact layer appears to impact cardiac function. There is also evidence that spongy myocardium is sensitive to reduced PO$_2$. During hypoxic exposures, there is a rapid-acting, negative inotropic effect on isolated spongy ventricular myocardium from Atlantic cod (Gadus morhua), where a drop from 100% air saturation to 40% resulted in a decline of 60% net work (Syme et al. 2013). Thus, the changes in blood PO$_2$ occurring in both ventricular layers of trout, and the negative inotropy observed with hypoxia, may result in a change to the dynamic function of the heart in terms of the contractile contribution of these two tissues to the cardiac cycle. The impact of changes in arterial and venous blood PO$_2$ during hypoxia on the performance of isolated spongy and compact myocardium from trout have not been explored.

In addition to changes in the inotropy of isolated myocardium, there is also a decline in heart rate that occurs in vivo during hypoxia for many fish species (Farrell 2007). Mechanistically, the drop in heart rate has been largely associated with an increase in vagal tone to the pacemaker cells of the heart (Short et al. 1979; Farrell 2007). However, hypotheses that this drop in heart rate occurs only as a result of neural regulation have not been well supported (Farrell 2007). Vagotomised Atlantic cod still experience a drop in heart rate during hypoxia (although bradycardia first occurs at a lower PO$_2$ than in fish with intact vagal innervation) (Mckenzie et al. 2009). As well, during severe hypoxia arrhythmias have been observed in European eel (Anguilla anguilla) treated with the pharmacological acetylcholine blocker atropine.
as a means of vagal inhibition (Iversen et al. 2010). This could possibly suggest that vagal suppression of heart rate during hypoxia is important, at least in part, for maintaining normal function of the myocardium and avoiding arrhythmias during severe hypoxia.

Stimulation of trout ventricular myocardium strips at high heart rates has occasionally resulted in a refractory heart beat (work produced in sequential heart beats differed dramatically) (Chapter 2). Further, there was a higher incidence of ventricular preparations becoming refractory at lower PO$_2$ (e.g. when preparations were stimulated at 90 beats per minute (BPM) in air saturation 46% of preparations were refractory versus 62% refractory at hypoxia of 10% air saturation). However, the relationship between level of hypoxia and maximal contraction rate (i.e. before becoming refractory) is not known and requires exploration to understand how hypoxia might limit cardiac performance.

To better understand the effect of oxygen availability on performance of compact and spongy myocardium, work output and maximum sustainable rates of muscle contraction were measured at the PO$_2$ each tissue routinely experiences in normoxia and hypoxia. It was hypothesized that 3.1) compact and spongy myocardium would experience different magnitudes of change in work output following exposure to the hypoxic PO$_2$ they normally encounter in vivo. Due to the smaller magnitude of change in PO$_2$ in venous blood during hypoxia relative to arterial blood, it was predicted that exposure to hypoxic conditions would result in less decline in spongy myocardium work output than in compact. It was also hypothesized 3.2) that the maximum sustainable contraction rate that the compact and spongy myocardium could follow would be less during hypoxic conditions. This prediction follows from observations of a potentially protective effect of the bradycardic responses to hypoxia in intact fishes, and the observation of more hearts becoming refractory at high hearts rates in hypoxia.
3.3 Methods

3.3.1 Animal care and tissue preparation

All aspects of animal handling were approved by the animal care committee at the University of Calgary following CCAC guidelines. Experiments were conducted on 11 juvenile, freshwater acclimated rainbow trout of mixed sex obtained from the Sam Livingston fish hatchery in Calgary, Alberta, Canada. All fish were exposed to a 16h:8h light:dark photoperiod, held in 100% air saturated waters near 14°C. The holding tank for fish was 1000l. Trout were fed ~1% body weight of commercial trout pellets daily (Martin Mills Inc. Classic sinking fish feed for salmonids). Fish were allowed to acclimate to these conditions for several months prior to experimentation.

Fish were euthanized with a sharp percussion to the cranium with a wooden baton followed by pithing of the brain and spinal cord. Immediately following euthanasia, hearts were dissected out of the fish, cut in half with a razor blade, and rinsed then placed into chilled saline (in mM: NaCl 132; KCl 2.6; CaCl$_2$ 2.7; MgSO$_4$ 1.0; NaH$_2$PO$_4$ 1.0; glucose 10; HEPES buffer 10, pH=7.8).

Dissections of compact and spongy myocardium preparations were conducted in the same manner as Chapter 2. However, preparations of each tissue (spongy and compact) were dissected out of the heart in a random order and the second preparation was not extracted until the completion of experiments with the first, rather than extraction occurring in immediate succession. During the completion of the experiments with the first dissected preparation (about 90 minutes), the ventricle half with the remaining myocardium tissue was pinned to the dissection dish to maintain tension on the muscle, the saline in the dish was replaced routinely,
dishes were held on a chilling plate to maintain the saline temperature near 14°C, and electrical stimuli were applied to the hearts once per 10 minutes.

3.3.2 Measuring work output

Work output was measured using the work-loop method in the same manner as described in Chapter 2. The optimal length for work output in the present study however, was determined at 50BPM. Strain cycles were symmetrical, set to have equal lengthening and shortening periods and stimulations were set to occur at 10% of the strain cycle.

To compare the work capacity of spongy and compact muscle, preparations were first subjected to 3 sets of 30 consecutive strain cycles and contraction at 100% air saturation. From these sets of strain cycles, the net, lengthening and shortening work were measured from the final strain cycle of each series and averages were calculated and used for analysis. A series of 30 cycles was used as work output largely stabilizes by the 30th strain cycle.

To measure the effects of PO₂, as the compact and spongy myocardium would experience in vivo, on contractile performance, preparations of both spongy and compact myocardium were then exposed in a random order to in vivo normoxic or hypoxic blood PO₂, as appropriate for each tissue. The normoxia PO₂ used for compact and spongy preparations were 65% air saturation (13 kPa) and 25% air saturation (5 kPa), respectively. The hypoxia PO₂ used were 25% air saturation (5 kPa) for compact and 10% air saturation (2 kPa) for spongy. These values were selected based on in vivo PₐO₂ and PᵥO₂ in rainbow trout exposed to hypoxic PₕO₂ of 60% air saturation (12kPa) (Gamperl et al. 1994a). For a rainbow trout, PₕO₂ of 60% air saturation is well above the PO₂ where cardiac failure occurs and above the PₕO₂ where hypoxic bradycardia occurs (Gamperl et al. 1994a).
PO$_2$ in the saline bathing the muscle was controlled by bubbling with mixtures of N$_2$ and atmospheric air using a gas mixing pump (Wosthoff M200, Bochum, Germany). PO$_2$ was monitored in the saline by a fibre-optic meter (Presens Fibox 3 LCD, Espoo, Finland). Once the desired PO$_2$ of the saline was achieved, the preparations were exposed to 1-3 sets of 30 consecutive strain and contraction cycles. However, these values were not used for analysis, only to assess and attain stability of the preparation at the new PO$_2$. Once stable, 3 more sets of 30 strain cycle sets were completed, and the net, lengthening and shortening work output of the final strain cycles were recorded and averaged for the analysis. After making recordings at a given PO$_2$, the preparations were then returned to 100% air saturation to avoid any cross-over effects of prolonged hypoxia exposure before being exposed to the remaining PO$_2$ to be tested (either hypoxic or normoxic, depending which was tested first) and the measurement set was repeated.

