THE EFFECTS OF CHRONIC HYPOXIA AND NITRIC OXIDE ON MYOCARDIAL CONTRACTILITY IN STEELHEAD TROUT (*ONCORHYNCHUS MYKISS*)

by

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Abstract

Environmental hypoxia has been intensifying and spreading in recent years due to climate change, and there is accumulating evidence that the cardiac function of hypoxia-intolerant fish is negatively affected by long-term exposure to low oxygen conditions. However, the cause of this reduced heart performance has not been conclusively identified, and it is unknown whether nitric oxide (NO)-mediated effects are involved. This thesis examined how chronic hypoxia (> 8 weeks at ~8 kPa O_2) influenced the contractility (i.e., work and power) of steelhead trout (*Oncorhynchus mykiss*) spongy myocardial strips upon exposure to graded hypoxia (21 – 1.5 kPa), and influenced NO-mediated performance. Hypoxic-acclimated strips produced less shortening power (by ~35%) as compared to those from normoxic-acclimated conspecifics, but experienced similar reductions in contractility during acute hypoxia and did not recover as well upon re-oxygenation. The effects of NO on myocardial function were not greatly affected by hypoxic-acclimation, but were found to be highly dependent on contraction frequency and strain.

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Dedication

This thesis is dedicated to Angelo and Bernadette Carnevale.

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List of Symbols, Abbreviations and Nomenclature

Abbreviation	Definition
%	Percent
[ ]	Chemical concentration
~	Approximately
ADMA	Asymmetric dimethylamine
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APD	Action potential duration
ATP	Adenosine triphosphate
$\beta_{1/2}$-AR	Type 1 and 2 adrenoreceptors
β_3-AR	Beta 3 adrenoreceptor
βARK	Beta-adrenergic receptor kinase
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CAT	Catecholamines
cGMP	Cyclic guanosine monophosphate
cMyBPC	Cardiac myosin binding protein C
CO_2	Carbon dioxide
cTnC-Cys35	Cardiac troponin C-cysteine 35
cTnI	Cardiac troponin I
DO	Dissolved oxygen
EE cell	Epicardial endothelial cell
‘eNOS’	Endothelial nitric oxide synthase
f_H	Heart rate
$G_{i/o}$	G-protein-coupled receptor
GTP	Guanosine triphosphate
H_2S	Hydrogen sulfide
Hb	Haemoglobin
Hz	Hertz
iNOS	Inducible nitric oxide synthase
J	Joules
K	Condition factor
K^+	Potassium
K_{ATP} channel	ATP-sensitive potassium channel
kPa	Kilopascals
L-NMMA	N^G-Methyl-L-arginine; NOS inhibitor
L_{opt}	Optimum length of myocardial strip
M	Molar, mol L^{-1}
MAP	Mean arterial pressure
MRs	Membrane receptors

ms	Milliseconds
N	Newtons
n	Sample size
N_2	Nitrogen gas
Na^+	Sodium
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
NO_2^-	Nitrite
nNOS	Neuronal nitric oxide synthase
NR	Nitrite reductase
O_2	Oxygen
ODQ	1H-[1,2,4]oxadiazole-[4,3-a]quinoxalin-1-one; sGC inhibitor
OMZ	Oxygen minimum zone
PDE	Phosphodiesterase
PKA	Protein kinase A
PKG	Protein kinase G
PLB	Phospholamban
PO_2	Partial pressure of oxygen
P_{out}	Output pressure
P-TIO	2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide
P_{ven}	Central venous pressure
P_wO_2	Water oxygen partial pressure
Q	Cardiac output
Q_{max}	Maximum cardiac output
ROS	Reactive oxygen species
RVM	Relative ventricular mass
RyR	Ryanodine receptor;
SE	Standard error of the mean
Ser16	Serine 16 residue of phospholamban
SERCA	Sarco-endoplasmic reticulum Ca^{2+}-ATPase pump
sGC	Soluble guanlyl cyclase
SNAP	S-nitroso-N-acetylpenicillamine
S-NO	S-nitrosylation
SNP	Sodium nitroprusside
SR	Sarcoplasmic reticulum
S_V	Stroke volume
S_{Vmax}	Maximum stroke volume
S_{Vrest}	Stroke volume at rest
S_W	Stroke work
Thiol	Thiol residue of phospholamban or RyR protein
V	Volts
W	Watt

Chapter One: Introduction

1.1. Overview

This thesis compares the contractile performance of isolated ventricular trabeculae from normoxic and hypoxic-acclimated steelhead trout (*Oncorhynchus mykiss*) during acute hypoxic exposure, after normoxic recovery, and when exposed to exogenous nitric oxide (NO) donors and inhibitors. Acclimation to hypoxia has been reported to diminish cardiac output (Q) in Atlantic cod (*Gadus morhua*) and trout due to significant reductions in stroke volume (S_V), irrespective of morphological changes, or humoral or neuronal regulation of venous (filling) pressure (Peterson and Gamperl, 2010a; 2010b; Moytka *et al.*, 2017). Since these studies used *in vivo* and *in situ* methods to study the effects of chronic hypoxia on fish heart function, the goal of my thesis was to use an *in vitro* approach (isolated cycling myocardial strips; Josephson, 1985; Syme and Josephson 1995; Harwood *et al.* 1998; Shiels *et al.* 1998; Harwood *et al.* 2002; Syme *et al.*, 2003) to investigate: 1) how chronic hypoxia affects the contractility (i.e., power and work) of the spongy myocardium under varying levels of oxygenation (Chapter 2); and 2) the potential role of NO in any observed changes in myocardial function (Chapter 3). Nitric oxide is a known regulator of cardiovascular function and cardiac energy metabolism in mammals during hypoxia / ischemia (Hendgen-Cotta *et al.*, 2008; Chouchani *et al.*, 2013), and more recently studies on hypoxia-tolerant fish have revealed similar results (Imbrogno *et al.*, 2001; Tota *et al.*, 2005; Garofalo *et al.*, 2009; Imbrogno *et al.*, 2014). However, it is not known if the responsiveness to exogenous NO is altered by hypoxic acclimation, or whether this cardiac regulation originates from nitric oxide synthase (NOS) dependent or independent

mechanisms (i.e., such as nitrite, NO_2^-). Measurements of twitch duration were also taken during the NO experiments (Chapter 3) to compare the effect of chronic hypoxic acclimation and NO on the temporal characteristics of myocardial contraction. This research is important as changes in the duration of contraction and relaxation with chronic hypoxia, and / or associated with activation of the NO signaling cascade, could potentially influence cardiac filling and Q.

In the below sections, 1) an overview of the extent and types of environmental hypoxia is provided; 2) our current understanding of the physiological and cardiovascular responses of fish subjected to this stressor is described; 3) I review the role of NO in the regulation of cardiac contractility during periods of oxygen deprivation; and 4) the main goals of this thesis are summarized.

1.2. Background

1.2.1. *Environmental Hypoxia*

Aquatic organisms live in habitats where environmental parameters can fluctuate over a number of time scales (e.g., diurnally and / or seasonally). Hypoxia (low water oxygen levels that negatively impact an organism's physiology) can occur in the marine environment due to natural fluctuations in tidal cycles, density layer stratification and shifting wind patterns, or in bodies of water that are isolated for prolonged periods (e.g., shallow tide pools) (Diaz and Rosenberg, 2008; Richards, 2011), and these events have become more severe and frequent in recent years due to accelerated climate change (Altieri and Gedan, 2015; Breitberg *et al.*, 2018). For example, the increased use of nitrogen-based

fertilizers along the coasts has resulted in the eutrophication of coastal areas and estuaries, and consequent algal blooms (Diaz and Rosenberg, 2008). When these masses of algae die and sink in the water column, they are decomposed by microbes, and their respiration reduces the available dissolved oxygen (DO) creating hypoxic zones (Altieri and Gedan, 2015). These naturally arising oxygen minimum zones (OMZ) occur from approximately 200 to 1000 meters deep and persist due to the lack of mixing with oxygen rich surface waters (Ramirez-Llodra *et al.*, 2010). The presence of a thermocline can also reduce mixing of the water column, resulting in prolonged hypoxia and mass mortalities of benthic fish and crustaceans (Diaz and Rosenberg, 2008). Estuaries are formed where freshwater rivers and streams mix with saltwater and are important nursery habitats. However, they have become increasingly eutrophic due to coastal nutrient pollution. Chesapeake Bay, for example, is the largest estuary in the USA and has been impacted by increased nutrient load and severe hypoxia since the 1950s. This has led to the degradation of its benthic habitats and a general decline in total fish production (Kemp *et al.*, 2005). Tide pools are another example of important microhabitats which are prone to becoming hypoxic and eutrophic due to their shallow nature (Richards, 2011). Finally, coastal areas and estuaries may become hypoxic due to the combined effects of rising air temperatures and nutrient loading (Altieri and Gedan, 2015), and ocean acidification associated with rising global CO_2 levels may act synergistically with hypoxia to constrain aerobic scope, and thus, limit the performance and survival of aquatic organisms (Pörtner and Peck, 2010; Pörtner *et al.*, 2017).

1.2.2. *Responses of Fish to Hypoxia*

Fish can respond to environmental hypoxia in a number of ways. Three common behavioural responses are a reduction of activity (Herbert and Steffensen, 2005; Poulsen *et al.*, 2011), a decrease in/or a cessation of feeding (Roberts *et al.*, 2011; Remen *et al.*, 2012; Sun *et al.*, 2012), and avoidance of hypoxic waters (Claireaux *et al.*, 1995; Pörtner and Peck, 2010; Hughes *et al.*, 2015). For example, two stocks of Atlantic cod (*Gadus morhua*) reside in the Estuary and Gulf of St. Lawrence (EGSL), which is often hypoxic year-round due to perpetual density stratification (Bugden, 1991), and these fish avoid areas where DO is less than 30% of air saturation (Chabot and Claireaux, 2008). However, fish are not always able to avoid hypoxic waters or reduce energy expenditures through the above mechanisms, and must respond physiologically to maintain homeostasis. Some fish, such as the crucian carp (*Carassius carassius*, Stecyk *et al.*, 2004) and goldfish (*Carassius auratus*, Imbrogno *et al.*, 2014), are very hypoxia tolerant and can produce ethanol, instead of lactate, as an end product of anaerobic metabolism (Stecyk *et al.*, 2004). Sculpins (Mandic *et al.*, 2009), flatfish (Wood *et al.*, 1979) and epaulette sharks (*Hemiscyllum ocellatum*, Routley *et al.*, 2002; Nilsson and Renshaw, 2004) are all examples of hypoxia tolerant marine fishes. Multiple sculpin species are able to inhabit shallow tide pools which are subject to large variations in oxygen saturation (Yoshiyama and Cech, 1994) due to their large gill surface area, low routine metabolic rates and high Hb-O_2 binding affinity (Mandic *et al.*, 2009). Flatfish employ a combination of reduced metabolic rate, increased O_2 binding affinity and enhanced cardiac pumping to cope with hypoxia and their natural behaviour of burying, or partially burying, themselves in benthic sediments (Nonnotte and

Kirsch, 1978; Wood *et al.*, 1979; Joaquim *et al.*, 2004; Mendonça *et al.*, 2007). Finally, the epaulette shark (*Hemiscyllum ocellatum*) is known to tolerate severe anoxia at high temperatures due to being naturally preconditioned (Routley *et al.*, 2002). This preconditioning arises based on exposure to slowly lowering tides in the spring. Under these conditions, the respiration of organisms can drive the oxygen saturation down to less than 18% of air saturation (Routley *et al.*, 2002). The shark's metabolism is depressed as a result of this graded hypoxic exposure, and it is able to maintain cerebral blood flow (due to cerebral vasodilation) and increase ventilatory frequency to assist in oxygen uptake (Nilsson and Renshaw, 2004).

Many marine fish, for example the Atlantic cod (Petersen and Gamperl, 2010a; Petersen and Gamperl, 2010b; Petersen and Gamperl, 2011) and steelhead (rainbow) trout (Gamperl *et al.*, 2001; Overgaard *et al.*, 2004a; 2004b; Moytka *et al.*, 2017), are considered to be hypoxia-intolerant [but see Faust *et al.* (2004) for an example of a hypoxia tolerant strain of rainbow trout]. These species have not developed the above-mentioned specific mechanisms to deal with hypoxia or anoxia, and normally rely on short-term physiological adjustments to maintain homeostasis. Decreased heart rate (bradycardia) and increased stroke volume (S_V) (Holeton and Randall, 1967; Sandblom and Axelsson, 2005; Perry and Desforges, 2006) are short-term responses to acute hypoxia, along with adjustments to ventilatory frequency, gill vascular resistance and blood flow, and elevated levels of circulating catecholamines (Van Raaij *et al.*, 1996; Farrell *et al.*, 2009; Gamperl and Driedzic, 2009). There are three general responses of fishes to hypoxia with regards to alterations in heart rate (f_H), S_V and Q (Gamperl and Driedzic, 2009). Rainbow trout and

Atlantic cod are characterized by a response pattern where S_V starts to increase well before the onset of hypoxic bradycardia (resulting in a significant increase in Q), and further increases in S_V once bradycardia is initiated maintain Q above normoxic levels until the fish reaches its hypoxic limit. Constriction of the vasculature and increases in central venous pressure (P_{ven}), are also significant components of the S_V response to acute hypoxia in these fish (Smith *et al.*, 2001; Sandblom and Axelsson, 2005; 2006; Gamperl and Driedzic, 2009). For example, rainbow trout exposed *in vivo* to moderate acute hypoxia (~35% air saturation) were able to maintain Q through increases in S_V due a 0.04 kPa (67%) increase in P_{ven} (Sandblom and Axelsson, 2005). Despite these short-term adjustments, unlike the hearts of hypoxia-tolerant fish fishes [i.e., crucian carp (Stecyk *et al.*, 2004), tilapia (Lague *et al.*, 2012), eels (Davie *et al.*, 1992) or hagfish (Cox *et al.*, 2010)], the hearts of hypoxia-intolerant species are unable to sustain cardiac function when exposed to a prolonged hypoxic insult. For example, exposure to severe hypoxia/anoxia (water PO_2 0.6 kPa at 10^{o}C) for 15 minutes decreased the routine and maximum Q, and scope for Q, of *in situ* Atlantic cod hearts by ~30, 60 and 70%, respectively (Petersen and Gamperl, 2010b). Further, when *in situ* rainbow trout hearts were exposed to 30 minutes of severe hypoxia (PO_2 1 kPa), Q consistently dropped throughout the experiment and was ~90% lower by the end of the trial (Faust *et al.*, 2004).

1.2.3. *Cardiovascular Responses of Fish to Chronic Hypoxia*

The majority of studies examining the effects of hypoxia on fish cardiac function have utilized acute hypoxic exposures / protocols, and the effects of chronic exposure to

low oxygen levels on the cardiac function of 'hypoxia-intolerant' fish has not been extensively investigated. However, recent studies shed considerable light on how chronic hypoxia affects their cardiorespiratory physiology. These studies exposed cod (Petersen and Gamperl, 2010 a, b and 2011) and steelhead trout (Moytka *et al.*, 2017) to chronic hypoxia (40% of air saturation) for more than 8 weeks, and examined how this treatment affected the fish's heart function and metabolic capacity (maximum metabolic rate and aerobic scope). These studies showed that chronic hypoxia significantly reduced cardiac pumping capacity (due to diminished S_V), but that this did not affect the fish's metabolic capacity, swimming performance (cod, Petersen and Gamperl, 2010a), or hypoxia (cod, Petersen and Gamperl, 2011) and thermal (trout, Moytka *et al.*, 2017) tolerance. Why the pumping capacity of hypoxic–acclimated individuals is limited is unknown, but additional studies have eliminated some potential explanations. Changes in humoral and/or neuronal regulation of venous (filling) pressure are unlikely to account for the reduced S_V observed in hypoxia-acclimated fish as this reduction in cardiac pumping capacity was also reported for in *in situ* Atlantic cod hearts (Petersen *et al.*, 2010b). Further, Moytka *et al.* (2017) ruled out changes in heart morphology as a cause of the observed reduction in S_V. These authors did not find any effects of chronic hypoxia (over 12 weeks at 40% air saturation) on trout relative ventricular mass (RVM), percent compact myocardium, maximum end-diastolic volume or gross ventricular morphology.

Given that there were no differences in gross cardiac morphology or maximum end-diastolic volume, it is possible that the reduced S_V (Q) of hypoxia-acclimated fish is related to the inherent properties of the muscle itself. Cardiac output (Q) is determined by the

difference between end-systolic and end-diastolic volume, and these two factors are dependent on / influenced by myocardial contractility and stiffness, respectively. To examine the contractility and stiffness of ventricular muscle, the work loop method (Josephson, 1985) can be used. For example, Syme *et al.* (2013) studied the interactive effects of graded (acute) hypoxia and elevated temperature (10 vs. 20^{o}C) on the work and power output of cod ventricular myocardial strips. These authors showed that shortening power decreased across all PO_2 levels (94.5 to 7 kPa) at 10^{o}C, whereas there was no change in shortening power but a dramatic increase in lengthening power (i.e., the muscle became stiffer) when PO_2 fell below 21 kPa at 20^{o}C. These data suggest that the heart of hypoxic-acclimated fish may either not fully eject blood into the circulation due to decreased contractility (i.e., end-systolic volume is not zero) or that an increase in myocardial stiffness makes it difficult to fill the ventricle (i.e., end-diastolic volume is constrained). Indeed, the data of Petersen and Gamperl (2010b) support both possibilities. These authors showed that the input pressure required to maintain resting *in situ* cardiac output was consistently higher by ~0.2 – 0.3 kPa in hypoxia-acclimated cod, and that maximum cardiac function was reduced in this group independent of input pressure.

1.2.4. *Nitric Oxide and Hypoxia*

NO is a gasotransmitter that has important cardiovascular effects (both on the vasculature and the myocardium), and regulates tissue oxygen supply and demand during oxygen deprivation (Fago *et al.*, 2012; Hansen and Jensen, 2010). Under normoxic

conditions, NO is produced from L-arginine and oxygen using the enzyme nitric oxide synthase (NOS), which catalyzes the reaction:

$$2\ \text{L-Arg} + 3\ \text{NADPH} + 3\ H^+ + 4\ O_2 \leftrightarrow 2\ \text{citrulline} + 2\ \text{NO} + 4\ H_2O + 3\ NADP^+$$

Mammals have three NOS isoforms (neuronal, inducible and endothelial NOS; nNOS, iNOS and eNOS, respectively: Alderton *et al.*, 2001), whereas fish lack the eNOS isozyme (Donald and Broughton, 2005); although recent studies have found evidence of 'eNOS-like' activity (McNeill and Perry, 2006; Angelone *et al.*, 2012; Imbrogno *et al.* 2014). In the teleost heart, NO induces negative inotropy under basal (resting) conditions. For example, exogenous NO diminished the S_V of goldfish hearts, providing evidence for basal tonic regulation of cardiac activity, whereas NOS and sGC inhibitors [N^G-Methyl-L-arginine (L-NMMA) and 1H-[1,2,4]oxadiazole-[4,3-a]quinoxalin-1-one (ODQ)] increased S_V (Imbrogno *et al.* 2014). In contrast, NO enhances the Frank-Starling response under increasing preload (Imbrogno *et al.*, 2014); i.e., it can increase the hearts sensitivity to filling pressure and the maximum achievable S_V. This study also showed that acute hypoxia enhanced this sensitivity.

Under hypoxic conditions, NO production via NOS is greatly reduced (Tota *et al.*, 2005; Lundberg *et al.*, 2008; Fago and Jensen, 2015), but nitrite (NO_2^-) from the fish`s diet or other sources can be converted into NO using deoxygenated myoglobin or hemoglobin, xanthine oxidoreductase, or other proteins (Rassaf *et al.*, 2007; Lundberg *et al.*, 2008; Jensen, 2009; Zweier *et al.* 2010). Given that similar downstream effectors and targets,

such as soluble guanylate cyclase (sGC), cyclic GMP (cGMP) and protein kinase G (PKG), are used in either NOS-dependent or -independent NO regulation of cardiac function (Layland *et al.*, 2002; Imbrogno *et al.*, 2017; Imbrogno and Cerra, 2017, Gattuso *et al.*, 2018), this may allow NO to play a role in the modulation of cardiac contractility during periods of oxygen limitation. This hypothesis is supported by the work of Imbrogno *et al.* (2014), who showed that sGC, but not NOS, inhibition affected the goldfish heart's Frank-Starling response under conditions of acute hypoxia. However, it is not known whether the regulation of NO in the teleost myocardium is altered following acclimation to hypoxia.

1.2.5. *Goals of My Thesis*

The Gamperl lab previously used *in vivo* (Petersen and Gamperl 2010a; Petersen and Gamperl 2011; Moytka *et al.*, 2017) and *in situ* (Petersen and Gamperl 2010b) approaches to study cardiac function in hypoxia-acclimated cod and trout. In this thesis, I used an *in vitro* method to further examine factors that could be contributing to the diminished cardiac function reported in hypoxia-acclimated steelhead (rainbow) trout. The goals of the second chapter of my thesis were to: 1) investigate whether hypoxia-acclimation affects myocardial contractility and stiffness in steelhead trout, and whether this depends on contraction frequency; and 2) compare the acute hypoxia tolerance, and recovery of contractile performance, of the myocardium from normoxia- and hypoxia-acclimated fish at different frequencies. The third chapter of my thesis compared myocardial performance between these acclimation groups as well, but expanded on the previous study by: 1) measuring the effect of the NO donor SNP on ventricular contractile

performance in both acclimation groups at different muscle strains and contraction frequencies; 2) examining whether hypoxic acclimation affects the contribution of two different pathways of NO production to myocardial function by exposing ventricular trabeculae to NOS and cGMP inhibitors L-NMMA and ODQ, respectively; and 3) comparing the effect of hypoxia, and SNP, L-NMMA and ODQ, on twitch duration between acclimation groups at varying contraction frequencies. In these studies, the use of different strains was particularly important as NO has opposite effects on cardiac function at rest (i.e., low strain; negative inotropy) and elevated stroke volumes (high strain; positive inotropy) (Tota *et al.*, 2005; Angelone *et al.*, 2012; Imbrogno *et al.*, 2014).

In these experiments, contractile performance was measured using the work-loop method (Josephson, 1985; Syme and Josephson 1995; Harwood *et al.*, 1998; Shiels *et al.*, 1998; Harwood *et al.*, 2002; Syme *et al.*, 2013)(see Figure 1.1. for a description). This allowed for the direct measurement of contractile performance at varying levels of oxygen saturation while the muscle underwent cyclical changes in length with each contraction (as it does *in vivo*), and for an examination of how contraction frequency (and muscle strain; see Chapter 3) influence myocardial function and physiology. Collectively, these measurements allowed me to obtain an accurate image of *in vivo* trout cardiac function and power production before and after hypoxic acclimation. While mimicking diastolic filling and the systolic ejection of blood, the immediate environmental conditions (i.e., oxygen content and/or concentration of dissolved compounds) of the spongy ventricular muscle were then manipulated to compare contractile responses between acclimation groups. The spongy myocardium comprises ~60-70% of the trout heart's total mass (Graham and

Farrell, 1989; Santer and Greer Walker, 1980) and normally receives low oxygen (venous) blood, whereas the compact outer layer is supplied with well-oxygenated arterial blood. This makes the spongy tissue more relevant for studying the effects of oxygen limitation on myocardial function.

Steelhead trout (*Oncorhynchus mykiss*) were used in this study because of their relative intolerance to hypoxia (Pedersen *et al.*, 2010), and the background information we have on the response of their physiology to hypoxia (e.g. Faust *et al.*, 2004; Gamperl *et al.*, 2004; Moytka *et al.*, 2017). Due to their relative hypoxia intolerance, this species may display a greater decline in contractile function when exposed to hypoxic acclimation / acute exposure. This species has also been reported to encounter hypoxia in nature (Scott and Crossman, 1964; Luecke and Teuscher, 1994; Matthews and Berg, 1997), adding to the ecological relevance of this study. Furthermore, rainbow (steelhead) trout are an important contributor to the aquaculture industry, and are often housed in sea-cages or pens where overcrowding, rapid temperature fluctuations and prolonged hypoxia exposure are common (Ellis *et al.*, 2002; Liu *et al.*, 2016). The trout used in Chapters 2 and 3 were acquired from the same original stock to avoid intra-specific differences in hypoxia tolerance which exists between trout populations (e.g. see Faust *et al.* 2004 vs. Gamperl *et al.*, 2004).

This research expands greatly on the limited amount of research on the cardiac response of hypoxia-intolerant fish to chronic hypoxia, an expanding and intensifying environmental condition experienced by fish in both fresh and saltwater. The degree of

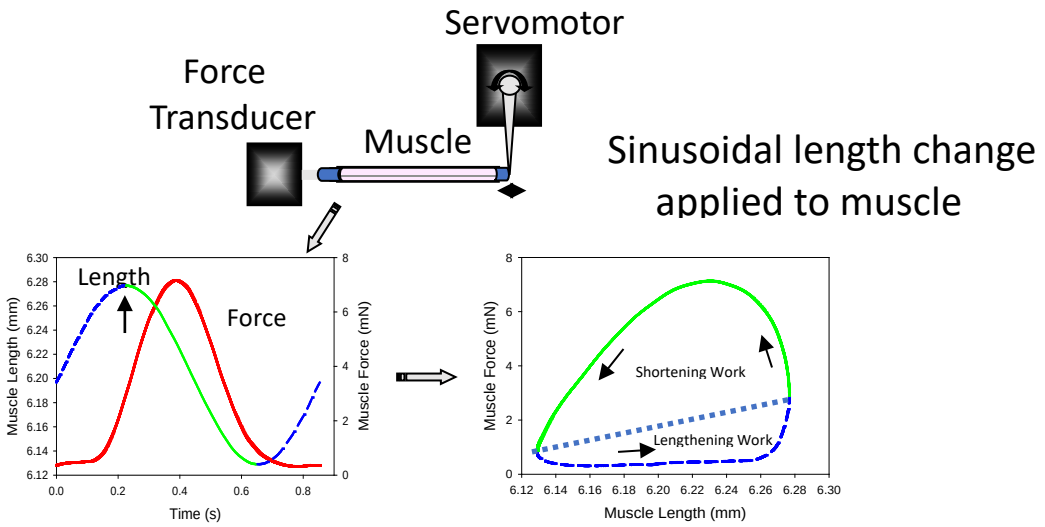

Figure 1.1. Schematic diagram showing the experimental set-up, the relationship between changes in muscle length and force development, and how myocardial work was determined. Muscle strips from the spongy myocardium were attached to the arm of a servomotor (300C-LR; Aurora Scientific, Aurora ON, Canada) on one end and to a force transducer (404A; Aurora Scientific) on the other. The servomotor was used to stretch the muscle to pre-determined lengths during each cycle (i.e., it determined the strain applied to the muscle; the ± % change from resting length), while the force transducer was used to measure the force produced by, and required to stretch, the myocardium. The relationship between muscle length (mm) and force (mN) over time is shown in the left panel. The servomotor must produce a certain amount of force to stretch the muscle to its maximum length. The resultant increase in muscle length is represented by the blue dotted line on both panels, and the force used to stretch the muscle is considered to be negative (i.e., lengthening) force. When the muscle has reached its maximum length, the stimulator (Isostim A320, World Precision Instruments, Sarasota, FL, USA) delivers an electric stimulus to contract the muscle (see black arrow on left panel), which shortens. The resultant change in length is represented by the green line, and the resultant force is positive (i.e., shortening force). The total force measured during the muscle cycle is represented by the solid red line in this graph, but is a sum of force developed by the muscle and that required by the servomotor to stretch it. To convert the force into work (in J), force measured by the servomotor (mN) is plotted against muscle length (mm) to produce a 'work loop'. The integral of the area of the 'work loop' represents the total work output of the muscle during a contraction cycle, where the area enclosed by the dotted blue line is 'lengthening work', and the area enclosed by the green line is 'shortening work', and the difference between the two is 'net work'. Power output (in W) is later calculated by multiplying work done per cycle by the contraction frequency (in Hz). Methods adopted from Josephson, 1985; Syme and Josephson 1995; Harwood *et al.*, 1998; Shiels *et al.*, 1998; Harwood *et al.*, 2002; Syme *et al.*, 2013.

plasticity in cardiac physiology and function is an important determinant of fish performance and survival, and may determine their ability to acclimate and/or adapt to hypoxic zones (Gamperl and Farrell, 2004; Gamperl, 2011). Therefore, a complete picture of the cardiac response of teleosts to reduced O_2 will be crucial in the near future. NO has a significant role in this response, due to its differing effects on cardiac function under basal function versus conditions of high S_V. This research presents the first measures of ventricular contractility in trout after hypoxic acclimation when exposed to exogenous NO, and NOS and sGC inhibitors. Currently, there are few to no studies which have explored the effect of exogenous NO, and blocking its production (L-NMMA) and signal transduction (ODQ), on twitch duration in fish cardiac muscle (especially after hypoxic acclimation), and this study compliments the mammalian literature in this area (Rouet-Benzineb *et al.*, 1999; Thompson *et al.*, 2009; Dungel *et al.*, 2017).

Co-Authorship Statement

The following statements clearly identify my contributions to the development, execution and preparation of this thesis:

1) Design and Identification of the Research Topics: The research ideas in this thesis were proposed by Dr. Gamperl and were modified after discussions with my committee members and Dr. Doug Syme (University of Calgary). Dr. Gamperl, Dr. Syme and I collaborated on the development of research methodologies, and Dr. Syme and Mr. Jordan Roberts trained me in nyocardial strip dissection / preparation and the use of the 'work loop' method for measurements of myocardial performance

2) Practical Aspects of the Research: All experiments were performed, and all raw data was collected, exclusively by me.

3) Data Analysis: Dr. Dave Schneider helped me to develop an appropriate statistical model, and I performed all statistical analyses using a combination of the R statistical package and SPSS.

4) Manuscript Preparation: I provided Dr. Gamperl with initial drafts of the sections of my thesis, and made subsequent edits based on the input of Drs. Gamperl and Dr. Syme.

Authorship for the future publication arising from **Chapter 2** is Christian Carnevale, Jordan C. Roberts, Devyn Ramsay, Douglas A. Syme, and A. Kurt Gamperl.

Authorship for the future publication arising from **Chapter 3** is Christian Carnevale, Douglas A. Syme, and A. Kurt Gamperl.

Chapter Two: Effects of Hypoxic-Acclimation on the Contractile Properties of Spongy Ventricular Myocardium Isolated from Steelhead Trout (*Oncorhynchus mykiss*)

2.1. Abstract

Cardiac stroke volume (S_V) is compromised in Atlantic cod and rainbow trout following acclimation to hypoxia (i.e., 40% air saturation; ~8 kPa) at 10-12^{o}C, and this is not due to changes in gross heart morphology or maximum achievable *in vitro* end-diastolic volume. To examine if this diminished S_V may be related to diminished myocardial contractility, we used the work loop method to measure work and power in strips of spongy myocardium from normoxia- and hypoxia-acclimated steelhead trout when exposed to decreasing PO_2 levels (21 to 1.5 kPa) at several frequencies (30 – 90 contractions min^{-1}) at 14^{o}C (their acclimation temperature). The work required to lengthen the muscle, as during filling of the heart, was strongly frequency dependent (i.e., increased with contraction rate), but was not affected by hypoxic acclimation or test PO_2. In contrast, while shortening work was less frequency dependent, this parameter and net work (and power): 1) were consistently lower (by ~30 – 50 and ~15%, respectively) in strips from hypoxia-acclimated fish; and 2) fell by approximately 40 – 50% in both groups from 20 to 1.5 kPa PO_2, despite the already reduced myocardial performance in the hypoxia-acclimated group. These results suggest that hypoxic acclimation reduces myocardial contractility, and in turn, may limit S_V (possibly by increasing end-systolic volume), but that this lowered performance does not improve the capacity to maintain myocardial function under oxygen limiting conditions. Interestingly, strips from these hypoxia-acclimated trout also showed a poorer recovery of net power when returned to normoxia. Both of these latter results are not consistent with data on 10^{o}C hypoxia-acclimated cod and suggest that a high degree of myocardial stunning was likely present in muscle strips from the hypoxia-acclimated trout

in this study, due to a more intense degree of hypoxic challenge (i.e., 14^{o}C; where water O_2 content is lower, but metabolic rate would be elevated). These data suggest that the effect of chronic hypoxia on myocardial performance may be highly dependent on the severity of this stressor.

2.2. Introduction

Hypoxia (low water oxygen levels) can occur naturally in aquatic environments due to biological oxygen demand, fluctuations in the tidal cycle, density stratification, changing wind patterns and the isolation of water bodies for prolonged periods of time (e.g., in tidepools) (Diaz and Rosenberg, 2008; Richards, 2011), and these events have become more frequent and severe in recent years due to accelerated climate change (Altieri and Gedan, 2015; Breitberg *et al.*, 2018). Consequently, research on the effects of acute and chronic environmental hypoxia is an important focus in the field of fish physiology. This research has demonstrated a number of impacts of hypoxia on fish behavior and physiology. Sculpins, which inhabit intertidal zones exposed to cyclical and frequent hypoxic episodes, display aerial surface respiration (ASR) and aerial emergence, along with a suite of physiological and biochemical adaptations (Richards, 2011). The epaulette shark (*Hemiscyllum ocellatum*) is able to depress its metabolism and increase ventilatory frequency in response to graded hypoxia (Nilsson and Renshaw, 2004). The freshwater Crucian carp (*Carassius carassius*), which has the ability to endure anoxia for a considerable amount of time (i.e., days), can maintain cardiac function and produce ethanol as an end-product of anaerobic metabolism instead of lactate to avoid self-acidosis (Stecyk

et al., 2004). Hypoxia intolerant fish such as the Atlantic cod (*Gadus morhua*) (Petersen and Gamperl, 2010a) and rainbow trout (*Oncorhynchus mykiss*) (Overgaard *et al.*, 2004a) also encounter hypoxic waters (Luecke and Teuscher, 1994; Matthews and Berg, 1997; Chabot and Claireaux, 2008). However, the effects of prolonged hypoxic exposure and hypoxic-acclimation on hypoxic-intolerant species such as these has only received limited attention (Hall *et al.*, 2009; Petersen and Gamperl, 2010a and b; Petersen and Gamperl, 2011; Anttila *et al.*, 2013; Cook *et al.*, 2013; Motyka *et al.*, 2016).

Recent studies on Atlantic cod and steelhead trout showed that exposure to chronic moderate hypoxia (> 6 weeks at 8.5 kPa O_2) greatly diminished the capacity of these fish to increase stroke volume (S_V), and thus, cardiac output (Q) when swum to exhaustion (Petersen and Gamperl, 2010a) or exposed to an acute incremental temperature increase until the fish's critical thermal maximum (Motyka *et al.*, 2017). Given that this reduced cardiac pumping capacity was also evident in *in situ* Atlantic cod hearts [i.e., eliminating the possibility that the decrease in S_V was largely due to alterations in neuronal or humoral regulation of cardiac function, afterload or venous return, Petersen and Gamperl, 2010b], and not related to changes in relative ventricular mass, % compact myocardium or maximum achievable *in vitro* stroke volume (Petersen and Gamperl, 2010b; Moytka *et al.*, 2017), other aspects related to cardiac function must be dysregulated following exposure to chronic hypoxia. Syme *et al.* (2013) investigated the interactive effects of graded (acute) hypoxia and elevated temperature (10 vs. 20^{o}C) on the work and power output of cod ventricular myocardial strips. These authors showed that shortening power decreased across all PO_2 levels (94.5 to 7 kPa) at 10^{o}C, whereas at 20^{o}C there was no change in

shortening power but a dramatic increase in lengthening power (i.e., the muscle became stiffer) when PO_2 fell below 21 kPa. These data suggest that the ventricular myocardium of hypoxic-acclimated fish can either not fully eject blood into the circulation due to decreased contractility (i.e., end-systolic volume rises above zero) or that an increase in myocardial stiffness makes it difficult to fill the ventricle (i.e., end-diastolic volume decreases). Indeed, this suggestion is supported by the results of Petersen and Gamperl (2010b) who showed that the input pressure required to maintain resting *in situ* cardiac output was consistently higher (by ~0.2 – 0.3 kPa) in hypoxia-acclimated cod, and that maximum cardiac function was reduced in this group independent of input pressure.