### 3.3.3 Determination of the maximum sustainable contraction rate

To measure the maximum rate at which the preparations could sustain stable contractions (analogous to the maximum heart rate before the heart would experience arrhythmia), the preparations were held at their optimal resting length and subjected to a series of stimulations at an increasing frequency. The stimulations increased in frequency from 50BPM up to 140BPM in increments of 10BPM. The preparations were first subjected to 30 stimulations at 50BPM to allow contractions to stabilize before increasing the rate of stimulation, where then only 10 stimulations occurred at each subsequent frequency. Isometric force was recorded during these contractions. The maximum contraction rate was then determined based on the stability of force during contractions, where at some (high) stimulation frequencies the force was not stable between successive contractions, but instead would oscillate between high and low values. The
maximum heart rate was taken as the highest rate the preparation could be stimulated where the developed isometric force during the final two successive twitches at that stimulation frequency remained within 10% of each other (Figure 3.1); stimulation at higher frequencies resulted in force oscillating by more than 10% between successive contractions. This measurement was completed on both the compact and spongy myocardium at 100% air saturation, the in vivo normoxic PO$_2$, and hypoxic PO$_2$.

3.3.4 Analysis

Following the completion of an experiment, preparations were removed from the apparatus and viewed under a microscope; tissue that was clearly non-viable was dissected away from the preparation. The preparation was then blotted on filter paper to remove surface moisture, and weighed (Mettler-Toledo MT5, Highston NJ, USA). Work was then expressed relative to muscle mass (J/kg) to facilitate comparison between tissue layers.

For all analyses of work output, the average work value and coefficient of variance was calculated from the three measures of work made under each experimental condition for each of lengthening, shortening and net work. If the coefficient of variance was greater than 15%, the triplicate work value most different from the other two was removed for analysis (this was done for 8 measurements out of 76 total measurements; for the other 68 all three values of work were included in the average).

For the comparison of mass specific work output between spongy and compact myocardium at 100% air saturation, the average work values (shortening, lengthening and net work output) were compared by means of two sample t-test, a P value of 0.05 was considered significant. Mass specific work output was calculated simply as the quotient of work and the
preparation mass. For this portion of the analysis, mass specific work output was used to allow direct comparisons between tissue layers.

Analyses of the effects of normoxic and hypoxic \( \text{PO}_2 \) on net, shortening and lengthening work (mass specific, \( J/kg \)) were conducted as mixed-effects models with the fixed effects as \( \text{PO}_2 \) (i.e. normoxia or hypoxia) and tissue (compact or spongy) with the interaction term between the two main effects included; a random intercept was included in the model for individual preparations, accounting for the non-independence of the repeated measures. Net and shortening work were log transformed to improve the fit of residuals to a normal distribution. Diagnostic residual plots were used to visually assess normality of residuals: histogram, QQ-norm and Pearson residual vs. Fitted plots. P values were estimated from the model using Satterthwaite estimation of denominator degrees of freedom, a P value of 0.05 was considered significant (Hrong-Tai Fai and Cornelius 1996; Kuznetsova et al. 2013).

The measures of maximum sustainable contraction rate were first compared at the in vivo \( \text{PO}_2 \) to determine the effect of normoxia and hypoxia exposure on the tissue layers. A mixed-effects model was used to evaluate this variable with the fixed effects as \( \text{PO}_2 \) and tissue layer, with the interaction term between the two main effects included and a random intercept term included for individual preparations. This model was also applied to the data set with the measures of maximum contraction in 100% air saturation included to determine if an effect of \( \text{PO}_2 \) on maximum heart rate exists above physiologically relevant \( \text{PO}_2 \).

Where models showed significance, post-hoc contrasts were conducted using Tukey’s correction. All analyses were conducted using R analysis software (R-core team 2015). Mixed effect models were conducted using the lme4 package with the P value estimates from the package lmerTest (Bates et al. 2005; Kuznetsova et al. 2013).
3.4 Results:

The trout used in this experiment had an average standard length of 19.1 cm ± 0.63 (mean ± SE), the average fork length was 20.9 ± 0.65, with an average mass of 105 ± 9 g. The ventricular masses were 0.108 g ± 0.0081 g, with a relative ventricular mass of 0.104% ± 0.006%.

The mass of compact preparations was 2.2 ± 0.37 mg, and 0.63 ± 0.078 mg for the spongy myocardium. The optimal length of compact preparations was 4.17 ± 0.39 mm, and 2.53 ± 0.23 mm in the spongy myocardium.

Mass specific net work output (P=0.96, Figure 3.2) and shortening work (P=0.17, Figure 3.2) were not significantly different between the compact and spongy tissue types at air saturation. However, lengthening work was significantly higher in spongy myocardium than in compact myocardium (P=0.013, Figure 3.2).

At the PO$_2$s experienced in vivo for fish in environmental normoxia and hypoxia, hypoxia resulted in a significant decline in net work (P=0.0015, Figure 3.3) and shortening work (P=0.0048, Figure 3.3). The post-hoc contrasts show PO$_2$ had a significant effect on net work in both compact (P=0.0012) and spongy myocardium (P=0.0081). However, the effect of hypoxic PO$_2$ was significant on the shortening work of the compact myocardium (P=0.00060), but not the spongy (P=0.20). The effect of normoxic vs. hypoxic PO$_2$ on lengthening work in either tissue type was not significant (P=0.17) (Figure 3.3). There was no difference between tissue types nor an interaction between PO$_2$ and tissue type for net work (tissue type: P=0.35, PO$_2$-tissue interaction P=0.18) and shortening work output (tissue type: P=0.24, PO$_2$-tissue type interaction: P=0.23). Lengthening work was significantly higher in the spongy vs. compact myocardium at
the *in vivo* PO$_2$ during both normoxia and hypoxia (P=0.035). The interaction of PO$_2$ and tissue type for lengthening work was insignificant (P=0.55).

Maximum sustainable contraction rate of compact and spongy myocardium showed a significant effect of PO$_2$ (P=0.00019) and a significant effect between compact vs. spongy myocardium (P=0.0066) (Figure 3.4). However, the interaction term between these factors was marginally not significant (P=0.060). The compact myocardium had a significantly higher maximum sustainable contraction rate in normoxia than in hypoxia (P=0.0002). In spongy myocardium, however, this difference was marginally not significant (P=0.095). In the analysis that included the maximum contraction rates also measured at air saturation, the effects of PO$_2$ remained significant (P=0.044), as did tissue type (P=0.039), and the PO$_2$-tissue type interaction remained insignificant (P=0.47). The maximum contraction rate was still significantly higher in normoxia vs. hypoxia in the compact myocardium (P=0.033). However, the maximum contraction rate at 100% air saturation was not significantly different from the hypoxia conditions in the compact myocardium (P=0.11). The contrasts from this analysis show none of the differences were significant between the PO$_2$ exposures for spongy myocardium (air saturation-normoxia P=0.82, normoxia-hypoxia P=0.57 and air saturation-hypoxia P=0.88).

3.5 Discussion:

3.5.1 Comparison of spongy vs. compact mass specific work output at air saturation

Mass specific work outputs between compact and spongy myocardium were directly compared, at the same PO$_2$ exposure, in order to provide some context to the contractile capability of these tissue layers. The same as for comparisons of work output unadjusted for muscle mass (Chapter 2), at 100% air saturation (which is above the physiological PO$_2$ either
tissue experiences) these two tissues produced similar mass-specific net work and shortening work outputs (Figure 3.2). This may reflect similarities within the contractile components of the tissues and may indicate neither tissue is superior at pumping blood.