To examine the hypothesis that the diminished S_V of hypoxia-acclimated fish is associated with alterations in myocardial performance (i.e., shortening and/or lengthening work and power), we acclimated steelhead trout (seawater-acclimated rainbow trout) to a PO_2 of approximately 8.5 kPa or normoxia (21 kPa) for 8 – 12 weeks, and used cycling strips from the spongy myocardium to measure the muscle's contractile performance at PO_2 levels of 21, 13.5, 10.5, 6.9, 4.2 and 1.5 kPa across a range of contraction frequencies (30 – 90 contractions min^{-1}). The PO_2 values of 13.5 and 6.9 kPa approximate the PO_2 values in the arterial and venous blood, respectively, of normoxic fish (Thomas *et al.*, 1994), 1.5 kPa is the blood PO_2 which has been suggested as the lower limit at which trout cardiac function can be sustained (Steffensen and Farrell, 1998; Farrell and Clutterham, 2003), and 10.5 and 4.2 kPa provide intermediate (additional) values for the examination of the relationship between PO_2 and myocardial function in hypoxic-acclimated fish.

2.3. Methods

2.3.1. *Experimental Animals*

Only female steelhead trout (*Oncorhynchus mykiss*) were available from our commercial supplier for these studies. The trout (mean 0.61 ± 0.03 kg; range 0.32 to 0.82 kg) were initially housed for approximately two months at the Dr. Joe Brown Aquatic Research Building (Ocean Sciences Centre, Memorial University of Newfoundland, St. John's NL, Canada) in a $3m^3$ tank supplied with aerated, 10-11ºC, seawater. Photoperiod was maintained at 12h light: 12h dark and the trout were fed a commercial salmon diet three times a week at a ration of 1.5% of body mass. All procedures were approved by MUN's Institutional Animal Care Committee, and followed the guidelines of the Canadian Council on Animal Care.

2.3.2. *Holding and Acclimation Conditions*

After the initial holding period described above, eighty trout were divided equally between two ~1.2 m^3 square tanks equipped with wooden lids to control for external stimuli (noise, human presence, etc.), that were supplied with 13-14ºC seawater at 10 L min^{-1} which had a water oxygen partial pressure (P_WO_2) of 19 – 20 kPa (i.e., ~95% air saturation). After a two-week acclimation period, the water oxygen level in the tank that was to be made hypoxic was slowly lowered to the desired value (8.4 ± 0.1 kPa; 40% air saturation) over a period of 2 weeks. This was accomplished by: (1) slowly reducing the flow to the tank to 5 L min^{-1} (i.e., allowing fish metabolism to partially reduce the water O_2 saturation); and (2) using a custom-designed control system (Electronic Workshop, Memorial

University of Newfoundland). This system monitored the oxygen saturation in the tank continuously by pumping tank water through an external circuit of tubing (Tygon Food, 6-419, Cole Parmer) that contained a galvanic oxygen electrode (CellOx 325, WTW, Weilheim, Germany) placed in a D201 flow cell (WTW). The oxygen probe was connected to an oxygen meter (Oxi 340, WTW), which was interfaced with an electronic controller that set the open/closed position of two solenoid valves: one that bubbled pure N_2 into the tank when O_2 reached the upper limit of 9.0 kPa; the other bubbling air into the tank when oxygen saturation levels reached 8 kPa. This allowed for control of the oxygen levels in the hypoxic tank within a narrow O_2 range ($\pm$ 0.5 kPa). During the acclimation period, fish in both tanks were fed daily with commercial trout pellets at 1% body mass. However, if the hypoxic fish failed to eat their full ration (as determined by a number of pellets accumulating on the bottom of the tank), the normoxic fish were only given the same amount of food. Ammonia and nitrite levels were also checked periodically to ensure that these compounds did not exceed 0.02 and 0.5 mg L^{-1}, respectively.

2.3.3. *Isolated Myocardial Strips*

Fish from each treatment (n = 9) were randomly selected from their holding tank following 8-12 weeks of acclimation, swiftly killed by cerebral percussion, measured for length and body mass, and had their hearts removed. The ventricle was separated from the bulbus arteriosus and atrium, weighed, cut in half lengthwise and rinsed in ice-cold physiological saline for marine teleosts (Petersen and Gamperl, 2010), pH 7.6 at 20ºC. This saline contained (in gl^{-1}): 10.5 NaCl; 0.49 $MgSO_4 \cdot 7\ H_2O$; 0.37 KCl; 0.33 $CaCl_2 \cdot 2\ H_2O$;

0.14 $NaH_2PO_4 \cdot H2O$; 1.84 sodium TES base ($C_6H_{14}NO_6SNa$); 0.59 TES acid ($C_6H_{15}NO_6S$); 1.0 glucose. The ventricular halves were pinned to the bottom of a temperature-controlled (4ºC) dissecting dish containing saline, and a small segment of spongy trabecular muscle (approx. 5 mm in length x < 1 mm^2 in cross section) was isolated from the inner (luminal) surface of the ventricle under a dissecting microscope. Muscle segments (3.1 ± 2.3 mg wet mass and 4.9 ± 1.5 mm resting length) were selected so that the majority of fibers ran parallel to the long axis of the preparation, and there was minimal branching along their length. Once dissected free, a short piece of 7-0 silk suture was tied to each end of the strip and the strips were attached to the arm of a servomotor (300C-LR; Aurora Scientific, Aurora ON, Canada) on one end and a force transducer (404A; Aurora Scientific) on the other end using the methods as described in Syme *et al.* (2013). The muscle segments were bathed in physiological saline, and the temperature was maintained at 14 ± 0.2ºC using Peltier thermoelectric modules and a temperature controller (TC-24-12; TE Technology, Traverse City, MI). A custom program built using LabView software (National Instruments, Austin, TX) controlled a 12-bit analog/digital converter card (PCI MIO 16E 4; National instruments) that regulated the stimulator and servomotor (5 kHz D/A output), and collected muscle force, muscle length (servomotor arm position) and stimulus signals (1 kHz A/D input).

Saline PO_2 was maintained at specific levels by bubbling a mixture of O_2 and N_2 gas delivered from a Wöstoff gas mixing pump (DIGAMIX 6KM301, Bochum, Germany) into a reservoir of saline that was then drained via stainless-steel tubing into the muscle chamber, and the PO_2 in the muscle chamber was continuously monitored using a

calibrated, fiber optic dipping oxygen probe (PSt3; PreSens, Regensburg, Germany) and oxygen meter (Fibox 3; PreSens). Complete turnover of the saline in the 30 mL bath occurred approximately once per min. The PO_2 of the bath was sequentially set at 21 ± 0.5, 13.5 ± 0.5, 10.5 ± 0.5, 6.9 ± 0.5, 4.2 ± 0.5 and 1.5 ± 0.5 kPa during the experiment, with muscle strips allowed to equilibrate for 10-15 min. at each oxygen level. Recovery of muscle work and power was also assessed 10-15 min. after the saline in the bath was returned to 21 kPa (normoxic conditions).

2.3.4. *Measuring Muscle Work and Power*

Initially, the length of each preparation was increased systematically until developed twitch force, elicited by a 1-ms supra-maximal shock (~10 v), was maximal. Stimulus shocks were applied using a stimulator (Isostim A320, World Precision Instruments, Sarasota, FL, USA) and platinum plates placed on either side of the preparation. Muscle length was then decreased by 5% to reduce stress on the preparation and to better mimic the normal function of cardiac muscle which is on the ascending limb of the force-length relationship. Further, Harwood *et al.* (1998) showed no significant difference in work output between muscles operating at 95% vs. 100% of the length giving maximal force. This resting length (L_{opt}) was then used for the remainder of experiments. In a few instances where passive force rose steeply when strain was applied to the muscle during work loop experiments, suggesting that muscle length was too long, muscle length was decreased slightly to maximize net work output.

Muscle work and power were measured using the work-loop method (Josephson, 1985). To measure work, muscle length was oscillated about L_{opt} in a sinusoidal trajectory by the servomotor, and this mimicked muscle strain experienced during the cardiac cycle. The amplitude of strain (related to stroke volume) was set at ± 5% of L_{opt} as this has been shown to elicit maximum work output in spongy ventricular muscle preparations (Syme *et al.*, 2013); an observation confirmed in our study. The proportion of the sinusoidal strain cycle that comprised shortening vs. lengthening was adjusted at each contraction frequency. This ensured that measurement conditions reflected changes in the proportion of the cardiac cycle occupied by systole vs. diastole as heart rate changed: i.e., approx. 30% of the cycle period was muscle shortening (the remaining 70% being lengthening) at 30 contractions min^{-1}, it was 50% of shortening at 50 and 70 contractions min^{-1}, and was 80% of shortening at 90 contractions min^{-1}. The timing of stimulation of the muscle relative to the imposed length changes (i.e., stimulation phase) was also adjusted at each frequency used (30 – 90 contractions min^{-1}, see below). The optimal phase values varied between preparations and frequency, and ranged from -10% to 25% across the range of frequencies used. These adjustments ensured that the preparations achieved maximum net work output under all measurement conditions.

Work (in J) provides a measure of the mechanical energy produced during each beat of the heart, and was calculated as the integral of muscle force with respect to length change. The work done by the servomotor to lengthen the muscle in each cycle (lengthening work; analogous to filling work in an intact heart), the work done by the muscle when it contracts during each cycle (shortening work; analogous to stroke work),

and net work (the difference between shortening work and lengthening work) were all measured. Power (in W) provides a measure of the sustained rate of mechanical energy produced, and was calculated as the product of the work done per cycle (shortening, lengthening, or net) and contraction frequency (Hz). Mass-specific work (J kg^{-1}) and power (W kg^{-1}) were calculated for each muscle strip based on the mass of each preparation.

Contractile performance was measured at 4 contraction frequencies (30, 50, 70, 90 contractions min^{-1}) at each of the PO_2 values noted above, and again once the strips had been returned to 21 kPa PO_2 (normoxia). These frequencies span the physiological range of heart rates reported in trout at similar temperatures (0.5-1.5 Hz) (Priede, 1974), with 1.5 Hz (90 contractions min^{-1}) also being the maximum contraction frequency that the strips could be subjected to before they were unable to maintain consistent work output at every cycle; i.e., above this frequency full mechanical restitution could not be achieved. At every combination of frequency and PO_2, thirty isometric contractions were performed prior to the measurements of work output to ensure the preparation was stable, and then a series of 30 consecutive work loops were recorded. The order in which contraction frequency and strain were applied (used) were randomized between strips to limit the effect of repeated measures of muscle performance on the results. Further, pre-experimental trials (2-3) determined that there was no decrement in function when the 2 hour protocol was performed under normoxic conditions. This is consistent with Roberts and Syme (unpubl.) who performed similar studies, and several studies using *in situ* trout hearts (e.g. Faust *et al.*, 2004).

At the conclusion of each experiment, the muscle strips were removed from the bath and trimmed of any tissue past the silk sutures and obviously dead tissue, blotted on filter paper to remove surface moisture, and weighed on a microbalance (Mettler UMT2; Mettler-Toledo, Columbus, OH). Vital staining was not performed to more accurately determine the amount of viable tissue. Thus, muscle mass is likely an overestimate of viable tissue mass, and mass-specific work values are thus likely to be underestimates of muscle performance.

2.3.5. *Data and Statistical Analyses*

A 20-point median filter was applied to every record of muscle force and length before analyses to eliminate background noise, and checks were made to ensure that the filter did not distort the traces. Performance of the working preparations was measured as the average work done during the final 10 contractions of every series of 30 contractions. The number of replicates (*n*) reported for this experiment refers to the number of trabeculae (strips) tested, each originating from a different fish. Relative power was calculated by treating the initial power output values under fully oxygenated conditions (i.e., normoxia) as maximum output, and calculating the percent change in power output as PO_2 was decreased.

A split-split plot mixed general linear model was performed, using the R statistical package (version 3.22), to examine the effects of the three controlled variables (acclimation condition, contraction frequency, and saline PO_2), and one random variable (strip), on muscle performance parameters. Ventricular trabeculae were not controlled for length.

This contributed to variability between strips in work done, but this was accounted for in the main model by including strip as a random factor. These analyses were followed by Holm-Sidak pairwise-comparisons. When significant interactions were present, a two-way analysis of variance was performed, followed by Holm-Sidak pairwise-comparisons, using SPSS (version 11.0). Main factors of acclimation and PO_2 (at each frequency), or acclimation and frequency (at each PO_2 level) were used. Fish mass, ventricular mass, relative ventricular mass (RVM in % = ventricle mass/body mass x 100), fish length and condition factor (K = mass / length^3 x 100) were compared between acclimation groups using two-tailed t-tests. All statistical analyses were performed with the level of statistical significance set at $P<0.05$. Graphs and figures were created using GraphPad Prism 5 (GraphPad Software, La Jolla, CA), and all data in the text and figures are expressed as means ± 1 standard error of the mean (S.E.).

2.4. Results

2.4.1. *Cardiac Morphometrics, Fish Size and Mortality*

Acclimation to hypoxia did not significantly ($P > 0.05$) affect the trout's length, ventricular mass or condition factor, but had a significant effect on fish mass and relative ventricular mass (RVM) (Table 2.1.). Hypoxic fish weighed ~100g (20%) less than the normoxic fish, whereas RVM was ~0.021% (27%) higher in hypoxic fish (Table 2.1.). Approximately 30% of the hypoxic-acclimated fish did not survive the first 3-4 weeks of exposure to water of 40% air saturation, whereas > 95% of the normoxic-acclimated group survived this period.

2.4.2. *Effects of Acclimation, Contraction Frequency and PO_2 on Myocardial Performance*

There were numerous acclimation, frequency and PO_2 effects, as well as interactions between these fixed factors (Table 2.2.). Thus, two-way ANOVAs were performed, where interactions were present, to identify significant differences between the acclimation groups as affected by PO_2 at every frequency, and at each frequency across PO_2 levels.

2.4.2.1. Mass-Specific Work

At a saline PO_2 of ~21 kPa (normoxia), the shortening work of strips from hypoxic-acclimated fish was comparable to that measured in the normoxic-acclimated group at the lowest frequency (30 contractions min^{-1}) (Fig. 2.1. A and B). However, there was a distinct negative effect of frequency on shortening work in the hypoxic-, but not normoxic-acclimated group at 21 kPa, so that at 90 contractions min^{-1} shortening work in the hypoxic group was only 73% of that at 30 min^{-1}, and only ~60% of that measured in the normoxic-acclimated group. Shortening work declined with decreasing PO_2 in both acclimation groups (Fig. 2.1 A and B), but interestingly, the fall in shortening work between 21 and 13.5 kPa was inversely related to contraction frequency in the hypoxia-acclimated group. This resulted in shortening work being: 1) the same at all contraction frequencies at 13.5 kPa; and 2) ~35% lower than measured in strips from normoxic-acclimated individuals. From 13.5 to 1.5 kPa, there was no effect of frequency on shortening work, and the depressive effect of PO_2 on shortening work was similar between acclimation groups.

Neither acclimation condition (normoxia vs. hypoxia), or PO_2, had a significant effect on lengthening work. However, there was a distinct frequency effect ($P < 0.05$) (Fig. 2.1. C and D). The work done by the servomotor to lengthen the muscle (i.e., negative work) increased with contraction frequency and was approximately 150% higher at 90 contractions min^{-1} vs. 30 contractions min^{-1} in both groups.

Due to the additive effects of shortening work and lengthening work on net work, and the number of interactions between parameters (see Table 2.2.), interpretation of the effects of acclimation, PO_2 and frequency on net work was difficult. Nonetheless, there are a number of apparent trends in the data. First, the effect of frequency on net work was qualitatively similar in strips from normoxic- and hypoxic-acclimated fish. Net work decreased by approximately 30 - 60% as contraction frequency increased from 30 to 90 contractions min^{-1}. Second, exposure to acute reductions in PO_2 had comparable effects on net work in both groups, where a decrease from 21 to 1.5 kPa resulted in a ~40% decrease in net work. Finally, strips from hypoxic-acclimated fish produced approximately 30 - 40% less mass-specific net work when compared to those from normoxic-acclimated fish at similar PO_2's and frequencies.

2.4.2.2. *Mass-Specific Power*

As expected, power was highly frequency dependent (Fig. 2.2.). This resulted in a positive (not negative, as seen for work) relationship between contraction frequency and shortening power in both acclimation groups (Fig. 2.2. A and B). Strips from hypoxic-acclimated fish produced, on average, approximately 30% less shortening power than

Table 2.1. Body and cardiac morphometrics for steelhead trout (n = 9 for each acclimation state) acclimated to either normoxia (water oxygen partial pressure of ~20 kPa) or hypoxia (8-9 kPa) for 8-12 weeks. Values are means ± SE and * indicates significant difference between acclimation groups ($P<0.05$).

	Animal Mass (g)	**Fork Length (cm)**	**Condition factor (K)**	**Ventricular Mass (g)**	**Relative Ventricular Mass (RVM %)**
Normoxia	678.6±34.5	35.2±0.7	1.55±0.04	0.53±0.04	0.079±0.003
Hypoxia	548.9±45.7*	33.2±0.9	1.48±0.07	0.54±0.04	0.100±0.006*

Table 2.2. Results of the split-split plot, mixed general linear model (Method 1) analysis that was used to examine the effects of acclimation group, contraction frequency and PO_2 on mass-specific shortening (s), lengthening (l) and net (n) work and power, as well as relative (Rel) shortening, lengthening and net power. Results of the split plot, mixed, general linear model (Method 2) analysis that examined the effects of acclimation group and frequency on the recovery (Rec) of shortening, lengthening and net power are also included.

Parameter	Method	Factor(s)	F	d.f.	P
sWork	1	Acclimation	2.987	1	0.103
		Freq.	7.636	3	$5.83x10^{-5}$
		PO_2	105.094	5	$<2x10^{-16}$
		Acclimation*Freq.	1.292	3	0.276
		Acclimation* PO_2	3.311	5	0.006
		Freq* PO_2	1.203	15	0.267
		Acclimation*Freq.* PO_2	0.947	15	0.512
lWork	1	Acclimation	0.00249	1	0.921
		Freq.	45.602	3	$<2x10^{-16}$
		PO_2	2.501	5	0.030
		Acclimation*Freq.	1.333	3	0.263
		Acclimation* PO_2	0.708	5	0.618
		Freq* PO_2	0.185	15	0.999
		Acclimation*Freq.* PO_2	0.082	15	1
nWork	1	Acclimation	3.46462	1	0.081
		Freq.	86.991	3	$<2x10^{-16}$
		PO_2	87.929	5	$<2x10^{-16}$
		Acclimation*Freq.	4.831	3	0.0026
		Acclimation* PO_2	5.092	5	0.00016
		Freq* PO_2	1.73	15	0.043
		Acclimation*Freq.* PO_2	0.57	15	0.897
sPower	1	Acclimation	2.7207	1	0.118
		Freq.	357.164	3	$<2x10^{-16}$
		PO_2	33.651	5	$<2x10^{-16}$
		Acclimation*Freq.	12.77	3	$6.05x10^{-8}$
		Acclimation* PO_2	1.862	5	0.1
		Freq* PO_2	0.454	15	0.961
		Acclimation*Freq.* PO_2	0.306	15	0.995
lPower	1	Acclimation	0	1	0.985
		Freq.	110.755	3	$<2x10^{-16}$
		PO_2	0.609	5	0.693
		Acclimation*Freq.	0.167	3	0.919
		Acclimation* PO_2	0.08	5	0.995
		Freq* PO_2	0.094	15	1
		Acclimation*Freq.* PO_2	0.018	15	1
nPower	1	Acclimation	3.3964	1	0.083
		Freq.	72.914	3	$<2x10^{-16}$
		PO_2	43.662	6	$<2x10^{-16}$
		Acclimation*Freq.	14.923	3	$3.02x10^{-9}$
		Acclimation* PO_2	3.41	6	0.003
		Freq* PO_2	0.437	18	0.979
		Acclimation*Freq.* PO_2	0.419	18	0.983

RelsPower	1	Acclimation	0.00409	1	0.842
		Freq.	36.092	3	$<2x10^{-16}$
		PO_2	118.977	5	$<2x10^{-16}$
		Acclimation*Freq.	9.033	3	$9.87x10^{-6}$
		Acclimation* PO_2	0.342	5	0.887
		Freq* PO_2	1.484	15	0.109
		Acclimation*Freq.* PO_2	0.556	15	0.907
RellPower	1	Acclimation	0.2875	1	0.599
		Freq.	0.045	3	0.987
		PO_2	5.823	5	$3.52x10^{-5}$
		Acclimation*Freq.	3.883	3	0.0094
		Acclimation* PO_2	0.647	5	0.664
		Freq* PO_2	0.671	15	0.813
		Acclimation*Freq.* PO_2	0.282	15	0.996
RelnPower	1	Acclimation	0.6273	1	0.439
		Freq.	7.102	3	0.0001
		PO_2	136.856	6	$<2x10^{-16}$
		Acclimation*Freq.	0.291	3	0.831
		Acclimation* PO_2	1.398	6	0.224
		Freq* PO_2	0.517	18	0.93
		Acclimation*Freq.* PO_2	0.144	18	0.999
sPowerRec	2	Acclimation	0.643	1	0.426
		Freq.	1.55	3	0.211
		Acclimation*Freq.	0.295	3	0.62
lPowerRec	2	Acclimation	0.132	1	0.718
		Freq.	0.199	3	0.897
		Acclimation*Freq.	0.295	3	0.829
nPowerRec	2	Acclimation	6.84	1	0.011
		Freq.	0.65	3	0.586
		Acclimation*Freq.	0.096	3	0.962

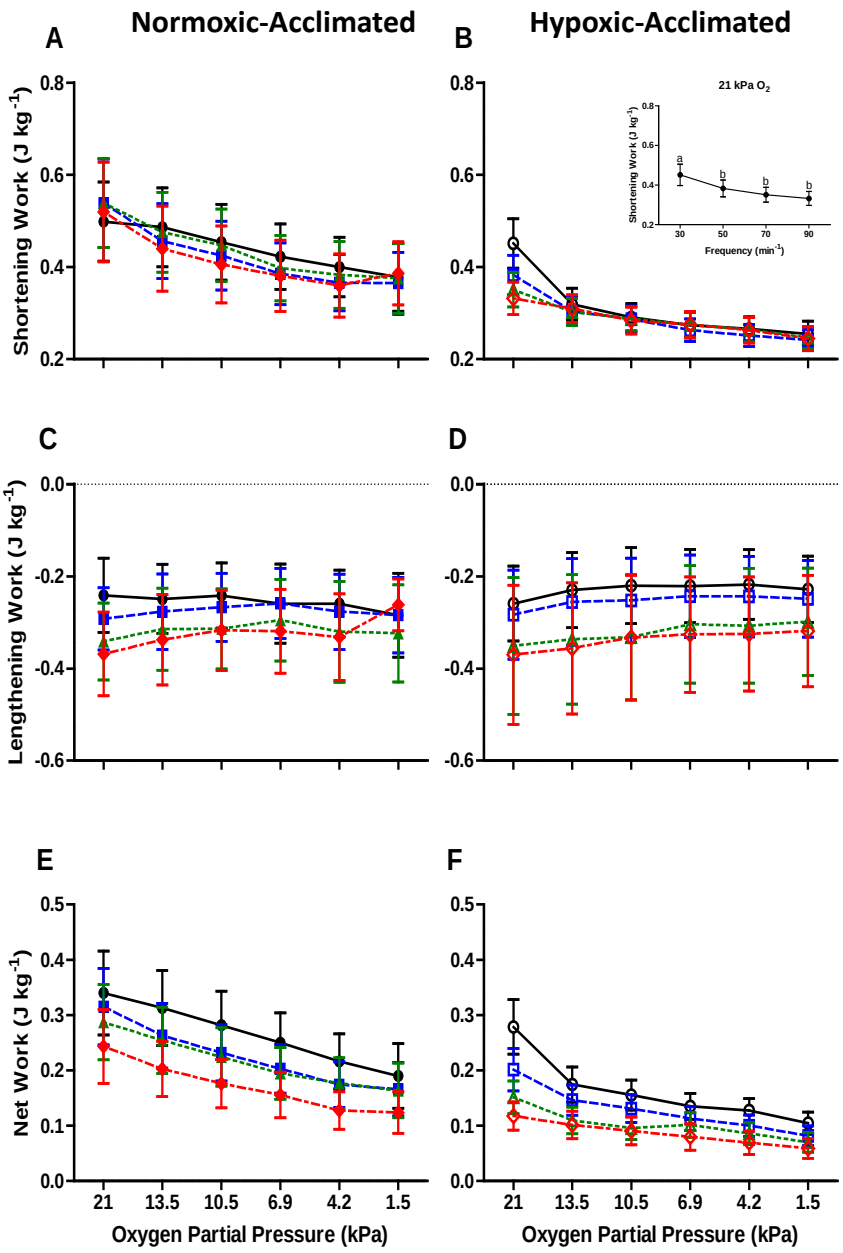

Figure 2.1. Shortening (A and B), lengthening (C and D) and net work (E and F) done by ventricular trabeculae from normoxic-acclimated (solid symbols; left panels) and hypoxic-acclimated (open symbols; right panels) steelhead trout when exposed to declining O_2 levels and four different contraction frequencies at 14ºC. Lengthening work is shown as a negative value to reflect the work that was done by the servomotor to stretch the muscle. The trabeculae were cycled at 30 (black ● and ○,—), 50 (blue ■ and □, ---), 70 (green ▲ and △, ···) and 90 (red ◆ and ◇,-·-·-) contractions min^{-1}. Values are means ± SE; n = 9. Insert shows the shortening work done by ventricular trabeculae from hypoxic-acclimated steelhead trout at 21 kPa O_2 at the four contraction frequencies. A one-way repeated measures ANOVA followed by Tukey's post-hoc tests showed that shortening work at 90 min^{-1} was significantly higher than at 30 min^{-1} at this PO_2.

normoxic-acclimated strips. In both acclimation groups there was an approximately 25% decrease in shortening power over the range of PO_2's studied. Acclimation condition and PO_2 had no significant effect on lengthening power (Fig. 2.2. C and D), but the negative relationship between frequency and lengthening power was even more pronounced in comparison to lengthening work (see Fig. 2.2. C and D). Net power increased with contraction frequency in both acclimation groups, but to a much greater extent in strips from normoxic-acclimated fish (Fig. 2.2. E and F). For example, net power increased by ~220% from 30 to 90 contractions min^{-1} in strips from normoxia-acclimated fish, whereas, this parameter only increased ~120% over this frequency range in strips from hypoxic-acclimated trout (Fig. 2.2.). The depressive effect of decreasing PO_2 on net power was relatively similar between the two acclimation groups (Fig. 2.2. E and F).

2.4.2.3. *Relative Myocardial Power*

To further emphasize some of the relationships between contractile performance, PO_2 and contraction frequency, and to remove variability in the results due to errors in estimating the viable mass of the preparations, relative power was also calculated (Fig. 2.3.). Relative shortening power declined by ~30% over the range of PO_2's studied at every frequency in the normoxic acclimated muscles. However, in the hypoxic-acclimated group, the reduction in shortening power as PO_2 was lowered was frequency dependent; decreasing by ~35% at 30 contractions min^{-1} but only by 15% at 90 contractions min^{-1}. This difference suggests that strips from hypoxic-acclimated fish were better able to preserve myocardial performance in the face of decreasing oxygen levels when stimulated at high frequencies. In both acclimation groups there was no significant effect of frequency on relative lengthening power (Fig. 2.3. C and D). Similarly, there was no effect of acclimation condition or frequency on relative net power, although power decreased by 40-50% in both groups as oxygen levels fell (Fig. 2.3. E and F).

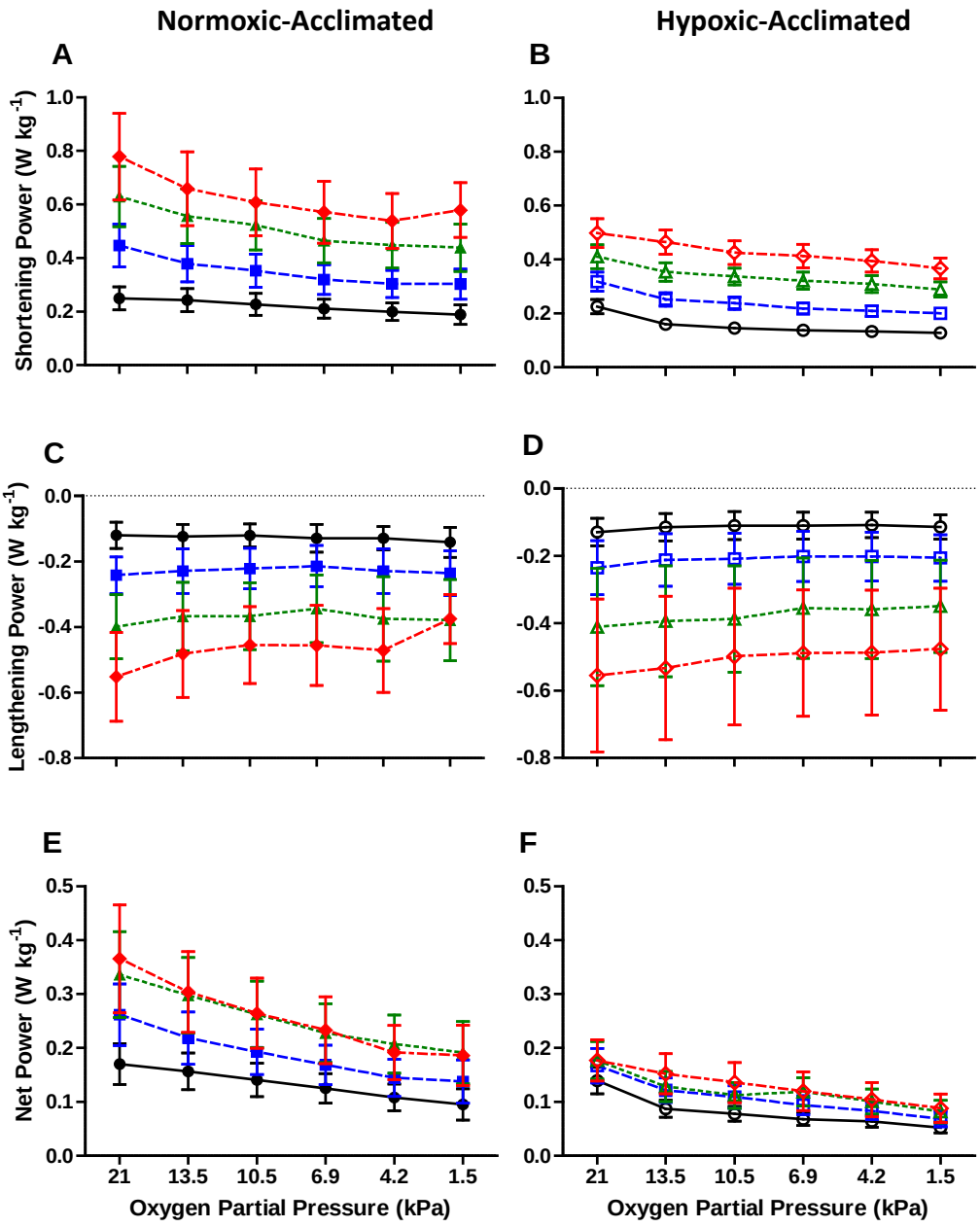

Figure 2.2. Shortening (A and B), lengthening (C and D) and net power (E and F) done by ventricular trabeculae from normoxic-acclimated (solid symbols; left panels) and hypoxic-acclimated (open symbols; right panels) steelhead trout when exposed to declining O_2 levels and four different contraction frequencies at 14ºC. Lengthening power is shown as a negative value to reflect the power that was done by the servomotor to stretch the muscle. The trabeculae were cycled at 30 (black ● and ○,—), 50 (blue ■ and □, - - -), 70 (green ▲ and △, ···) and 90 (red ◆ and ◇,– – –) contractions min^{-1}. Values are means ± SE; *n* = 9.

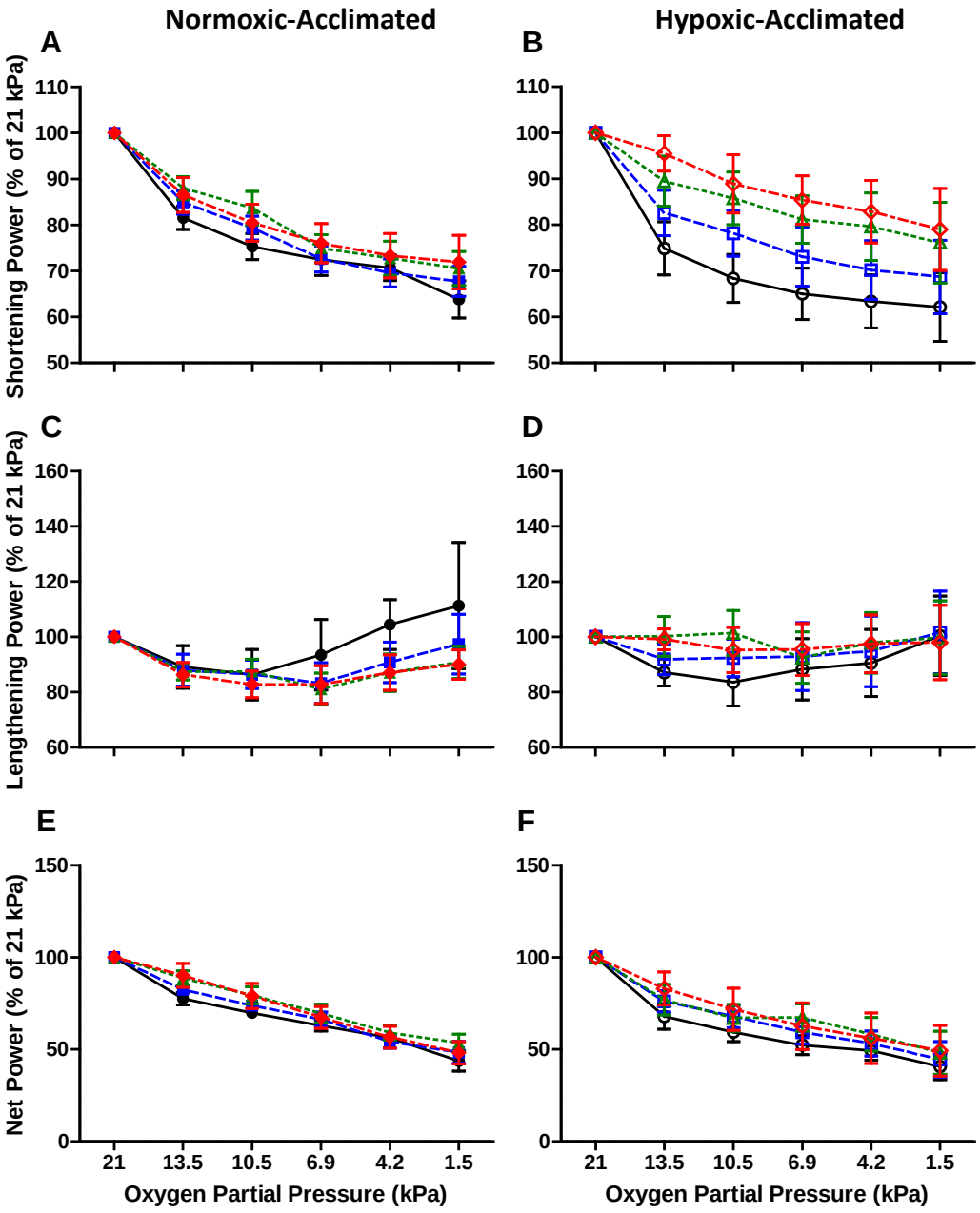

Figure 2.3. Relative shortening (A and B), lengthening (C and D) and net power (E and F) done by ventricular trabeculae from normoxic-acclimated (filled in symbols; left panels) and hypoxic-acclimated (open symbols; right panels) steelhead trout when exposed to declining oxygen levels and four different contraction frequencies at 14ºC. Values of power are expressed relative to the values initially obtained at 21 kPa PO_2 for each preparation. Lengthening power is shown as a negative value to reflect the power that was used by the servomotor to stretch the muscle. The trabeculae were cycled at 30 (black ● and ○,—), 50 (blue ■ and □, ---), 70 (green ▲ and △, ···) and 90 (red ◆ and ◇,-·-·) contractions min^{-1}. Values are expressed as % change relative to the power output measured at normoxia for each strip. Values are means ± SE; n = 9.