Alternatively, as discussed in Chapter 2, the mass specific values obtained may be biased based on the quality of dissection and other factors specific to each tissue. The mass of preparations was affected by the ability to remove non-viable tissue; identification of this tissue was difficult to ascertain, particularly from preparations of compact myocardium, which may suggest the mass specific work outputs here are underestimates. The average mass specific net work output obtained here from spongy ventricular myocardium (about 0.30 J/kg) was similar to values obtained from previous studies involving this tissue (0.2J/kg, Shiels et al. 1998). However, the mass specific work output generated by compact myocardium (about 0.29 J/kg) was only about 25% of what had previously been obtained from rainbow trout compact myocardium (1.3J/kg, Harwood et al. 1998; Harwood et al. 2002). This likely reflects the much higher strain used in the experiments completed by Harwood et al. (1998), who used a peak-to-peak strain of 24%, compared to only 10% used in the present study. The same strain values were applied to the compact and spongy myocardium in the present study to allow a direct comparison of work capacity. With the same strain applied, both tissue types produced similar amounts of net work output at air saturation. This lends support to the use of mass specific work values for the \textit{in vivo} \textit{PO}_2 exposure analysis.

The strain value used in the present study may also provide some explanation as to why the compact myocardium had a lower lengthening work when compared to spongy myocardium (Figure 3.2). Because it is located further from the lumen of the heart, the compact myocardium routinely operates at a higher strain and perhaps longer muscle lengths than spongy. As such, the
elastic elements in compact myocardium may have a lower stiffness to compensate for the large strains and lengths, leading to less work required to lengthen compact myocardium than spongy when compared at the same strain.

3.5.2 Changes to work output during hypoxic conditions

The loss in net work output observed during exposure to hypoxic conditions in both compact and spongy myocardium was mediated through a decrease in shortening work (Figure 3.3). The lengthening work was not affected by changes in PO$_2$ exposure (Figure 3.3). These patterns of change in the net work and its components during hypoxia matched what has previously been observed in ventricular spongy myocardium of Atlantic cod at 10°C during hypoxia (Syme et al. 2013).

Both tissues exhibited a similar extent of decrease in work output following exposure to hypoxic conditions (Fig. 3.3), despite the PO$_2$ change from normoxia to hypoxia being much greater in compact than in spongy myocardium. This could perhaps indicate the contractile properties of compact myocardium are less sensitive than spongy to changes in PO$_2$ as experienced during exposure to the P$_a$O$_2$ of normoxic and hypoxic blood. Previously, however, it was shown that the changes in work output between the two tissues were largely similar across identical ranges of PO$_2$ exposures (Chapter 2), suggesting the spongy and compact muscles actually have similar sensitivities to change in PO$_2$. In agreement, although not statistically significant, there was a trend toward a larger decline in net and shortening work from the compact versus spongy myocardium in hypoxia (Figure 3.3), where the extent of the drop in PO$_2$ was also larger in compact versus spongy myocardium, which would be consistent with the similar PO$_2$-work relationship seen previously. However, results seen here indicate that despite
large differences in the magnitude of change between \( P_aO_2 \) and \( P_vO_2 \) during hypoxia, the pumping mechanics (i.e. relative contribution of the compact vs. spongy layers) of the rainbow trout heart during mild hypoxia are somewhat maintained. Exposure of these tissues to the blood \( PO_2 \) experienced at more extreme hypoxic insults would elucidate if tissue differences in performance might occur with larger changes in \( PO_2 \). Further, changes to pumping mechanics are difficult to predict without a much more comprehensive analysis, as even small changes to the contractile performance of the ventricular tissue could potentially change the dynamic of heart function.

The clear decline in net work output during hypoxic exposure and work output appears to contrast with the \textit{in vivo} cardiac performance during hypoxia. It has been shown in several species, including Atlantic cod and rainbow trout, that cardiac output is largely maintained during hypoxic conditions above critical values (Fritsche and Nilsson 1989; Gamperl et al. 1994a). This indicates additional factors are allowing cardiac output to remain constant during hypoxia, despite a decline in the capacity of the muscle to perform work. The increase in adrenergic tone associated with hypoxia may be involved in this relationship (Perry and Reid 1994; Reid et al. 1998). With direct exposure of isolated rainbow trout ventricular myocardium to epinephrine, isometric twitch force substantially increases (Keen and Vianzon 1993; Overgaard and Gesser 2004). Additionally, the vasoconstriction associated with adrenergic stimulation is thought to promote atrial filling and enhance cardiac output (Petersen et al. 2013). Thus, we must be cautious in interpreting the decline of myocardial work in hypoxia to be associated with a loss of cardiac function \textit{in vivo}; perhaps the potential for work is reduced in hypoxia, but it still remains adequate to maintain cardiac function \textit{in vivo}. In order to address the
discrepancy between tissue isolate performance and \textit{in vivo} cardiac output, a more complete emulation of humoral and mechanical conditions occurring during hypoxia is needed.

3.5.3 \textit{Maximum sustainable contraction rate}

The observation that spongy myocardium has the potential for a higher maximum heart rate than compact myocardium (Figure 3.4) is difficult to interpret biologically. These two tissue layers must contract in a synchronized manner in the ventricle. Therefore, it seems unlikely the spongy myocardium would ever function at high heart rates where the compact layer is refractory. The maximum sustainable heart rate of the compact myocardium could set the maximum heart rate of the ventricle, despite the ability of spongy to go faster. Using atropine to elevate heart rate, the \textit{in vivo} maximum heart rate for rainbow trout at 14°C (the temperature used in the present study) is between 75-80bpm (Ekström et al. 2014). This is close to the maximum heart rate of compact myocardium that was obtained here, which supports the notion that the heart does not operate at rates that exceed the abilities of the compact (slowest) layer.

The lower maximum contraction rate in compact vs. spongy myocardium could be reflected in differences in the properties of the cardiac action potential. A repolarizing efflux of K\textsuperscript{+} is important for mediating the duration of action potential in rainbow trout myocardium (Vornanen et al. 2002). This repolarizing K\textsuperscript{+} current and the action potential duration have exhibited plasticity with thermal acclimation (Haverinen and Vornanen 2009). A PO\textsubscript{2} related acclimation effect on the K\textsuperscript{+} current could affect the action potential duration and resting membrane potential between compact and spongy myocardium. Having increased action potential duration or decreasing resting membrane potential could reduce the maximum contraction rate in compact myocardium.
In addition to the difference in maximum contraction rate between the two tissues, exposure to hypoxic PO\textsubscript{2} resulted in a reduction of maximum contraction rate for the compact myocardium when compared to normoxic exposures. However, this difference was only present between the normoxia and hypoxia conditions but not between 100% air saturation and hypoxia. The mean maximum contraction rates were quite similar between 100% air-saturation (84BPM) and normoxia (86BPM), while the maximum contraction rate in compact myocardium during hypoxic exposure was only 77BPM. This may indicate maximum contraction rate only decreases in hypoxia but does not get higher above physiological normoxic PO\textsubscript{2}. Alternatively, the lack of significant difference between the hypoxic P\textsubscript{o}O\textsubscript{2} and 100% air saturation could also indicate any decline in the maximum contraction rate associated with hypoxic exposure is relatively minor at the P\textsubscript{o}O\textsubscript{2} tested here. Future studies exploring the maximum contraction rates of myocardium might be better served by increasing the number of frequencies tested (i.e. a smaller increment between frequencies), thereby increasing the resolution of this test. As well, this relationship could be further investigated by exposing compact myocardium to lower PO\textsubscript{2} as would be experienced in more severe hypoxia to determine if this effect becomes more pronounced in the compact myocardium.

Maximum contraction rates were not significantly different for spongy myocardium between any PO\textsubscript{2} tested in the present study. This may indicate spongy myocardium is able to maintain normal contraction rates at low PO\textsubscript{2} exposures, and thus the scope of heart rate increase is preserved during exposure to hypoxic P\textsubscript{o}O\textsubscript{2}. During environmental hypoxia, the need to maintain a high scope for heart rate increase might be limited with reflex bradycardia occurring (Farrell 2007). However, the P\textsubscript{o}O\textsubscript{2} of trout declines during exercise, while heart rate increases
(Farrell and Clutterham 2003; Stevens and Randall 1967). This could potentially necessitate the ability to contract at elevated rates in low $P_vO_2$.