2.4.3. *Effects of Acclimation Condition and Contraction Frequency on Recovery*

The recovery of shortening, lengthening and net power upon the return to a saline PO_2 of 21 kPa was compared between the acclimation groups at the four contraction frequencies (Fig. 2.4.). Neither frequency nor acclimation had a significant effect on the recovery of lengthening or shortening power ($P > 0.05$) (Table 2.2., Fig. 2.4.). In contrast, the recovery of net power was consistently ~20% lower in trabeculae from hypoxic-acclimated fish ($P<0.05$), and this difference reached statistical significance at 30 contractions min^{-1} (Table 2.2., Fig. 2.4.).

2.5. Discussion

2.5.1. *Effects of Chronic Hypoxia on Fish Size and Cardiac Morphometrics*

Acclimation to chronic hypoxia had no effect on fish mass, length, condition factor, ventricular mass or RVM in prior studies on Atlantic cod (mixed sex, > 6 weeks at 8.5 kPa O_2 at 10^oC; Petersen and Gamperl, 2010; 2011) and rainbow trout (sexually immature female trout, > 8 weeks at 8.5 kPa O_2 at 10^oC; Moytka *et al.*, 2017) in our lab. However, in the current study hypoxic-acclimated steelhead trout weighed significantly less and their RVM was significantly greater (Table 2.1.). It is not known why hypoxia-acclimated fish from this study did not grow as fast as normoxic-acclimated individuals, despite our attempt to provide equal amounts of food to both acclimation groups. Hypoxia has been shown in numerous studies to decrease appetite and growth rate (Chabot and Dutil 1999; Chabot and Claireaux, 2008; Yang *et al.*, 2013), and a previous study on rainbow trout acclimated to hypoxia (10.5 kPa for 28 days) reported ~50% reductions in food intake and growth rate as compared to normoxic individuals when fed to satiety (Glencross, 2009). The one difference between this study and Petersen and Gamperl (2010a; 2010b; 2011) and Motyka *et al.* (2017) was the temperature at which the fish were held (14 vs.

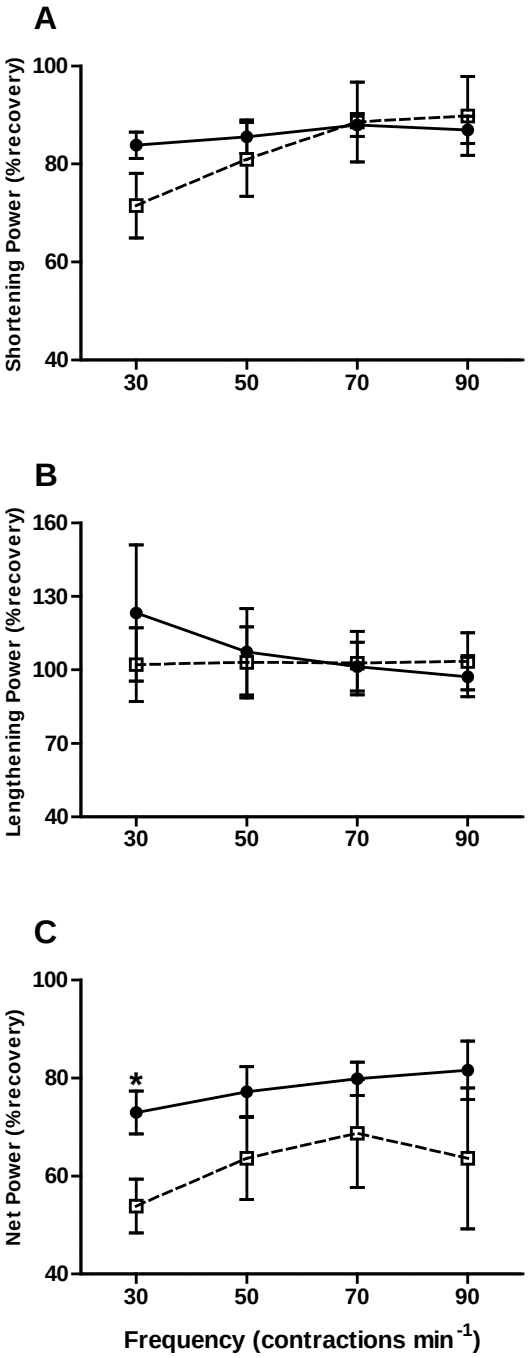

Figure 2.4. Recovery of shortening (A), lengthening (B) and net power (C) in myocardial trabeculae from normoxic-acclimated (●, solid lines) and hypoxic-acclimated (□, dotted lines) steelhead trout at 14ºC and four contraction frequencies. Values are expressed as power measured at 21 kPa PO_2 after exposure to reduced levels of oxygen relative to the power output measured initially at 21 kPa. *Significant difference ($P < 0.05$) between normoxic- and hypoxic-acclimated groups at a particular frequency. Values are means ± SE; $n = 9$.

10°C respectively), and it is possible that the higher temperature used in this study contributed to the lower growth rate reported. Basal metabolic rate increases, and water oxygen content decreases, at higher temperatures, and so the hypoxic challenge was actually more severe in the current experiment. Both of these effects could have significantly constrained the trout's aerobic scope, and this parameter has a large influence on the growth rate of fish (Claireaux *et al.*, 2000). Further, it is very likely that this temperature difference explains the higher RVM in hypoxia-acclimated fish in this experiment. Ventricle size has been linked to cardiac work in prior studies on rainbow trout (Houlihan *et al.*, 1988; Farrell *et al.*, 1990), and the temperature-dependent increase in cardiac workload associated with rearing at 14°C (i.e., increased metabolic demand and decreased oxygen availability) might have required the ventricle to be larger relative to body mass to maintain oxygen delivery under hypoxic conditions. Indeed, the data of Anttila *et al.* (2015) suggest that there is an interactive effect of temperature and hypoxia on cardiac morphology. These authors exposed Atlantic salmon (*Salmo salar*) and Arctic char (*Salvelinus alpinus*) to daily, overnight bouts of hypoxia (65% oxygen saturation) for 4 weeks at 14.9 vs. 7.7°C, and showed that although RVM was not different at either temperature, the percentage of compact myocardium was significantly higher in hypoxia- vs normoxia-acclimated fish at 14.9 but not 7°C. Alternatively, it is conceivable that the heart is protected during periods of impaired growth associated with reduced food intake. Gamperl *et al.* (unpublished) food deprived Atlantic cod for 8 – 10 weeks at 10°C, and showed that while the fish weighed significantly less, they had a greater RVM and maintained similar amounts of ventricular protein as compared to fed cod.

2.5.2. *Frequency Dependence of Myocardial Performance*

The net work and power produced by the ventricular trabeculae of normoxic-acclimated trout working in normoxic saline (21 kPa PO_2) averaged 0.300 J kg^{-1} and 0.280 W kg^{-1}, respectively, across the four contraction frequencies employed (Figs. 2.1. E and 2.2. E). While these values fall within the range reported for teleosts (Harwood *et al.*, 1998; 2002; Shiels *et al.*, 1998; Syme *et al.*, 2013), they are considerably higher than values reported in two of those studies: trabeculae from the lumen of the ventricle of rainbow trout produced 0.13 J kg^{-1} and 0.12 W kg^{-1} at 12^{o}C (Shiels *et al.*, 1998) and trabeculae from the spongy myocardium of Atlantic cod produced 0.15 J kg^{-1} and 0.09 W kg^{-1} at 10^{o}C (Syme *et al.*, 2013). Our protocol was specifically designed to maximize work and power (including the adjustment of the proportion of the strain cycle that comprised muscle shortening and lengthening), and was conducted at warmer temperatures, and these differences may explain the higher values reported in our study. In contrast, our values for work and power were less than those reported by Harwood *et al.* (1998, 2002) (0.9-1.4 J kg^{-1} and 0.8-1.2 W kg^{-1} at 15^{o}C). This may be due to differences in myocardial performance between ventricular tissue layers. Based on the description of where their myocardial strips were dissected from (ventral apex of the heart), it is probable that the preparations in those studies were from the compact layer vs. the spongy layer in the present study. The compact layer has a higher content of contractile proteins, and this might contribute to higher work vs. the spongy myocardium (Poupa *et al.*, 1981).

Contractile force (and thus work) was expected to decrease substantially with increasing contraction frequency due to the well-known negative force-frequency relationship shown for isometrically contracting muscles (Driedzic and Gesser, 1985, 1988; Hove-Madsen, 1992; Keen *et al.*, 1994; Shiels and Farrell, 1997; Mercier *et al.*, 2002) and cycling muscle strips (Harwood *et*

al., 1998; 2002). However, a strong negative relationship between shortening work and contraction frequency was only evident in strips from hypoxic-acclimated trout at a test PO_2 of 21 kPa (normoxia)(see below), and not at any PO_2 in normoxic-acclimated trout. This is an interesting observation, which is consistent with previous studies that have suggested that work production in cycling fish cardiac muscle may not mirror isometric relationships (Harwood *et al.*, 1998; Shiels *et al.*, 1998; Syme *et al.*, 2013). In addition, the sine wave was distorted in the current study to account for changes in the duration of systole and diastole as heart rate increased, and this could have affected this relationship as well.

Shortening power increased almost 4-fold in strips from normoxic-acclimated trout when frequency was increased from 30 to 90 contractions min^{-1}, whereas net power only doubled across the same frequency range and did not change between 70 and 90 contractions min^{-1} (Fig. 2.2. E). The latter results are consistent with Shiels *et al.* (1998) and Harwood *et al.* (2002) for rainbow trout, where net power of ventricular strips tested at 12 – 15^{o}C peaked at approximately 60 contractions min^{-1}. The difference in the effect of frequency on shortening vs. net power, and the apparent limit in net power at higher frequencies, is explained by the fact that lengthening work and power also increased with contraction frequency (Figs. 2.1. and 2.2. C). This latter effect would have offset the positive effect of frequency on shortening power. The increase in lengthening power with frequency is in agreement with observations for ventricular muscle from trout (Harwood *et al.*, 2002) and cod (Syme *et al.*, 2013), and likely reflects a failure to achieve complete mechanical restitution (relaxation) between beats at high heart rates (Harwood *et al.*, 1998).

2.5.3. *Impact of Acute and Chronic Hypoxia on Myocardial Work and Power Output*

2.5.3.1. Reduced Contractility Under Normoxic Conditions

When tested at 21 kPa PO_2 (normoxic conditions), net work and power were 30 – 50% and ~15% lower, respectively, in ventricular trabeculae from hypoxic-acclimated trout as compared to those from normoxic-acclimated conspecifics (Figs. 2.1. and 2.2. E vs. F). This effect was not due to increased stiffness of the myocardium, as lengthening work was similar between the two acclimation groups, but to a decrease in shortening work (Fig. 2.1. C vs. D). As such, this study sheds light on the possible mechanism(s) underlying the reduction in S_V and Q reported in hypoxic-acclimated rainbow trout (Moytka *et al.*, 2017) and Atlantic cod hearts (Petersen and Gamperl, 2010a; 2010b; Petersen and Gamperl, 2011). There are at least three potential explanations for the poor shortening work and power output of the ventricular trabeculae from hypoxic-acclimated fish when cycled under normoxic conditions. First, these experiments lacked any tonic adrenergic stimulation and hypoxia-acclimated hearts may have been more dependent on catecholamines to achieve maximum force production. Adrenaline is known to produce positive inotropy and chronotropy in the heart (Farrell, 1986; Gamperl *et al.*, 1994b; Shiels and Farrell, 1997) and a change in the number or specific binding of cardiac β-adrenergic receptors (β_1, β_2 or β_3) may have occurred with chronic hypoxia in this experiment. Moytka *et al.* (2017) reported increased numbers of β_2-adrenoreceptors with low binding affinity, in combination with a reduced number of β_3-adrenoreceptors, in the hearts of steelhead trout following hypoxic acclimation (12 weeks at ~8 kPa and 10^{o}C) when compared with normoxic fish. Adrenergic stimulation was omitted from these experiments so that direct comparisons could be made with Chapter 3 (Carnevale *et al.*, submitted).

Second, it is possible that our hypoxic acclimation conditions were severe enough to induce myocardial-remodelling and/or myocardial necrosis. Mitochondrial function is often depressed in

fish experiencing chronic hypoxia and/or upon recovery (Forgan and Forster, 2010) due to a myriad of factors, including: the reversible inhibition of electron transport chain complexes by nitric oxide which reduces the rate of oxygen consumption; and an enhancement of reactive oxygen species (ROS) production during reoxygenation, and consequent mitochondrial leak, due to the incomplete reduction of O_2 during NO production, (Solien *et al.*, 2005; Dedkova and Blatter, 2009; Zaobornyj and Ghafourifar, 2012). It is possible, that to reduce oxygen diffusion distances and enhance oxidative respiration, mitochondrial volume may have increased in the cardiomyocytes of fish acclimated to hypoxia, and that this resulted in fewer myofibrils per volume of myocardium (Farrell *et al.*, 2011). A reduction in the volume of myofibrils would lead to a loss of force development and mass-specific work. However, a reduction (not increase) in myocardial mitochondrial volume has been reported in hypoxia acclimated rats (Costa *et al.* 1988, Nouette-Gaulain *et al.*, 2005) and flounder (*Platichthys flesus*, Lennard and Huddart, 1992) due to an increase in the number of smaller mitochondria, and evidence for increased muscle mitochondrial volume following acclimation to hypoxia is lacking in studies on other fish species (Johnston and Bernard, 1982; Johnston *et al.*, 1983; Cook *et al.*, 2013; Moytka *et al.*, 2017). Further, while 3 weeks of severe hypoxia (5 kPa O_2 at 21^{o}C) did result in myocardial necrosis in flounder (Lennard and Huddart, 1992), no changes in myocardial ultrastructure were evident in snapper exposed to a 6 week period of moderate hypoxia (PO_2, 10.2-12.1 kPa at 21^{o}C) (Cook et al., 2013). While we cannot exclude the possibility that myocardial necrosis did occur in the hypoxia-acclimated fish in this experiment, the results of Cook *et al.* (2013), and the finding that there was no myocardial damage or limitations in myocardial energetic or enzymatic function after exposure of trout hearts to acute severe hypoxia (<30 min, perfusate $PO_2 \leq 1$ kPa) (Faust *et al.*, 2004; Overgaard *et al.*, 2004a; Overgaard *et al.*, 2004b), suggest that it is unlikely.

Third, the ventricular trabeculae of hypoxic-acclimated trout may have been 'stunned.' Mechanical dysfunction that persists after reoxygenation and/or reperfusion, termed 'myocardial stunning,' can occur without the presence of irreversible damage to the myofibrils (Bolli and Marbán 1999), and has been reported in several studies on acute hypoxic effects on cardiac function in trout (Gamperl *et al.*, 2001; Faust *et al.*, 2004; Overgaard *et al.*, 2004a; 2004b). Although the mechanisms mediating myocardial stunning in fish have not been directly studied, research on mammals suggests that two factors are largely responsible for the loss of myocardial function following periods of oxygen deprivation / the loss of coronary blood flow. These are the production of ROS and calcium overload (Bolli and Marbán, 1999). With regards to ROS production post-ischaemia / hypoxia, there are several mechanisms through which myocardial stunning can occur, such as protein denaturation, sarcoplasmic reticulum dysfunction, and interference of ion transport. However, the most plausible explanation is damage to the contractile proteins (such as troponin I and α-actinin). Oxidation of the thiol groups of contractile proteins often results in a reduction in the myofilaments' responsiveness to calcium (Bolli and Marbán, 1999). Calcium overload has also been reported to be a component of myocardial stunning in mammals (Bolli and Marbán 1999); this increase in Ca^{2+} levels caused by a rise in intracellular Na^{+} during ischemia that results in enhanced Na^{+}-Ca^{2+} exchange during reperfusion, and the activation of phospholipases and other degradative enzymes (Bolli and Marbán 1999). Nonetheless, Ca^{2+} overload is unlikely to be the cause of the diminished force (work) production in myocardial strips from hypoxia acclimated trout. There was no increase in muscle stiffness (lengthening work) in strips from hypoxic-acclimated trout under normoxic or hypoxic conditions which would be expected if there was an increase in intracellular Ca^{2+} levels.

In this study, net work was 30-50% lower for the myocardial strips from hypoxic-acclimated trout as compared to the 28% decrease in *in situ* maximum stroke volume in hearts from hypoxic- vs. normoxic-acclimated Atlantic cod when tested under oxygenated conditions at 10^{o}C (Petersen and Gamperl, 2010b). As discussed before, this heightened level of myocardial dysfunction (stunning) was likely due to the more severe hypoxic conditions used in this experiment.

2.5.3.2. *Myocardial Performance During Acute Hypoxia*

When bath PO_2 was reduced from 21 to 13.5 kPa, the decrease in myocardial performance (i.e., shortening work and relative power) in strips from normoxic-acclimated animals was similar at all contraction frequencies. In contrast, there was a strong inverse relationship between contraction frequency and the decrease in work and power in the hypoxic-acclimated group (Figs. 2.1. and 2.3.). This effect was not likely due to differences in the accumulation of anaerobic byproducts as: (1) the strips only performed 30 contractions during each measurement and 13.5 kPa is well above typical venous PO_2 levels (~4 – 5 kPa; Thomas *et al.*, 1994, Steffensen and Farrell, 1998; Farrell and Clutterham, 2003); (2) lactate production is generally higher in muscles when exercised at higher intensities for shorter durations (Wood, 1991; Medbø and Tabata, 1993) which is at odds with the pattern of changes in performance observed, and (3) the myocardium of hypoxic-acclimated red snapper has a greater capacity for anaerobic metabolism (i.e., LDH:CS activity), and mitochondria from hypoxia-acclimated fish are also less affected by decreasing oxygen concentration and are able to maintain ATP synthesis better at lower oxygen saturations (Cook *et al.*, 2013). While we do not have an explanation for this finding, it may be related to the observations of Syme *et al.* (2013; Gesser and Rodnick, 2019), who reported PO_2-related decreases

in myocardial work and power even when oxygen levels were well above physiological values. These authors suggested that this result was due to a mechanism other than reduced energy supply, possibly oxygen sensing mechanisms, such as H_2S and NO (Fago *et al.*, 2012). H_2S molecules accumulate in the trout myocardium when oxygen levels are reduced (Whitfield *et al.*, 2008), and have been shown in rat myocardium to protect against ischemia while causing negative inotropy (Geng *et al.*, 2004; Yong *et al.*, 2008). NO also has the capability to regulate excitation-contraction coupling and negatively affect contractility (Seddon *et al.*, 2007; Imbrogno *et al.*, 2017; Imbrogno and Cerra, 2017), and nitrite which can be converted into NO through an array of proteins (Fago and Jensen, 2015; Jensen *et al.*, 2015; Imbrogno *et al.*, 2017; Imbrogno and Cerra, 2017)]. However, it is unlikely that NO was involved in this rapid reduction in shortening work and power when the hearts of hypoxic-acclimated fish were initially exposed to lowered PO_2. Exogenously applied SNP (a NO donor; 10^{-9} to 10^{-4} M) only had a modest negative inotropic effect (10-20%) on ventricular shortening power in both groups, and there was no difference in the concentration dependency of this effect (Chapter 3). Further Jensen *et al.* (2015) showed that exposure to 24 h of hypoxia does not affect nitrite levels in the trout heart.

In the normoxic group, there was a 40-50% reduction in net work and power across the four tested frequencies as the saline PO_2 was lowered from 21 to 1.5 kPa (Figs. 1-3 E), and myocardial performance (net power) recovered to ~75 – 80% of pre-hypoxic levels upon the return to 21 kPa (Fig. 2.4.). Comparisons of the effects of reduced PO_2 between the current study and the literature are difficult due to the different methodologies and species used; however, this loss of myocardial/cardiac performance upon acute hypoxic exposure, and the degree of recovery, are similar to those reported in other studies. Maximum twitch force was reduced by approximately 50% in isometrically contracting eel (*Anguilla anguilla*) myocardial strips when paced at 40

contractions min^{-1} and exposed to 10.5 kPa of O_2 for 30 minutes (Joyce *et al.*, 2016). The cardiac output of *in situ* rainbow trout hearts was reduced by 50% when exposed to 15 minutes of severe hypoxia (< 1 kPa) at a P_{out} of 50 cm H_2O (f_H ~60 beats min^{-1}) and Q_{max} recovered to 65-75% of pre-hypoxic levels (Gamperl *et al.*, 2001). Finally, the maximum cardiac output (Q_{max}) of *in situ* Atlantic cod hearts was ~65% lower following exposure to 15 minutes of severe hypoxia (saline PO_2 ~0.6 kPa) at 60 beats min^{-1} and the recovery of Q_{max} was 83% (Petersen and Gamperl, 2010b). In contrast, the lack of an effect of hypoxic acclimation on the loss of myocardial force and power when the strips were exposed to acute hypoxia (Figs. 2.1. and 2.3.), and the poorer recovery (by ~20%) despite the lower absolute values of work and power produced by strips from hypoxia-acclimated fish (Fig. 2.4.), were not anticipated/predicted. This is because all previous studies that have used hypoxia-acclimated fish, or populations that were inherently hypoxia-tolerant, report improved myocardial / heart performance during hypoxia and/or recovery once normoxia was re-established. For example, (1) Driedzic *et al.* (1985) showed that hearts from hypoxia-acclimated (4-6 weeks, PO_2 4 – 4.7 kPa) eel pout (*Zoarces viviparous*) were better able to sustain peak tension during anoxia when bath Ca^{2+} levels were elevated; (2) Petersen and Gamperl (2010b) showed that while the Q_{max} of hearts from hypoxia-acclimated Atlantic cod (~8 – 9 kPa for 6 – 12 weeks) was lower than normoxic-acclimated individuals, it fell less when exposed to severe hypoxia, and recovered better (by 10%) when returned to normoxic conditions; (3) Joyce *et al.* (2015) showed that myocardial muscle from hypoxia-tolerant sea bass (*Dicentrarchus labrax*) developed 3-5 times more isometric force as compared to strips from hypoxia-sensitive fish when exposed to acute hypoxia; and (4) Faust *et al.* (2004) showed that *in situ* hearts from a hypoxia-tolerant strain of rainbow required twice the duration of severe hypoxia (~1 kPa), and an elevated workload during hypoxia, before they experienced a 'typical' degree of hypoxia-induced loss of function.

The reasons for this discrepancy are not known, but collectively, these studies point to the severity of hypoxia during acclimation as a key determinant of myocardial dysfunction, and suggest that hypoxic-acclimation cannot realize the improvements in myocardial function under oxygen limiting situations that is achieved through adaptation to hypoxia (i.e., where certain populations or individuals are inherently hypoxia tolerant).

2.5.4. *Perspectives*

This is the first study to compare contractility in isolated ventricular trabeculae from normoxic- and hypoxic-acclimated rainbow trout and strongly suggests that the diminished S_V reported in chronically hypoxic trout and cod (Petersen and Gamperl, 2010a; 2010b; 2011; Moytka *et al.*, 2017) results from an increase in end-systolic volume, i.e., a decrease in ejection fraction. This is proposed because, while lengthening work (i.e., related to ventricular stiffness) was not affected by hypoxic-acclimation, shortening work and power, and thus the ability to eject blood from the heart, were substantially reduced. This study also showed that the response of the myocardium of hypoxia-acclimated trout to a decrease in PO_2 is highly frequency dependent. This is an interesting / novel finding, and we hypothesize that this phenomenon may involve some form of 'oxygen sensing'. This suggestion would be in-line with the findings of Syme *et al.* (2013) for Atlantic cod and Gesser and Rodnick (2019) for rainbow trout. These authors showed that myocardial function was very PO_2-dependent at partial pressures well above those that would be encountered by the heart *in vivo*, and the existence of oxygen sensing mechanisms is well established in the cardiovascular system (Fago *et al.*, 2012).

In this study, we report that acclimation of trout to a level of hypoxia close to their limit of tolerance (i.e., as suggested by the ~30% mortality in the hypoxic-acclimated group) results in an

increase in relative ventricular mass, no improvement in the capacity of the myocardium to perform during hypoxic conditions (i.e., shortening and net work / power) and a reduced capacity to recover function following hypoxic exposure. These results are in clear contrast to previous studies on cod and trout exposed to a less severe hypoxic-insult (Petersen and Gamperl, 2010 a, b; Petersen and Gamperl, 2011; Moytka *et al.*, 2017) and suggest that: 1) the impacts of chronic hypoxia on myocardial performance are highly dependent on the severity of the hypoxic challenge; and 2) that cardiac remodelling does occur under extreme hypoxic conditions to compensate for the loss of myocardial / heart function. Further, when all our previous studies on hypoxic-acclimation and fish cardiac function (present study; Petersen and Gamperl, 2010 a,b; Petersen and Gamperl, 2011; Moytka *et al.*, 2017) are compared to the results of experiments on normally 'hypoxia-tolerant' species where individuals (Joyce *et al.* 2015) or populations (Faust *et al.*, 2004) show a degree of inherent hypoxia tolerance, the data suggest that alterations in the genotype are a pre-requisite for substantial improvements in myocardial hypoxia tolerance (i.e., acclimation to hypoxia does not produce this phenotype). Understanding these aspects of the relationship between water O_2 levels and heart function, and the mechanisms involved, are key to determining whether fishes will be able to tolerate prolonged environmental hypoxia

Chapter Three: Effects of Nitric Oxide Modulation on the Contractility of Spongy Myocardium Isolated From Normoxic- and Hypoxic-Acclimated Steelhead Trout (*Oncorhynchus mykiss*)

3.1. Abstract

Nitric oxide (NO) is a gasotransmitter that is key to the fish's physiological response to low oxygen conditions (hypoxia), including that of the cardiovascular system. Thus, we measured the duration of contraction and relaxation, and work and power (using the work loop method), in spongy myocardial strips from 14^{o}C normoxia and hypoxia-acclimated (40% air saturation) trout exposed to increasing concentrations of the NO donor SNP (10^{-9} to 10^{-4} M) at several frequencies (20 – 80 contractions min^{-1}) and at two strains (8 and 14%). Further, we examined the influence of: 1) nitric oxide synthase (NOS) produced NO (using 10^{-4} M L-NMMA); and 2) sGC mediated, NOS-independent, NO effects (i.e., after blockade with 10^{-5} M ODQ), on myocardial contractility. Hypoxic acclimation resulted in an 8-10% increase in the duration of relaxation, and net power values that were ~35% lower. However, it only had minor impacts on the effects of SNP or the two blockers on myocardial function. SNP had considerable effects on myocardial function (a decrease in the duration of relaxation and of the contraction cycle, reduced net power), and NOS blockade and sGC inhibition resulted in decreases and increases in net power, respectively. However, the most surprising result from this study was the degree that contraction frequency and strain impacted NO-mediated myocardial function. For example, relative net power at 8% strain decreased by ~30% at 20 min^{-1} with 10^{-4} M SNP, but increased by ~20% at 80 min^{-1}. Further, this effect was magnified when muscle strain was increased to 14%: net power decreasing less at 20 and 40 min^{-1} and increasing by up to 70% at 80 min^{-1}. This study on trout: suggests that hypoxic acclimation only has minor effects on NO-mediated myocardial function in fishes; is the first to report the highly frequency dependent nature of NO effects on myocardial contractility; and supports previous work suggesting that the effects of NO on the heart (myocardium) are finely tuned spatio-temporally.

3.2. Introduction

Nitric oxide (NO) is a gaseous signalling molecule (i.e., a gasotransmitter) that is key to the physiological response to low oxygen conditions and is particularly important in mediating cardiovascular adjustments to acute and chronic hypoxia (Cosby *et al.*, 2003; Lundberg and Weitzberg, 2005; Tota *et al.*, 2005; Maher *et al.*, 2008; Imbrogno *et al.*, 2014; Imbrogno *et al.*, 2018). In the heart, NO is predominantly produced from L-arginine by nitric oxide synthase (NOS) enzymes, of which there are three isoforms (nNOS, iNOS, eNOS), in a reaction that also requires O_2 and NADPH (Alderton *et al.*, 2001; Tota *et al.*, 2005; Imbrogno and Cerra, 2017, Imbrogno *et al.*, 2018). In addition, mitochondrial nitrate reductases and deoxygenated myoglobin can produce NO from nitrite (NO_2^-) (Shiva, 2013; Dungel *et al.*, 2017). Nitric oxide's effects on fishes include dilation of the vasculature (Tota *et al.*, 2005; Jensen, 2009; Costa *et al.*, 2015) and in most cases (with the exception of haemoglobinless icefish; Garofalo *et al.*, 2009) a decrease in basal cardiac mechanical performance associated with negative inotropy, but an enhancement of the Frank-Starling response (Tota *et al.*, 2005; Imbrogno *et al.*, 2011; Angelone *et al.*, 2012; Imbrogno *et al.*, 2018). This former effect is partially mediated by NO's stimulation of the enzyme soluble guanylyl cyclase (sGC), which in turn produces the secondary messenger cGMP (Tota *et al.*, 2005; Imbrogno and Cerra, 2017; Imbrogno *et al.*, 2018). cGMP has two major effects: 1) the activation of protein kinase G (PKG) results in the phosphorylation of sarcolemmal K_{ATP} and L-type Ca^{2+} channels and proteins in the myofilament / troponin complex, and this results in reduced Ca^{2+} entry into the cells and a decrease in myocardial contractility; and 2) the activation of phosphodiesterase 2 (PDE2) which leads to the local depletion of cAMP within the microenvironment around L-type calcium channels, and decreased Ca^{2+} influx (Dittrich *et al.*, 2001; Imbrogno and Cerra, 2017; Zhang *et al.*, 2017; Imbrogno *et al.*, 2018). However, not all of the effects of NO on basal cardiac

function are mediated by cGMP. For example, NO produced by nNOS or eNOS ('eNOS'; Imbrogno *et al.*, 2011) can also result in the S-nitrolysation of L-type Ca^{2+} channels, K_{ATP} channels, myofilament proteins and the ser16 of phospholamban (PLB) associated with the SERCA (sarco-endoplasmic reticulum Ca^{2+}-ATPase), all of which reduce myocardial contractility. In addition, NO production can lead to decreased phosphodiesterase 3 (PDE3) activity which results in an increase in cell cAMP levels and the activation of protein kinase A (PKA). The latter phosphorylates PLB, which in turn increases SERCA activity and Ca^{2+} re-uptake into the sarcoplasmic reticulum, and ß-adrenergic receptors which results in their desensitization (Figure 3.1.). With regards to the Frank-Starling mechanism, the effects of NOS and NO are separated into an early phase and a slow phase. In the early-phase response to increased pre-load, autocrine NO produced by nNOS increases Ca^{2+} uptake into the sarcoplasmic reticulum through the S-nitrolysation of thiol groups of PLB which is associated with SERCA 2a, and this decreases myocardial stiffness and allows the heart to easily fill. During the subsequent slow-phase, NO produced by 'eNOS' sustains the increase in Ca^{2+} transient and force generation through S-nitrosylation of thiol residues of the ryanodine receptors (RyR). This enhances Ca^{2+} release from the sarcoplasmic reticulum, and thus, myocardial force development (Imbrogno and Cerra, 2017a and b; Imbrogno *et al.*, 2018) (Figure 3.2.). NO also competitively binds to electron transport chain complexes, and this results in an inhibition of mitochondrial respiration. This is an important effect during, and after, a period of oxygen limitation (hypoxia), as it protects ATP stores, regulates (limits) the rate of respiration, increases myocardial O_2 utilization efficiency during hypoxia, and reduces ROS production and mitochondrial proton leak during re-oxygenation (Cleeter *et al.*, 1994; Moncada and Erusalimsky, 2002; Erusalimsky and Moncada, 2007; Shiva *et al.*, 2007; Misfeldt *et al.*, 2009; Pedersen *et al.*, 2010; Chouchani *et al.*, 2013).

Since oxygen is required for the production of NO by NOS, its sources and effects are altered under oxygen limiting conditions. This has been well documented in mammals (Cosby *et al.*, 2003; Lundberg and Weitzberg, 2005; Maher *et al.*, 2008; Dungel *et al.*, 2017). However, only recently have similar experiments been performed to understand the role that NO plays in modulating fish cardiac function, and its sources under varying oxygen conditions. These studies have largely focused on extremely hypoxia-tolerant fish such as goldfish (*Carassius auratus*) and crucian carp (*Carassius carassius*). For example, Imbrogno *et al.* (2014) showed that while acute hypoxia (~10 kPa O_2) did not affect the magnitude of NO's negative inotropic effect on basal cardiac function in goldfish or the impact of L-NNMA (NOS blocker) or sGC inhibition on myocardial contractility, NO's ability to enhance the sensitivity of the Frank–Starling response was inhibited by ODQ and P-TIO (an inhibitor of sGC and a NO scavenger, respectively), but not L-NMMA. These latter data suggest that NOS-independent pathways of NO production, such as nitrite conversion to NO mediated via deoxygenated myoglobin or haemoglobin (Tota *et al.*, 2005; Lundberg *et al.*, 2008; Fago and Jensen, 2015), predominate in S_V control under conditions of oxygen limitation (Imbrogno *et al.*, 2014). Indeed, recent studies on crucian carp found that the concentration of nitrite was significantly higher in the hearts of fish exposed to anoxia (1, 3 or 5 days at 0.02 kPa O_2) or one day of deep hypoxia (~0.25 kPa O_2) when compared to fish maintained under normoxic conditions (Sandvik *et al.*, 2012; Hansen *et al.*, 2016), and these authors suggested that nitrite was acting as a NO donor for cytoprotection under anoxia / re-oxygenation. However, to our knowledge, only two studies have examined the physiological effects of NO on the hearts of 'hypoxia-intolerant' fish, and their results were contradictory. Pedersen *et al.* (2010) were able to demonstrate an effect of NOS inhibition and nitrite on the O_2 consumption and O_2 utilization efficiency of rainbow trout ventricular rings when exposed to 9% O_2, but no effect on myocardial

contractility. This latter finding is questionable, however, as these compounds also had no effect on goldfish myocardial contractility in this study, and this result is in direct contradiction with other research on this species (e.g. Cameron *et al.*, 2003; Imbrogno *et al.*, 2014). Conversely, Atlantic salmon (*Salmo salar*) perfused working hearts exposed to the NOS substrate L-arginine (10^{-7} and 10^{-6} M) suffered significant reductions in Q and S_V, while pre-treatment with NOS inhibitor L-NMMA (10^{-6} M) significantly increased Q and S_V in these fish (Gattuso *et al.*, 2002).