**3.6 Conclusion:**

There was a negative inotropic effect of declining $PO_2$ as occurs during environmental hypoxia on both compact and spongy myocardium, where shortening work was reduced in hypoxic $PO_2$. During exposure to relevant hypoxic $PO_2$, work output dropped to a similar extent in the compact and spongy myocardium, despite the magnitude of the drop in $PO_2$ in compact myocardium being much larger than in spongy. For fish exposed to hypoxia, this may indicate pumping mechanics are preserved to some extent during minor environmental hypoxia, although the absolute level of performance would be reduced. As rainbow trout maintain cardiac output over the range of $PO_2$ examined, the drop in shortening work for these ventricular tissues indicates additional factors are affecting cardiac output during hypoxia, or that even the reduced performance in hypoxia remains adequate to maintain cardiac output.

As well, compact myocardium showed a declining maximum sustainable contraction rate at hypoxic $PO_2$ compared to normoxic $PO_2$. Further work needs to be done to confirm the maximum contraction rate of compact myocardium declines during hypoxia by measuring this parameter at lower $PO_2$ as would be experienced in more severe hypoxia.
3.7 Figures

Figure 3.1 Force trace at increasing stimulus frequencies

Isometric twitch force from spongy myocardium in a series of stimulations at increasing frequency; only data at 90, 100 and 110 BPM are shown. The twitches at the stimulus frequencies of 90 and 100 BPM were not considered refractory (i.e. force is stable or oscillates less than 10% between successive contractions), while the preparation became refractory at 110 BPM (i.e. force oscillates more than 10% between successive contractions). Maximum contraction rate for this preparation is defined as 100 BPM, the highest frequency that contractions were stable.
Figure 3.2 Work output of compact and spongy myocardium

Mass specific work output of spongy and compact myocardium at 100% air saturation. Asterisk indicates significant difference between tissue types (P<0.05).
Figure 3.3 Work output of spongy and compact myocardium during exposure to hypoxia

Mass specific work output (net, shortening and lengthening) of spongy and compact myocardium at the PO$_2$s experienced by the respective tissue *in vivo* when fish are in environmental normoxia and hypoxia. The PO$_2$ exposures were as follows: compact normoxia (65% air saturation), compact hypoxia (25% air saturation), spongy normoxia (25% air saturation), spongy hypoxia (10% air saturation). Asterisk indicates significant difference between tissue layer/condition (P<0.05).
Figure 3.4 Maximum contraction rate of spongy and compact myocardium across PO₂

Maximum contraction rate sustained by spongy and compact myocardium without being refractory (see Methods for details of calculation) at 100% air saturation and at the PO₂'s experience by the respective tissues in vivo when fish are in environmental normoxia and hypoxia. The PO₂ exposures were as follows: compact normoxia (65% air saturation), compact hypoxia (25% air saturation), spongy normoxia (25% air saturation), spongy hypoxia (10% air saturation). Asterisk indicates significant difference between maximum contraction rate between PO₂ exposures (P<0.05).
Chapter Four: Effects of adrenergic tone on the contractile performance and maximum contraction rate of compact and spongy myocardium from rainbow trout (*Oncorhynchus mykiss*) during hypoxia
4.1 Abstract:

Hypoxia results in an increase to circulating catecholamines for some fish, though to be important for maintaining cardiac function. The salmonid ventricle is composed of an outer compact layer and an inner spongy myocardium, and evidence suggests higher β-adrrenergic receptor (AR) density in spongy vs. compact myocardium. This may affect the contractile response associated with adrenergic stimulation between tissues. To test whether these two tissue layers exhibit different adrenergic responses, and if these responses are modified in hypoxia, work output and maximum contraction rate were measured in isolated preparations of spongy and compact myocardium from rainbow trout (*Oncorhynchus mykiss*) in 100% air saturation (20.2kPa) and 10% air saturation (2kPa), with exposure to low (5nM) and high (500nM) levels of epinephrine. It was hypothesized 4.1) high epinephrine would increase the work output of compact and spongy myocardium compared to low epinephrine. 4.2) Spongy myocardium exposed to high epinephrine would exhibit a larger increase in work output than the compact myocardium, due to the expected tissue type differences in β-AR density, and this effect would be observed in 100% air saturation and at 10% air saturation. 4.3) It was also hypothesized that high epinephrine would result in an increased maximum contraction rate for both myocardial tissue layers, in 100% air saturation and 10% air saturation. Hypoxia exposure with low epinephrine resulted in a decline in the maximum contraction rate of compact myocardium, which was reversed in high epinephrine, but in contrast to expectations had no effect in spongy myocardium. Also in contrast to expectations, in 100% air saturation, high epinephrine resulted in an increase in work output from compact but no significant increase in spongy myocardium. However, in 10% air saturation, spongy myocardium maintained a higher proportion of work output in high concentrations of epinephrine vs low, while compact did not. These results
indicate high epinephrine is important for maintaining inotropy in spongy myocardium during hypoxia.

4.2 Introduction:

Environmental hypoxia causes a decline in the partial pressure of oxygen ($P_{O_2}$) of blood in fish (Thomas et al. 1994; Gamperl et al. 1994a). Exposure to acute hypoxia can also elicit an endocrine stress response (Perry and Reid 1992). For example, rainbow trout (Oncorhynchus mykiss) exposed to a drop in water $P_{O_2}$ ($P_wO_2$) to 4.6kPa experience an increase in circulating epinephrine (from 1.9nM to 300nM) and norepinephrine (from 1nM to 100nM) (Perry and Reid 1992). Increases in these circulating catecholamines are important in the primary stress response of fish and are known to affect physiological parameters related to oxygen delivery (Iwama et al. 1999; Tuurala et al. 1989).

Elevated circulating catecholamines result in an increase in cardiac contractility and cardiac output (Farrell et al. 1986; Gamperl et al. 1994b) mediated through the stimulation of adrenergic receptors on the myocardium and in the vasculature (Vornanen 1998; Zhang et al. 1998). Likewise, direct exposure to epinephrine increases the work output of isolated rainbow trout hearts, and isolated ventricular myocardium (Farrell et al. 1986; Sheils et al. 1998). Through the use of pharmacological agents, the roles of the different adrenergic receptors have been characterized for their effects on circulation and the heart (Petersen et al. 2013). The adrenergic receptor (AR) subtypes currently identified in fish are the $\alpha$-AR, $\beta_1$ -AR, $\beta_2$ -AR, and $\beta_3$ -AR (Nickerson et al. 2003). Activation of different receptor subtypes can elicit different and sometimes opposing effects on the myocardium and vasculature (Imbrogno et al. 2015), with the
overall effects of catecholamine exposure on the inotropy and chronotropy of the myocardium likely reflecting adrenergic receptor subtypes present on the cell surfaces.

While the effects of catecholamines on cardiac function in hypoxia have been investigated to some extent (see above), the specific impacts on the different tissue layers of the heart are not known. The ventricle of rainbow trout is divided into two morphologically distinct myocardial tissues: the outer compact layer of myocardium perfused with arterial blood supplied by a coronary artery, and the inner spongy layer only receiving oxygen through the returning venous blood (Farrell and Jones 1992). This anatomical configuration creates a situation where the spongy myocardium is constantly exposed to poorly oxygenated venous blood. In hypoxic conditions the PO$_2$ of venous blood drops even further (Thomas et al. 1994). However, the spongy layer has a 14% higher surface density of $\beta$-AR than the compact myocardium in coho salmon (Oncorhynchus kisutch) (Gamperl et al. 1998), which may allow for a greater adrenergic response during hypoxia, when catecholamines are released. This could allow for maintained cardiac function despite the reduced oxygen availability in the spongy myocardium.