Hypoxia (low water oxygen levels that negatively impact an organism's physiology; Richards, 2011) occurs naturally in fresh and marine waters, and accelerated climate change has increased the severity and duration of hypoxic episodes in both ecosystems (Diaz and Rosenberg, 2008; Richards, 2011; Altieri and Gedan, 2015; Gedan *et al.*, 2017; Breitberg *et al.*, 2018). Many studies have investigated the effect of short-term (acute) hypoxia on fish cardiac physiology, but few have considered chronic hypoxia's effect(s) on fish cardiac function (see reviews by Gamperl and Driedzic, 2009; Gamperl, 2011). Nonetheless, recent studies have shown that: 1) Atlantic cod (*Gadus morhua*) and steelhead trout (*Oncorhynchus mykiss*) acclimated to moderate chronic hypoxia (> 6 weeks at 8.5 kPa O_2; 40% air saturation) had a significantly diminished capacity to increase S_V, and thus Q, when swum to exhaustion or when exposed to an acute incremental temperature increase until the fish's critical thermal maximum, respectively (Petersen *et al.* 2010a; Moytka *et al.*, 2017); 2) that this loss of *in vivo* pumping capacity was mirrored by the performance of *in situ* cod hearts [(i.e., eliminating the possibility that the decrease in S_V was largely due to alterations in neuronal of humoral regulation of cardiac function, afterload or venous (filling) pressure (Petersen and Gamperl 2010b)]; and 3) this loss of function was not related to gross morphological changes that limited the filling capacity of the heart (Moytka *et al.*, 2017). This

research strongly suggests that other aspects of cardiac function must be dysregulated / altered following exposure to chronic hypoxia.

To examine the potential role of NO in the response of the steelhead trout (*Oncorhynchus mykiss*) heart to chronic low oxygen, we acclimated fish to hypoxia (PO_2 ~8.0 kPa) or normoxia (PO_2 ~21 kPa) at 13-14^{o}C for 10 – 14 weeks, and used cycling strips (e.g. see Josephson, 1985; Syme *et al.*, 2013) from the spongy myocardium to measure contractile performance at increasing concentrations of the NO donor sodium nitroprusside (SNP; 10^{-9} – 10^{-4} M) across a range of frequencies (20 – 80 contractions min^{-1}) and at two strains (8 and 14%). In addition, we measured the same parameters under NOS (L-NMMA, 10^{-5} M) and sGC (ODQ, 10^{-4} M) inhibition, followed by SNP (10^{-4}), to investigate: 1) the importance of NO produced through NOS-dependent and independent pathways in the control of myocardial function in this species; and 2) the ability of NO to alter myocardial performance after blockade of sGC/cGMP-mediated pathways.

3.3. Methods

3.3.1. *Experimental Animals and Holding / Acclimation Conditions*

The female steelhead trout (*Oncorhynchus mykiss*) used in this study comprised a portion of the fish that were acclimated to normoxia (~21 kPa O_2) and hypoxia (~8.0 kPa) in Chapter 2 (Carnevale *et al.*, submitted). These fish were sampled from 10 – 14 weeks after the fish reached the desired level of hypoxia and averaged 1.19 ± 0.6 kg (range 0.64 to 1.46). All details with respect to their husbandry and acclimation conditions can be found in the methods section of Chapter 2.

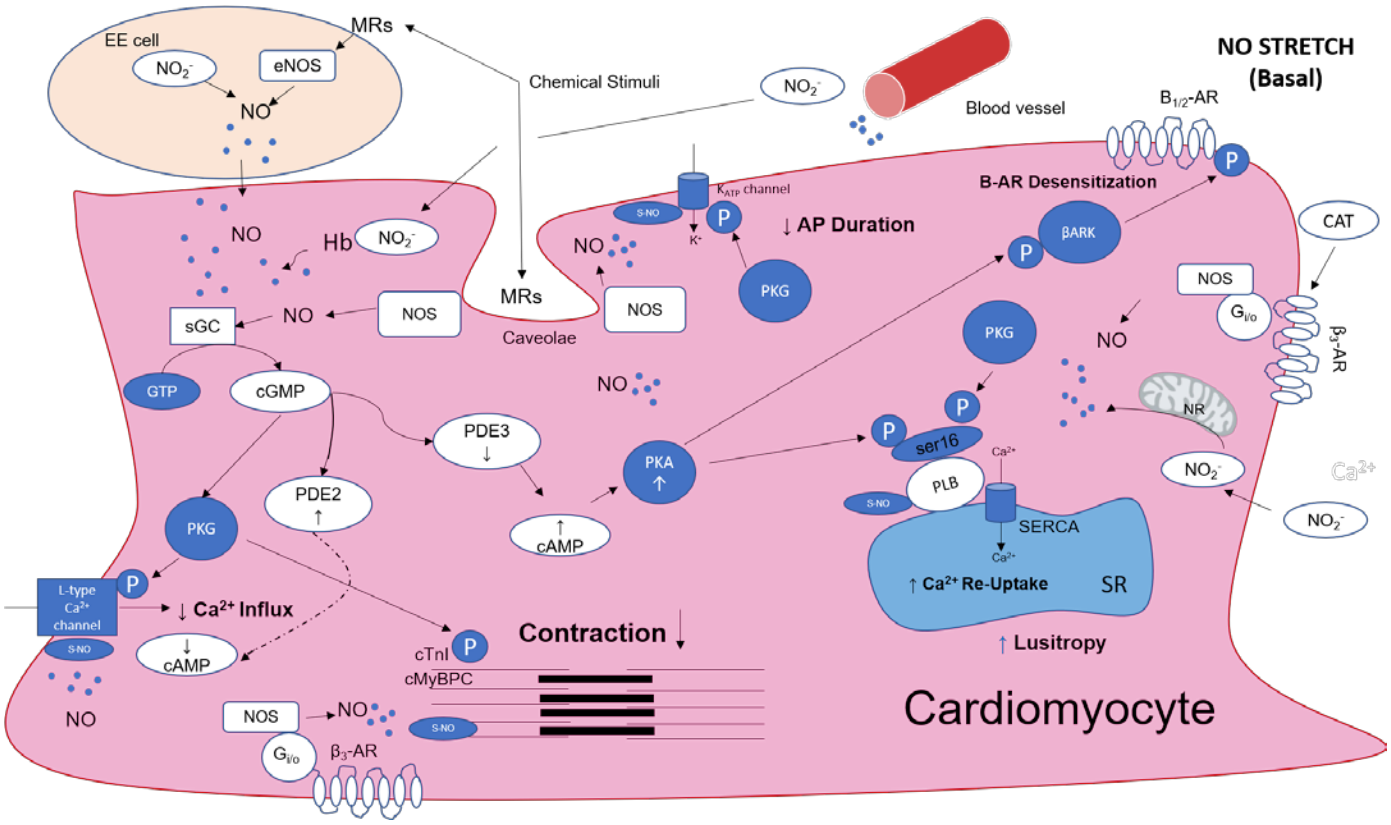

Figure 3.1. Schematic diagram of the sources of nitric oxide (NO), and the identified or potential role(s) of endocrine, paracrine and autocrine NO as a regulator of teleost cardiomyocyte function under basal conditions; i.e., the shown mechanisms / pathways are not dependent upon stretch to induce NO production (for these pathways see Figure 3.2.). NO and /or NO_2^- can be transported in the blood to the myocardium, and the latter can be converted into NO by the cardiomyocytes via deoxygenated myoglobin or mitochondrial nitrite reductase (endocrine effect). NO or NO_2^- can also be produced by the EE or the endothelium of coronary vessels and diffuse into cardiomyocytes (paracrine effect). Finally, various chemical stimuli can activate membrane bound receptors (MRs) on the cardiomyocyte (e.g., β_3-AR) and lead to the production of NO through nitric oxide synthases (i.e., autocrine effect). NO can exert its cellular effects through sGC activation, which converts GTP to cGMP. This molecule can directly bind to PDE2 in the immediate vicinity (microdomain) of L-type Ca^{2+} channels, and this decreases local cAMP levels and results in a reduction in Ca^{2+} influx into the cardiomyocyte. However, the main cGMP-dependent effects are mediated through its stimulation of the enzyme PKG. PKG can phosphorylate multiple downstream effectors, such as L-type Ca^{2+} channels, K_{ATP} channels, the Ser16 of PLB and the contractile proteins cTnI and cMyBPC. Phosphorylation of these contractile proteins results in a reduction in myofilament sensitivity to Ca^{2+} and a decrease in contractile force. The phosphorylation of K_{ATP} channels opens these channels, allowing K^+ to enter the myocyte, and this decreases AP duration and inotropy. Further, phosphorylation of L-type Ca^{2+} channels reduces Ca^{2+} influx, and of the Ser16 residue on the PLB protein enhances SERCA Ca^{2+} reuptake into the SR and increases lusitropy. NO also has several cGMP-independent effects that are mediated by the S-nitrosylation of various proteins. These include the L-type Ca^{2+} channel, the K_{ATP} channel, several contractile proteins (including cMyBPC, and cTnC-Cys35 and -Cys84), and all these effects decrease myocardial contractility. Finally, cGMP-mediated decreases in PDE3 activity, and the

subsequent increase in cAMP, can stimulate PKA, and this results in the phosphorylation of βARK and $\beta_{1/2}$ ARs (causing β-adrenergic receptor desensitization) and the Ser16 of PLB. Collectively, these responses lead to a decrease in myocardial contractility. **Note**: we have not specifically indicated whether the cardiomyocyte NOS is eNOS or nNOS, as while some studies have suggested that there is eNOS activity in fish cardiomyocytes based on the binding of antibodies specific for mammalian eNOS, there is no molecular evidence that this gene/enzyme exists in fishes. This diagram is based on previous reviews on fish (Imbrogno *et al.*, 2011, Imbrogno *et al.*, 2017; Imbrogno and Cerra, 2017, Gattuso *et al.*, 2018), as well as on the role of NO in the control of mammalian cardiomyocyte function (Fischmeister *et al.*, 2006; Shiva, 2013; Zhang *et al.*, 2017). $\beta_{1/2}$-AR, Type 1 and 2 adrenoreceptors; β_3-AR, beta 3 adrenoreceptor; βARK, beta adrenergic receptor kinase; Ca^{2+}, calcium; cAMP, cyclic adenosine monophosphate; CAT, catecholamines; cGMP, cyclic guanosine monophosphate; cMyBPC, cardiac myosin binding protein C; cTnC-Cys35; cardiac troponin C-cysteine 35; cTnI – cardiac troponin I; EE cell, epicardial endothelial cell; $G_{i/o}$, G-protein-coupled receptor; GTP, guanosine triphosphate; K_{ATP} channel, ATP-sensitive potassium channel; MRs, membrane receptors; NO, nitric oxide; NOS, nitric oxide synthase; NO_2^-, nitrite; NR, nitrite reductase; PDE2, phosphodiesterase 2; PKA, protein kinase A; PKG, protein kinase G; PLB, phospholamban; Ser16 – serine 16 residue of phospholamban; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase pump; sGC, soluble guanylyl cyclase; S-NO, S-nitrosylation; SR; sarcoplasmic reticulum.

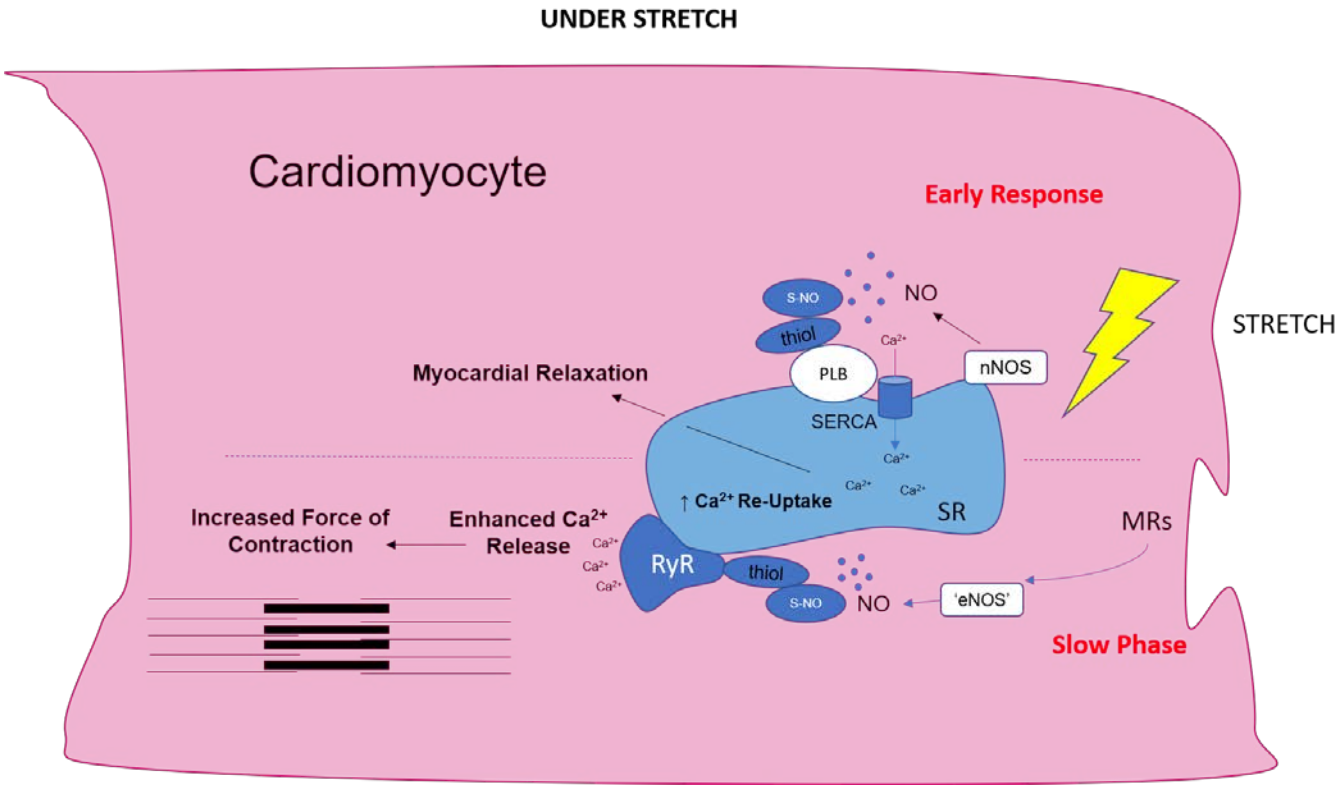

Figure 3.2. Schematic diagram of how autocrine NO is involved in the enhancement of the Frank-Starling response in the teleost heart. There are two phases of the response to stretching of the myocyte, which are proposed to activate both constitutive NOS enzymes ('eNOS' and nNOS). In the early response to stretch, nNOS associated with the SR is activated which produces NO. This NO results in the S-nitrosylation of a thiol residue of PLB, an enhancement of SERCA activity, and increased Ca^{2+} re-uptake into the SR which results in myocardial relaxation (e.g. important for filling the ventricle). The slow phase of this response begins while the myocardial fibres are being stretched, as 'eNOS' is activated and produces NO. This results in S-nitrosylation of a RyR thiol residue, and results in enhanced Ca^{2+} release from the SR. This results in a greater force of contraction by the myofilaments and positive inotropy at a time when blood is being ejected from the heart. Note: 'eNOS' is used as while some studies have suggested that there is eNOS activity in fish cardiomyocytes based on the binding of antibodies specific for mammalian eNOS, there is no molecular evidence that this gene/enzyme exists in fishes. This figure is based on information contained in Imbrogno *et al.*, 2011, Gattuso *et al.*, 2016, and Imbrogno and Cerra, 2017a and b. Ca^{2+}, calcium; 'eNOS', endothelial nitric oxide synthase; MRs, membrane receptors; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PLB, phospholamban; RyR, ryanodine receptor; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase, sGC, soluble guanylyl cyclase, S-NO, S-nitrosylation; SR, sarcoplasmic reticulum; thiol – thiol residue of phospholamban or RyR protein.

3.3.2. *Isolated Myocardial Preparations*

Fish from each treatment (n = 10 normoxic, n = 5-6 hypoxic) were haphazardly selected from their holding tank, swiftly killed by cerebral percussion, measured for length and body mass, and had their hearts removed. After the ventricle was separated from the bulbus arteriosus and atrium, the ventricle was weighed, cut lengthwise, and rinsed in ice-cold physiological saline for marine teleosts (Peterson and Gamperl, 2010), 7.6 pH at 20ºC. This saline containing (in gl^{-1}): 10.5 NaCl; 0.49 $MgSO_4 \cdot 7H_2O$; 0.37 KCl; 0.33 $CaCl_2 \cdot 2H_2O$; 0.14 $NaH_2PO_4 \cdot H2O$; 1.84 sodium TES base ($C_6H_{14}NO_6SNa$); 0.59 TES acid ($C_6H_{15}NO_6S$); 1.0 glucose. The ventricular halves were then pinned to the bottom of a temperature-controlled (4ºC) dissecting dish containing saline, and a small segment of spongy trabecular muscle (approx. 5 mm in length x < $1mm^2$ in cross section) was isolated from the inner (luminal) surface of the ventricle using a dissecting microscope. Muscle segments (7.4 ± 4.6 mg wet mass and 5.6 ± 1.3 mm resting length) were selected so that the majority of fibers ran parallel to the long axis of the preparation, and there was minimal branching along their length. Once dissected free, a short piece of 7-0 silk suture was tied to each end of the strip and the strips were attached to the arm of a servomotor (300C-LR; Aurora Scientific, Aurora ON, Canada) on one end and to a force transducer (404A; Aurora Scientific) on the other end using methods as described in Syme *et al.* (2013). The muscle segments were subsequently bathed in physiological saline, with the temperature maintained at 14 ± 0.2ºC using Peltier thermoelectric modules and a temperature controller (TC-24-12; TE Technology, Traverse City, MI). A custom program built in LabView software (National Instruments, Austin, TX) controlled a 12-bit analog/digital converter card (PCI MIO 16E 4; National instruments) that regulated the stimulator and servomotor (5 kHz D/A output), and collected muscle force, muscle length (servomotor arm position) and stimulus signals (1 kHz A/D input).