The work output of ventricular muscle in the absence of epinephrine decreases rapidly following exposures to lower PO$_2$ (Syme et al. 2013, Chapter 2 and 3). However, high levels of epinephrine may allow cardiac output to be maintained during hypoxia and help prevent cardiac collapse (Hanson et al. 2006). Adrenergic stimulation can also restore the contractile capability of isolated compact ventricular strips from rainbow trout during anoxia (Gesser et al. 1982), where above physiological exposure (5$\mu$M) to epinephrine resulted in an increase of isometric twitch force. Potential differences in adrenergic receptor availability on spongy and compact myocardium may suggest a differential response to epinephrine in hypoxia.
In addition to effects of catecholamines on force production by the myocardium, there is also an impact on heart rate, where catecholamines tend to promote tachycardia. As evidence, depression of the heart rate in rainbow trout occurs with the injection of propranolol, a $\beta_1$-AR and $\beta_2$-AR antagonist (Petersen et al. 2013). Additionally, during exercise, circulating epinephrine levels increase concurrently with heart rate (Butler et al. 1986). While there is a rapid decline in heart rate following administration of epinephrine in intact fish, in addition to an increase in stroke volume and overall increases to cardiac output (Gamperl et al. 1994b), this decline has been partially attributed to changes in vascular pressure and a reflex increase in vagal tone resulting in bradycardia, not necessarily through a direct effect of epinephrine on the heart or a change in the ability of myocardium to contract at higher rates (Gamperl et al. 1994b).

Previously it has been observed that there may be a decline in maximum contraction rate of isolated compact myocardium during exposure to hypoxia (Chapter 3). At rates above the maximum contraction rate, sequential stimuli produce twitches that differed in developed force, suggesting these ventricular strips were refractory. Since adrenergic stimulation is thought to increase the calcium current in myocardial cells, this could result in faster calcium transients and allow for an increase in the maximum contraction rate (Vornanen 1998). It might also suggest a role for adrenergic stimulation in protecting the heart from the depressive effects of hypoxia on maximal contraction rate.

The aim of this study was to measure the effect of low and high levels of epinephrine on the contractile performance of spongy and compact myocardium in both air saturation (above physiological $\text{PO}_2$) and under hypoxic conditions (physiologically relevant $\text{PO}_2$ during hypoxia). It was hypothesized that 4.1) exposure to high levels of epinephrine would result in an increase in the work output of compact and spongy myocardium, compared to low epinephrine levels. 4.2)
Spongy myocardium exposed to high levels of epinephrine would exhibit a larger increase in work output than the compact myocardium, in both air saturation and in hypoxia, due to potential tissue type differences in β-AR density. It was also hypothesized that high epinephrine would result in an increase in the maximum sustainable contraction rate for both myocardial tissue layers, and provide some protection from the depressive effects of hypoxia on heart rate.

4.3 Methods

4.3.1 Animal handling and tissue extraction

The following experiments were conducted on tissue isolates of 12 juvenile rainbow trout of mixed sex. Rainbow trout used in the present study were handled in the same manner as described and were obtained from the same source as in Chapter 3. As well, tissue dissections were conducted as previously described. See Chapter 2 for description of euthanasia procedures, and dissections.

As described in Chapter 3, dissections and experiments were first completed for one tissue type (~90min) while the remaining ventricular tissue was left pinned under tension in chilled saline, and the saline was replaced every 20 minutes and electrical stimulus was regularly applied every 10 minutes. Air was bubbled in to the saline to maintain a high PO₂ exposure until the second tissue sample was extracted. After completion of experiments on the first tissue type (spongy or compact, in random order), the second tissue type was then dissected from the heart for experimentation.
Following attachment of the myocardium preparations to the servomotor and force transducer, length and voltage were optimized to maximize the work output from the preparations, as described previously. All experiments were carried out at 14°C.

4.3.2 Epinephrine exposure

Stocks of epinephrine (Sigma-E4375) were made up in saline and frozen at -10°C for a maximum of 1 week before use; stocks were thawed just prior to use during experiments, and kept on ice wrapped in aluminum foil to minimize exposure to light. With the PO$_2$ of the saline set at 100% air saturation (20.2 kPa), an aliquot of epinephrine stock was added to the saline bath to bring the total saline concentration to 5nM, which is similar to blood concentrations in unstressed rainbow trout (Perry and Reid 1992). The saline was allowed to circulate for 1 minute after the addition of epinephrine before measurements of work output and maximum contraction rate were collected. All measurements of contractile performance at a given PO$_2$ were completed within 10 minutes of epinephrine addition to reduce the effects of photodegradation on epinephrine concentration.

For measures of contractile performance, spongy and compact preparations were subjected to 3 sets of 30 consecutive strain cycles and contraction at air saturation. The 3 sets of strain cycles were conducted in immediate succession, with averages of net, lengthening and shortening work from the last cycle in each set calculated and used for analysis. A series of 30 cycles was used as work output largely stabilizes by the 30$^{th}$ strain cycle. Maximum contraction rate was then assessed by measuring isometric force while the muscle was stimulated at successively increasing rates. The maximum contraction rate was selected as that just before the muscle became refractory, as described in Chapter 3.
Following the completion of these measurements, the saline in the chamber was replaced to avoid the accumulation of epinephrine. The PO$_2$ was then lowered to 10% air saturation (2kPa) by mixing N$_2$ gas with atmospheric air using a gas mixer (Wosthoff M200, Bochum, Germany). PO$_2$ was monitored in the saline by a fibre-optic oxygen meter (Presens Fibox 3 LCD, Espoo, Finland). This low PO$_2$ is likely experienced *in vivo* in the venous blood (and thus spongy myocardium) during mild hypoxia and in arterial blood (and thus compact myocardium) during more severe hypoxia (Thomas et al. 1994). Once the proper PO$_2$ was achieved in the saline, fresh epinephrine stock was added, restoring the low exposure level (5nM), and the measurements for work output were repeated.

Preparations were then returned to 100% air saturation and the same series of measurements were repeated but with the addition of a high dose of epinephrine (total saline concentration 500nM), following the same protocols of replacing the saline between series of measurements, and with the PO$_2$ at 100% and then 10% air saturation. This concentration of epinephrine is similar to that experienced in trout stressed by hypoxia (Perry and Reid 1992).

4.3.3 Analysis:

In total, 12 compact myocardium preparations and 11 spongy myocardium preparations were used for these experiments. During the experiments 3 spongy preparations were damaged due to incorrect lengthening commands being applied to the servomotor, preventing further collection of data from these preparations, however, all data collected prior to the damage were still used in the analysis. Work was expressed relative to muscle mass. Wet masses were obtained using the same procedure described in Chapter 3. The mass adjusted work values were used in the present study, as unadjusted values of work were more different between spongy and
compact myocardium than adjusted values, suggesting the unadjusted values were biased by non-viable tissue in the dissections. As noted, the average of the work outputs from the last strain cycle of the three sets of 30 strain cycles was used for analysis. In instances where the coefficient of variance (CV) for these three values was greater than 15%, the work value that differed the most from the other two was excluded; this occurred in 19 out of 89 total observations, where exclusion of the outlier then reduced the CV to less than 15%. In instances where the CV remained higher than 15% even after the exclusion of a value, that work value was still excluded from analysis; this occurred for 2 instances.

Analyses were conducted as mixed effects models, with a random intercept term included in all models for individual myocardial preparations to control for preparation specific performance (i.e. the non-independence of repeated measures on individual tissue preparations).

Work (net, lengthening and shortening) was compared between low and high epinephrine exposures at 100% air saturation for both tissue types, with the fixed effects of tissue type, epinephrine concentration and an epinephrine- tissue type interaction. Values of net, lengthening and shortening work were log transformed to improve the model fit for the assumption of normal distribution of residuals. Diagnostic residual plots including histogram, QQ-norm and Pearson residual vs. fitted plots were used to visually assess normality of residuals and other assumptions of the mixed effects models. The same fixed effects structure was used to compare the proportion of net, shortening and lengthening work output in hypoxia relative to that produced in air saturation (i.e. work output at 10% air saturation / work output at 100% air saturation) during exposure to low and high epinephrine levels. The fixed effects for these analyses were epinephrine exposure, tissue type and the interaction between tissue type and epinephrine exposure.
Analyses of maximum sustainable contraction rate were conducted comparing the effects of low and high epinephrine doses within and between tissue types at 100% air saturation to determine if there was an effect of epinephrine on contraction rate, and how it differs between tissue types. This analysis was conducted as a mixed effects model with the fixed effects of epinephrine level, tissue type, and an epinephrine tissue type interaction. Additionally, the maximum contraction rates were compared between high and low epinephrine levels at the low PO\textsubscript{2} for both tissue types, with the same fixed and random effect as in 100% air saturation, to determine if the there was an effect of epinephrine on maximal contraction rate in hypoxia. The maximum contraction rates were also compared between tissue types at 100% air saturation and at 10% air saturation with the low epinephrine dose. This was completed to confirm whether PO\textsubscript{2} had an effect on the maximum contraction rate and if the response was different between tissue types. The fixed effects for this mixed effects model were PO\textsubscript{2}, tissue type and the PO\textsubscript{2} tissue type interaction. Finally, a test was completed to compare the maximum contraction rates between 100% air saturation and 10% air saturation in high epinephrine to determine whether high epinephrine exposure during 10% air saturation was able to restore the maximum contraction rate. The fixed effects for this mixed effects model were treatment (100% air saturation low epinephrine, 10% air saturation high epinephrine), tissue type and the treatment tissue type interaction. All analysis were conducted using R software, and mixed effects models were assessed using the lme4 package (Bates et al. 2005; R-core team 2015). P value estimates were obtained from the package lmerTest using Satterthwaite estimation of degrees of freedom (Hrong-Tai Fai and Cornelius 1996; Kuznetsova et al. 2013). Where significant differences were found, Tukey’s correction factors were applied to post-hoc contrasts.
4.4 Results

4.4.1 Trout size and preparations

The mass of the trout used in the present study was 189±11 g, with a fork length of 22.8±0.69cm and a total length of 24.8 ±0.75cm. Preparations of spongy myocardium had a mass of 1.9± 0.42 mg, and compact preparations were 2.7± 0.40 mg. The optimal length for the spongy preparations was 3.57±0.41 mm and 4.77±0.32 mm in the compact.

4.4.2 Effects of epinephrine on work output

There was no significant difference between the work produced by compact and spongy myocardium in 100% air saturation, when compared at similar concentrations of epinephrine (P=0.71) (Figure 4.1). At 100% air saturation, high epinephrine levels led to a significantly higher net and shortening work output (P=0.0028, P=0.029, respectively), but only for the compact myocardium (net P=0.0031, shortening P=0.045), not the spongy myocardium (net P=0.14, shortening P=0.24). The effect on lengthening work was marginally not significant (P=0.052), with a general trend toward a decline in lengthening work with high epinephrine tone, particularly in the compact myocardium (Figure 4.1). The interactions of tissue type and epinephrine exposure were not significant (net P=0.27, shortening P=0.56, lengthening P=0.23).

The proportion of net and shortening work output maintained in 10% air saturation, relative to what is produced in 100% air saturation, was greater in high versus low levels of epinephrine (net P=0.0197, shortening P=0.01), (Figure 4.2), however the effect was significant only in the spongy myocardium (net P=0.025, shortening P=0.029), not the compact myocardium (net P=0.34, shortening P=0.14). However, the difference in proportion of net and shortening
work output maintained was not significantly different between tissue types (net $P=0.12$, shortening $P=0.43$), with no significant tissue type-epinephrine exposure interaction (net $P=0.22$, shortening $P=0.43$). The proportion of lengthening work maintained in 10% air saturation was not significantly different between tissue types ($P=0.76$), epinephrine exposure ($P=0.63$) or the interaction ($P=0.61$).

### 4.4.3 Effects of epinephrine on maximum contraction rate

Exposure to 10% air saturation resulted in a significant reduction in the maximum contraction rate at low epinephrine levels ($P=0.0058$) (Figure 4.3), but the effect was only significant in compact myocardium ($P<0.0001$), not spongy myocardium ($P=0.14$). There was not a significant PO$_2$-tissue type interaction ($P=0.38$).

High (vs low) epinephrine did not appear to affect the maximum contraction rate at air saturation, for either spongy or compact myocardium ($P=0.13$) (Figure 4.3). However, during 10% air saturation, high epinephrine resulted in a significantly higher maximum contraction rate ($P=0.023$) but again the effect was significant only in compact myocardium ($P=0.0056$), not the spongy myocardium ($P=0.66$). In both 100% and in 10% air saturation, as well as low and high epinephrine, spongy myocardium had a significantly higher maximum contraction rate than compact myocardium (100% air saturation $P<0.0001$, 10% air saturation $P<0.0001$).

Finally, maximum contraction rate with 100% air saturation and low epinephrine were not significantly different from those with 10% air saturation and high epinephrine ($P=0.70$), the conditions that might be anticipated in a normoxic vs. hypoxia stressed fish. The maximum contraction rate under these conditions was significantly higher in spongy myocardium vs. compact myocardium ($P<0.0001$), but there was no significant interaction between tissue type
and treatment (P=0.51), showing there was no significant difference in the change to maximum contraction rates between tissue types.

4.5 Discussion

4.5.1 Effects of epinephrine on work output

High epinephrine exposure resulted in increased shortening work from compact myocardium in 100% air saturation (Figure 4.1), as predicted. However, high epinephrine exposure did not have the predicted effect of increasing inotropy in the spongy myocardium at 100% air saturation (Figure 4.1). The physical euthanasia method used in the present study may have elicited a release in circulating epinephrine, and this could have resulted in an attenuation of the adrenergic contractile response upon subsequent exposure to epinephrine. Gamperl et al (1994c) however, found no change to β-AR surface levels on myocardium following repeated exposure to epinephrine. The present results were in contrast to results of Shiels et al. (1998), where spongy myocardium of rainbow trout increased work output with high epinephrine exposure in well oxygenated conditions. This discrepancy may be explained by the 20-fold difference in the concentration of the high adrenaline dose used in the present study (500 nM) vs. the study by Shiels et al. (10 µM). We chose 500nM as a concentration similar to that reported in salmonids during hypoxic stress. Thus, it appears that when oxygen is not limiting, contractility of the spongy myocardium is not enhanced by the levels of epinephrine in the circulation during a hypoxic stress response.

Additionally, the significant effect of high epinephrine on the inotropy of compact myocardium, but not spongy myocardium, appears to contrast with the expectation that there would be an even higher inotropic response in spongy vs. compact myocardium based on the
higher $\beta$-AR surface receptor density in spongy vs compact myocardium of adult wild coho salmon (Gamperl et al. 1998). It has been noted, however, that the hearts of coho salmon have a 2.8x higher $\beta$-AR surface receptor density than those of hatchery reared rainbow trout (Gamperl et al. 1998). Thus, the lower $\beta$-AR densities for both spongy and compact myocardium from the hatchery-reared trout, as used in the present study, may have resulted in a reduction or even reversed any difference between tissue types in their adrenergic inotropic response. Similarly, $\beta$-AR surface receptor is likely not the only factor contributing to intensity of adrenergic contractile response. This is supported by the observations that there were no significant differences in work output between tissue types following exposure to high epinephrine (tissue type-epinephrine interaction), and that both tissue types exhibited a similar proportional change to work output following exposure to high epinephrine (compact average 15% increase in net work and spongy average 16% increase in net work). This may suggest the $\beta$-AR densities were somewhat variable in the spongy myocardium, leading to higher variance and an inability to detect an effect of high epinephrine on work at air saturation.

Unlike the responses to epinephrine observed in 100% air saturation, during 10% air saturation high epinephrine exposure resulted in significantly more net work and shortening work from the spongy myocardium, but not compact (Figure 4.2). This reversal in tissue specific responses to epinephrine in 100% vs. 10% air saturation may indicate changes to adrenergic sensitivity in hypoxia. Increases in both cyclic AMP production (a down stream effector in the signal transduction pathway of $\beta_2$-AR activation) and receptor density during acute hypoxia (<60 minutes) have been observed in erythrocytes of rainbow trout (Reid et al. 1993). The present results may indicate that such changes occur in the spongy myocardium during hypoxia as well, but perhaps not, or to a lesser extent, in compact myocardium. The effect of high epinephrine on
contractile function of spongy myocardium during 10% air saturation observed in the present study is consistent with the improved hypoxia tolerance of cardiac function in rainbow trout with high epinephrine exposure (Hanson et al. 2006), as in both instances the myocardium maintained contractile function better with elevated epinephrine.

Surprisingly, work output of compact myocardium was not significantly affected by epinephrine during 10% air saturation (Figure 4.2), even though it was in 100% air saturation (Figure 4.1). This contrasts with observations of increased developed isometric twitch force of compact myocardium during anoxia in high epinephrine (Gesser et al. 1982). This discrepancy may again be the result of above physiological epinephrine exposures being used in that study (Gesser et al. 5µM, present study 500nM). The difference in response between 100% air saturation and 10% air saturation may reflect differences in β-AR subtypes present on the compact myocardium, which could generate PO$_2$ dependent responses to adrenergic stimulation. Both β2 -AR and β3 -AR have been identified in the ventricular myocardium of rainbow trout (Motyka et al. 2016; Petersen et al. 2013). β3 -AR stimulation acts on myocardium to decrease inotropy (Imbrogno et al. 2015), which would result in a decrease to net work output. The β3 -AR signal transduction pathway involves the stimulation of nitric oxide synthase to increase nitric oxide (NO) production in the cell (Imbrogno et al. 2015). In normoxia, NO rapidly oxidizes, supressing the cellular effects of this molecule (Fago et al. 2012; Hill et al. 2010). In the hypoxic conditions, however, NO would likely be stabilized to a greater degree, allowing the β3 -AR response to be augmented. This may result in the positive and negative inotropic effects of these two receptor subtypes (β2 -AR and β3 -AR, respectively) being largely balanced during exposure to 10% air saturation in compact myocardium, resulting in no net effect of adrenergic stimulation on work output.
4.5.2 Effects of epinephrine on Maximum contraction rate

The compact myocardium showed a decline in the maximum contraction rate between 100% air saturation and 10% air saturation, when exposed to low levels of epinephrine (Figure 4.3). Mechanistically, a change to maximum sustainable contraction rate likely reflects modifications to the cardiac action potential. Reduction in developed force between subsequent contractions suggests that the cells are refractory and unable to reach the same levels of intracellular calcium during each contraction. Refractory myocardium may be caused by a reduction of the calcium current as a result of L-type Ca$^{2+}$ channel inactivation, or other disruptions in Ca$^{2+}$ handling (Shiels et al. 2002). It has been shown that there are rapidly O$_2$ sensitive L-type Ca$^{2+}$ channels in rat myocardium (Scaringi et al. 2013). Changes in Ca$^{2+}$ currents sensitive to PO$_2$ could potentially affect the ability of the myocardium to sustain high contraction rates. As well, ischemic ventricular arrhythmia in mammals is thought to be associated with a reduction of intracellular K$^+$ through depression of the Na/K pump (Janse and Wit 1989). A change in current or activation/deactivation associated with reduced PO$_2$ for any of the ion channels involved in the action potential could provide insight into mechanisms driving a change of maximum sustainable contraction rate in the compact myocardium.

*In vivo*, hypoxic bradycardia in fish is largely mediated by changes in vagal tone (Short et al. 1979; Farrell 2007; Mckenzie et al. 2009). However, from the present study it appears there is a decline in the potential maximum contraction rate during hypoxia that may be intrinsic to the compact myocardium. This corresponds with what has been observed in vagotomised Atlantic cod, that still experience bradycardia during deep hypoxia, and in European eel where arrhythmias have been observed following atropine treatment in hypoxia (Mckenzie et al. 2009;
Iversen et al. 2010). As well, in mammals, ischemia caused by coronary blockage can result in arrhythmias of the myocardium (Janse and Wit 1989). A functional role for hypoxic bradycardia, and suppression of maximal contractile rate of myocardium, may be evident. In some species, hypoxic bradycardia could serve as a means to preserve cardiac function by suppressing heart rate to ensure arrhythmias do not occur as a result of the hypoxic decline in maximum sustainable contraction rate. These fish could maintain cardiac output during hypoxic bradycardia largely through increases in stroke volume. Research exploring changes to the action potential of the myocardium during hypoxia and analysis of ventricular ion channel function during hypoxia would further our understanding of how and why hypoxia impacts heart rate.

High epinephrine, however, appears to reverse the hypoxia induced decline in maximum contraction rate for the compact myocardium, where the maximum contraction rate of compact myocardium in 10% air saturation with high epinephrine was not different from that in 100% air saturation (Figure 4.3). This may support the presence of β3-AR on compact myocardium. Ventricular β3-AR stimulation, through the use of a subtype specific agonist (BRL-37344), results in an increase in heart rate of adult rainbow trout (Petersen et al. 2013). Additionally, in mammals, β3-AR stimulation results in a briefer action potential, which could reduce the refractory period of the myocardium (Gauthier et al. 1996) and result in an increase to the maximum sustainable contraction rate. Thus, β3-AR stimulation and higher levels of stabilized NO in 10% air saturation could explain both the positive effect of epinephrine on maximum contraction rate and the lack of an inotropic response for compact myocardium.

As observed previously, there was no effect of PO2 on maximum contraction rate of the spongy myocardium in the absence of epinephrine (Chapter 3, Figure 4.3). As well, spongy myocardium had a higher maximum contraction rate than the compact myocardium (Chapter 3).
Exposure to high epinephrine levels did not have a significant effect on maximum contraction rate for spongy myocardium at any PO$_2$ (Figure 4.3). The maximum contraction rate of the spongy myocardium thus appears very insensitive and stable. This could be reflective of a lower $\beta_3$-AR subtype density on spongy myocardium, resulting in spongy responding differently to PO$_2$ and epinephrine than compact. The observed increase in inotropy, but not maximum contraction rate, in response to epinephrine in the spongy myocardium is more indicative of higher levels of $\beta_2$ -AR (Vornanen 1998; Shiels et al. 2002). Chronic hypoxia leads to an increase in $\beta_2$ -AR and decrease in $\beta_3$ -AR in the ventricles of rainbow trout (from samples containing both spongy and compact) (Motyka et al. 2016). As spongy myocardium is routinely exposed to low PO$_2$ of venous blood, this might suggest $\beta_2$ -AR are expressed at high levels and $\beta_3$ -AR are supressed on the spongy myocardium, and this is reflected in the differential responses of inotropy and contraction rate in spongy and compact myocardium in response to epinephrine and hypoxia.

4.6 Future directions and conclusions

Further research is needed to clarify the mechanistic bases of the adrenergic responses. This could involve the use of $\beta$ -AR subtype specific pharmacological agents (Hanson et al. 2006; Petersen et al. 2013). Additionally, experiments involving norepinephrine exposure may clarify the overall effect of catecholamine release on contractile performance and maximum contraction rate. This may be particularly important, as norepinephrine has a higher binding affinity to $\beta_3$ -AR and norepinephrine levels are also highly elevated during hypoxia (Perry and Reid 1992; Imbrogno et al. 2015).

The adrenergic response in compact myocardium appears to be PO$_2$ dependent and may reflect a balance in $\beta$ -AR subtype activity. The increase in maximum contraction rate in
response to epinephrine for compact myocardium suggests elevated circulating catecholamines may be important to preserve the ability to the raise heart rate during hypoxia in trout, given that the maximum contraction rate of compact myocardium declined in hypoxia. Additionally, high epinephrine exposure during acute hypoxia appears to result in increased inotropy for the spongy myocardium, preserving a higher capacity for work output compared to what would be available at levels of epinephrine present in unstressed animals. These results may suggest increased adrenergic tone during hypoxia causes an increase in the contractile contribution of the spongy myocardium to the cardiac output, relative to compact myocardium (although overall performance of both myocardial tissue types declines). As the spongy myocardium makes up ~70% of the rainbow trout ventricle, the ability to preserve a larger portion of work output during hypoxia via increased epinephrine release would likely be reflected in enhanced cardiac output. The effect of high epinephrine in enhancing the work output of spongy myocardium and enhancing the ability of compact myocardium to work at higher heart rates, will contribute to the maintenance of cardiac output during hypoxia in rainbow trout.
4.7 Figures:

![Figure 4.1: Work output from spongy and compact myocardium with low and high epinephrine in 100% air saturation](image)

Figure 4.1 Work output from spongy and compact myocardium with low and high epinephrine in 100% air saturation

Net, shortening and lengthening work output from compact and spongy myocardium at 100% air saturation with low (5nM) and high (500nM) levels of epinephrine. Asterisk indicates significant differences between low and high epinephrine exposures (P<0.05). Data are means ±SE. Standard errors were calculated from group means (tissue type and epinephrine exposure).
Figure 4.2 Work output from spongy and compact myocardium in low and high epinephrine in hypoxia

Net, shortening and lengthening work output during 10% air saturation, expressed as a proportion (%) of work produced at 100% air saturation, from compact and spongy myocardium with low (5nM) and high (500nM) levels of epinephrine. Asterisk indicates significant differences between low and high epinephrine exposures (P<0.05). Data are means ±SE. Standard errors were calculated from group means (tissue type and epinephrine exposure).
Figure 4.3 Maximum contraction rate of spongy and compact myocardium with low and high epinephrine

Maximum contraction rate (BPM) of spongy and compact myocardium at 100% air saturation and 10% air saturation and exposure to low (5nM) and high (500nM) levels of epinephrine.

Asterisk indicates significant difference between low and high epinephrine or between 100% air saturation and 10% air saturation (indicated with bracket for compact myocardium) (P<0.05).

Data are means ±SE. Standard errors were calculated from group means (tissue type at an epinephrine exposure).
5.1 Limitations

Conclusions made in this thesis about the nature of compact and spongy myocardium were limited by differences among ventricular isolates caused by dissection. For example, the variability in the size dimensions of the myocardium preparations, an artefact of dissection, could have resulted in diffusion limitations in larger preparations and thus lead to even further depression of local PO₂ exposure for non-surface regions. As preparations of compact myocardium were often larger than those of spongy myocardium, the results could again reflect a diffusion limitation in the compact myocardium and therefore an overestimation on the effects of hypoxia. As well, the trimming of non-viable tissue from the myocardium preparations could have also potentially introduced bias between tissues types where measurements of work output were adjusted by mass, as was completed in chapters 3 and 4.

As well, both steelhead and fresh water acclimated rainbow trout were used in the experiments conducted for this thesis, results seen within one group may not extend to the other. It has previously been shown that different sources of hatchery-reared trout can have different hypoxia tolerances (Faust et al. 2004). The difference in hypoxia tolerances between different trout populations could potentially have a basis in cardiac performance during hypoxia or differences in adrenergic sensitivity. Thus these results may be to some extent specific to trout populations examined.

5.2 Conclusions

This thesis explored effects of acute and chronic hypoxia on the contractile performance of the compact and spongy myocardium. Consistent across the experiments presented here, both
tissue types exhibited a decline in net work output following exposure to acute hypoxia. The results of Chapter 4 however, suggest high levels of epinephrine present in the circulation during hypoxia may allow the spongy myocardium to maintain a higher proportion of contractile performance during acute hypoxia.

Following hypoxia acclimation, there was a loss of inotropy in the spongy myocardium. This may be associated with the decline in cardiac output observed for hypoxia acclimated trout in vivo. Surprisingly, hypoxia acclimation did not have an effect on the reduction in inotropy when working in acute hypoxia. Thus, it appears the constant exposure of the spongy myocardium to low PO$_2$ in the venous blood did not confer an enhanced ability to function in low PO$_2$. These results may suggest the chronic exposure to low PO$_2$ (occurring in venous blood in hypoxia acclimation), appears to only have detrimental effects on the spongy myocardium.

Compact and spongy myocardium exposed to the PO$_2$ as they would experience in blood in vivo during environmental hypoxia showed similar declines in work output. This was despite the larger drop in PO$_2$ experienced in the compact myocardium vs. spongy. These results suggested the contractile performance of both spongy and compact myocardium decline to a similar extent during mild environmental hypoxia, and therefore, the relative contractile contribution of both tissue types to myocardial function may not change to a large degree. However, in more severe hypoxia there is an increase in circulating catecholamines (Perry and Reid 1992). Exposure to high levels of epinephrine during hypoxia resulted in an increase in the inotropy of spongy myocardium, but not compact myocardium. Therefore, during more severe acute hypoxia where there is an increase in epinephrine exposure, there may be an increase in the relative contractile contribution of spongy myocardium.
It was also noted that compact myocardium exhibited a decline in the maximum contraction rate between 100% air saturation and 10% air saturation. This was not observed, however, between 100% air saturation and mild hypoxic P\textsubscript{a}O\textsubscript{2} (25% air saturation) (although paradoxically, a decline in maximum contraction was present between normoxic P\textsubscript{a}O\textsubscript{2} (65% air saturation) and mild hypoxic P\textsubscript{a}O\textsubscript{2} (25% air saturation)). However, with exposure to high epinephrine, the maximum contraction rate in hypoxic compact myocardium was restored to the same levels as in 100% air saturation. Thus, it appears increased circulating epinephrine during hypoxia may help to preserve the scope of heart rate increase for compact myocardium, perhaps preventing arrhythmias during hypoxia, and also to increase the contractile contribution of spongy myocardium to maintain cardiac function.

Of note, maximum contraction rate in the spongy myocardium was insensitive to changing PO\textsubscript{2} or epinephrine exposure. The lack of sensitivity of maximum contraction rate to changing PO\textsubscript{2} in spongy myocardium suggests this tissue is able to maintain high contraction rates during hypoxia. Although the need to maintain high contraction rates may be limited in environmental hypoxia due to reflex hypoxic bradycardia, intense exercise causes a decline in P\textsubscript{v}O\textsubscript{2} (Farrell and Clutterham 2003) while heart rate increases (Stevens and Randall 1967). This may support a need for the ability of spongy myocardium to elevate heart rate in hypoxic P\textsubscript{v}O\textsubscript{2}.

The effects of both acute and chronic hypoxia on the contractile performance of myocardium observed here suggest overall, hypoxia results in a decline in ventricular performance that is not mitigated by acclimation. Therefore, an integrated response of extrinsic factors influencing cardiac function (e.g. neural, hormonal, and other mechanical feedback) may be necessary for survival during acute and chronic hypoxia in rainbow trout.
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