PO_2 was maintained at 21 kPa for all experiments by circulating air delivered from a Wöstoff gas mixing pump (DIGAMIX 6KM301, Bochum, Germany) into a reservoir of saline that was then drained via stainless-steel tubing into the muscle chamber, and the level of O_2 in the muscle chamber was continuously monitored using a calibrated, fiber optic dipping oxygen probe (PSt3; PreSens, Regensburg, Germany) and oxygen meter (Fibox 3; PreSens). Complete turnover of the saline in the 30 mL bath occurred approximately once per min.

3.3.3. Optimization of Strip Performance

Initially, the length of each preparation was increased systematically until developed twitch force, elicited by a 1-ms supra-maximal shock (~10 v), was maximal; the electrical stimuli delivered using a stimulator (Isostim A320, World Precision Instruments, Sarasota, FL, USA) and platinum plates placed on either side of the preparation. Muscle length was then decreased by 5% to reduce stress on the preparation, and to better mimic the function of cardiac muscle which is normally on the ascending limb of the force-length relationship. Further, Harwood *et al.* (1998) showed that there was no significant difference in work output between rainbow trout cardiac muscle operating at 95% vs. 100% of lengths that gave maximal isometric force.

Thirty isometric cycles were then run on each strip so that distortion and stimulation phase could be calculated to best represent myocardial contraction and relaxation at each frequency to be used during the experiments (20, 40, 60 and 80 contractions min^{-1}). Distortion (in %) represents the amount of time the muscle was shortening in proportion to the entire cycle, with larger values indicating longer shortening times and vice versa. Distortion was calculated for each strip at each frequency using the equation:

$$Distortion\ (\%) = [period\ of\ contraction\ (ms) / \frac{1}{period\ of\ cycle\ (Hz)}] * 100\%$$

The stimulation phase (%) was also calculated at each frequency to ensure that the muscle was stimulated at the appropriate point in the cycle using the distortion previously calculated and the equation:

$$Stimulation\ Phase\ (\%) = \frac{[100\% - Distortion\ (\%)]}{2}$$

This resulted in the distortion and phase being customized for each myocardial strip; the mean values for these parameters are shown in Table 3.1.

3.3.4. *Experimental Protocols*

After optimization, and the calculation of distortion and stimulation phase, muscle strips were allowed to rest for 15 minutes (with only periodical stimulations to maintain activity). Then the strips were given one of two experimental treatments.

3.3.4.1. *SNP Dose-Response Curve*

In this experiment, the performance of strips from 8 normoxic- and 6 hypoxic-acclimated fish was measured under control conditions, and after being sequentially exposed to increasing concentrations of the NO donor sodium nitroprusside (SNP; ranging from 10^{-9} to 10^{-4} M). The strips were allowed to equilibrate for 10 minutes at each [SNP], and each experiment lasted ~2 hours.

3.3.4.2. *Effects of NO Inhibitors*

In this experiment, the performance of the strips (8 from normoxic- and 5 from hypoxic-acclimated fish) was measured under control conditions, and then following sequential exposure

to the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA, [10^{-5} M]), the specific soluble guanylyl cyclase (sGC) inhibitor 1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one (ODQ, [10^{-4} M]) to eliminate cGMP effects mediated through NOS-independent pathways, and finally 10^{-4} M SNP to investigate what proportion of the changes in SNP-mediated myocardial function were mediated by sGC/cGMP-mediated pathways. Thirty minutes were allowed after the addition of L-NMMA and ODQ, and 5 minutes after SNP addition, before contractile parameters were measured. This experiment took approximately 1.5 hours to complete, and the concentrations used for these pharmacological agents were based on prior fish studies which employed the same compounds (Small *et al.*, 1990; Joa *et al.*, 2000; Imbrogno *et al.*, 2001; Gattuso *et al.*, 2002; Pellegrino *et al.*, 2003; Tota *et al.*, 2005; Pedersen *et al.*, 2010; Imbrogno *et al.*, 2014). All chemicals were from Sigma-Aldrich Chemical Co. (St. Louis, MO., USA).

In both experiments, isometric contractions were initially used at every frequency and strain to measure twitch duration. Twitch duration (in milliseconds) was measured over 3 time-frames: time for force to rise from 10 to 90% of maximal, time for force to fall from 90 to 10% of maximal force, and time from 10% of maximal force during contraction to 10% of maximal force during relaxation. These measurements represent the duration of systole (contraction), diastole (relaxation) and a full contraction cycle, respectively (Harwood *et al.*, 1998; Syme *et al.*, 2013).

At each measurement point in the experiments, myocardial work and power were also measured at 4 frequencies (20, 40, 60, 80 contractions min^{-1}) and at two strains (8 and 14%) by exposing the strips to a train of 30 stimulations and measuring the work and power (see below) produced in the last few contractions. The contraction frequency and strain order were randomized in each strip to limit the effect of repeated measures on muscle performance. The trabeculae were unable to contract faster than 1.33 Hz (80 contractions min^{-1}), as full mechanical restitution could

Table 3.1. Mean stimulation phase and distortion (values in % ± S.E.) used for the individual ventricular trabeculae (n = 27) used to generate the SNP dose-response curves and examine the effects of various pharmacological agents on myocardial performance.

Frequency (contractions min^{-1})	Distortion (%)	Stimulation Phase (%)
20	32 ± 0.5	34 ± 0.3
40	58 ± 0.8	21 ± 0.4
60	80 ± 1.0	10 ± 0.5
80	92 ± 0.4	4 ± 0.2

not be achieved, so three slower frequencies (0.33 Hz, 20 min^{-1}; 0.66 Hz, 40 min^{-1}; 1.0 Hz, 60 min^{-1}) were also employed to allow comparisons of muscle performance at 4 equally separated contraction frequencies. Three of these frequencies fall within the physiological range of heart rates reported in trout at a similar temperature (0.66-1.33 Hz) (Priede, 1974), with 0.33 Hz (20 contractions min^{-1}) below this range. The muscle amplitudes (strains) chosen (8% and 14%) were used to approximate those for fish hearts operating at resting (0.4 ml g^{-1}) and maximum (1.3 ml g^{-1}) S_V (Syme *et al.*, 2013). Syme *et al.* (2013) measured the distance between markers (pieces of filter paper) placed on the surface of hearts from anaesthetized ('resting') Atlantic cod to determine the change in muscle strain between end-systolic and end-diastolic volume. This value was 8% of muscle length. Treating the ventricle as a sphere when filled with blood: 1) the equation $V = \frac{4\pi r^2}{3}$ was used to calculate the 'heart's radius at resting (0.4 ml g^{-1}) and maximum (1.3 ml g^{-1}) stroke volume; 2) the circumference of the heart was then calculated for each condition using the equation, circumference = $2\pi r$; and 3) the strain at maximum stroke volume (S_{Vmax}) was calculated as 8% x [circumference (S_{Vmax}) / circumference (S_{Vrest})]. These calculations resulted in a strain value at S_{Vmax} of just over 12%, but a muscle strain of 14% was used in these experiments to ensure that all strips were operating at maximum strain due to the inherent variability between fish and the varying orientation of trabeculae *in vivo* (Sanchez-Quintana *et al.*, 1995).

3.3.5. *Measurements of Myocardial Performance*

In these experiments, myocardial contractility was measured using the work loop method (Josephson, 1985) as the muscle strips were cycled through length changes at the various frequencies and strains. Cyclic changes in length and activation simulate muscle movement and contractions in a beating heart and allow for the measurement of the ability of trabeculae to produce

work and power. Sinusoidal strain was imposed on the muscle by the servomotor, with strain amplitude (analogous to stroke volume) and the rate of cycling and activation (analogous to heart rate) selected so that the preparations worked under conditions of low (8%; i.e., + 4%) and high (14%; i.e., + 7%) strain. Two strain values were chosen to determine whether the effects of SNP or the pharmaceutical blockers varied when the length change experienced by the muscle was increased (i.e., analogous to a change in S_V). The order in which contraction frequency and strain were applied (used) were randomized between strips to limit the effect of repeated measures of muscle performance on the results. Further, pre-experimental trials (2-3) determined that there was no decrement in function when the 2-hour protocol was performed under normoxic conditions. This is consistent with Roberts and Syme (unpubl.) who performed similar studies, and several studies using *in situ* trout hearts (e.g. Faust *et al.,* 2004). Work (in J) provides a measure of the mechanical energy produced during each beat of the heart and was calculated as the integral of muscle force with respect to length change. The work done by the servomotor to lengthen the muscle in each cycle (lengthening work; analogous to filling work in an intact heart), the work done by the muscle when it contracts during each cycle (shortening work; analogous to stroke work), and net work (the difference between shortening work and lengthening work) were all measured. Power (in W) provides a measure of the sustained rate of mechanical energy produced and was calculated as the product of the work done per cycle (shortening, lengthening, or net) and contraction frequency (Hz). Mass-specific work (J kg^{-1}) and power (W kg^{-1}) were calculated for each muscle strip based on the mass of each preparation (see below). In both experiments, power measured prior to the introduction of any drugs served as a reference point for the calculation of relative changes in this parameter.

At the conclusion of each experiment, the muscle strips were removed from the bath and trimmed of any tissue past the silk ties and obviously dead tissue, blotted on filter paper to remove surface moisture and weighed on a microbalance (Mettler UMT2; Mettler-Toledo, Columbus, OH). Vital staining was not performed to more accurately determine the amount of viable tissue. Thus, muscle mass is likely an overestimate of viable tissue mass and mass-specific work values are probably underestimates of muscle performance.

3.3.6. *Data and Statistical Analyses*

A 20-point median filter was applied to every record of muscle force and length before analyses to eliminate background noise, and checks were made to ensure that the filter did not distort the traces. Performance of the working preparations was measured as the average work done during the final 10 contractions of every series of 30 contractions. The number of replicates (*N*) reported for this experiment refers to the number of trabeculae (strips) tested, each originating from a different fish.

Fish mass, ventricular mass, relative ventricular mass (RVM [%]; ventricle mass/body mass x 100), length and condition factor (K) were compared between acclimation groups using two-tailed t-tests and GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

Split-plot, mixed, general linear models and the R statistical package (version 3.22) were used to examine the effects of the three controlled variables [acclimation condition, stimulation frequency and drug (L-NMMA, ODQ, SNP)] and one random variable (strip) on measures of twitch duration (10 - 90%, 90 - 10% and 10 - 10% of maximal isometric force). Ventricular trabeculae were not controlled for length. This contributed to the inherent variability between strips and this was accounted for in the main model by including strip as a random factor. When

significant interactions were present (which was in nearly all cases) a number of two-way ANOVAs were performed to identify significant main effects. These included: 1) between stimulation frequency and SNP concentration (or drug type) within each treatment group. 2) between the treatment groups (hypoxia vs. normoxia) and SNP concentration (or drug type) within a stimulation frequency; and. 3) between the treatment groups and stimulation frequency within an SNP concentrations or drug type. Finally, when significant differences were identified between main effects, these analyses were followed by Holm-Sidak pairwise-comparisons and Dunnett's tests using SPSS (version 11.0).

A split-split plot, mixed, general linear model (followed by two-way ANOVAs when interactions were present), was performed using the R statistical package to examine the effects of the four controlled variables [acclimation condition, stimulation frequency, muscle strain, and drug (SNP, L-NMMA and/or ODQ] and one random variable (strip) on muscle performance parameters The two-way ANOVAs that were performed were: 1) between SNP concentration (or drug type) and acclimation group, at each frequency and within each strain; 2) between stimulation frequency and acclimation group, at a particular SNP concentration (or drug type) and within each strain; 3) between SNP concentration (or drug type) and strain, at each frequency and within each acclimation group; and 4) between stimulation frequency and strain, at a particular SNP concentration (or drug type) and within each acclimation group. As above, these analyses were followed by Holm-Sidak pairwise-comparisons and Dunnett's tests using SPSS (version 11.0) to identify significant differences between main effects.

All statistical analyses were performed with the level of statistical significance set at $P < 0.05$. Graphs and figures were created using GraphPad Prism 5 and all data in the text and figures

are expressed as means ± 1 standard error of the mean (S.E.).

3.4. Results

3.4.1. *Cardiac Morphometrics and Fish Size*

Hypoxic acclimation did not significantly ($P < 0.05$) affect trout mass, length, condition factor, ventricular mass or relative ventricular mass in either experiment. However, hypoxic-acclimated trout tended to weigh less (by 13%) and have a greater RVM (by 18%) (P values = 0.25 and 0.13, respectively)(Table 3.2.), and this is in agreement with Chapter 2 where hypoxic-acclimated trout had significantly greater RVM values.

3.4.2. *Effects of Acclimation, Frequency and SNP Concentration on Twitch Duration*

Acclimation condition had no effect on the period of contraction (10 – 90% of maximal force), but SNP and frequency both had significant ($P < 0.05$) effects on this parameter (Table 3.3.). As frequency increased from 20 to 80 contractions min^{-1}, the period of contraction decreased from approximately 280 to 220 ms in both acclimation groups (Fig. 3.3. A and B). Over the range of [SNP], contraction duration decreased by ~10% across all frequencies tested in both acclimation groups. There were significant differences in the duration of relaxation (90 – 10% of maximal force) between acclimation groups (Fig. 3.3. C and D). Prior to the addition of SNP, the period of relaxation was longer in strips from hypoxic-acclimated fish (by ~10%), and decreased with each increase in frequency; the difference between 20 and 80 contractions min^{-1} ~240 ms. The addition of SNP did not change the qualitative nature of these two relationships. However, SNP resulted in a decrease in the duration of relaxation. This effect was most evident at the lower [SNP] and more pronounced at lower frequencies. The period from 10% of maximal force during contraction to

10% of maximal force during relaxation was also longer before SNP addition in strips from hypoxic-acclimated fish (by approx. 8%; Fig. 3.3. E and F). Both acclimation groups saw a reduction in the 10 – 10% period as SNP concentration increased, and the effects of [SNP] and frequency mirrored that seen for the duration of 90 – 10% of maximal force. The 10 – 10% twitch duration decreased by ~20% at 20 contractions min^{-1} in both groups while only decreasing by ~5% at 80 contractions min^{-1} as [SNP] was increased to 10^{-4} M.

3.4.3 *Effects of Acclimation Condition, Strain, Frequency and SNP Concentration on Myocardial Performance*

There were numerous acclimation treatment, strain, frequency and SNP effects, as well as interactions between these fixed factors (Table 3.3.). Thus, two-way ANOVAs were performed to ask specific questions with regards to main effects (e.g. acclimation, strain, frequency and [SNP]) (see methods section). For clarity, graphs of shortening, lengthening and net work were omitted, and the presentation of these data is limited to the analysis presented in Table 3.3. Further, there are no symbols showing statistical differences on the figures of myocardial power as they would have been too cluttered; significant ($P < 0.05$) differences (effects) are described in the below text.

3.4.3.1. *Shortening Power*

The relationship between contraction frequency and shortening power was positive in both acclimation groups, and values for this parameter were approximately 50 to 60% higher at 14 vs. 8% strain. Further, shortening power produced by the myocardium of hypoxic-acclimated fish prior to SNP addition was 20 – 25% less than measured in strips of normoxic-acclimated fish at each frequency (Figure 3.4.). It is difficult to see the concentration dependent effects on SNP on

shortening power using the absolute data (Fig. 3.4), and so the data for relative shortening power are also presented (Fig. 3.5.). Relative shortening power decreased in both groups as SNP concentration increased, however, the decrease was not proportional to the increase in [SNP]. In all groups, the largest decrease in relative shortening power occurred when 10^{-9} SNP was added to the chamber. In addition, a further large decrease was seen at the lowest frequency (20 contractions min^{-1}) when 10^{-8} M SNP was applied. Increasing the [SNP] more only resulted in relatively minor / small decreases in shortening power. Although hypoxic acclimation did not appear to have an effect on the response of shortening power to [SNP], contraction frequency and strain had considerable effects. At 20 contractions min^{-1} and 8% strain the decrease in relative shortening power was approximately 30% at the highest [SNP] (10^{-4} SNP) in both acclimation groups, whereas, it only decreased by approximately 10% in both groups at 80 contractions min^{-1}. There also was a significant ($P < 0.05$) interaction between SNP and strain on relative shortening power in both acclimation groups (Table 3.3.; Fig. 3.5.). For example: 1) there was a 30% reduction in relative shortening power at 20 contractions min^{-1} in both groups as [SNP] increased at 8% strain, but only a 20% reduction at the same frequency when strain was 14%; 2) there was an ~ 10% decrease in relative shortening power at 80 contractions min^{-1} in both groups at 8% strain as [SNP] increased, but this parameter increased slightly when muscle strain was 14%.

3.4.3.1. *Lengthening Power*

There was no effect of acclimation on lengthening power, but the effect of frequency was highly significant; the power required by the servomotor to stretch strips from both acclimation groups increased by 4-fold as frequency was increased from 20 to 80 contractions min^{-1} (Fig. 3.6.).

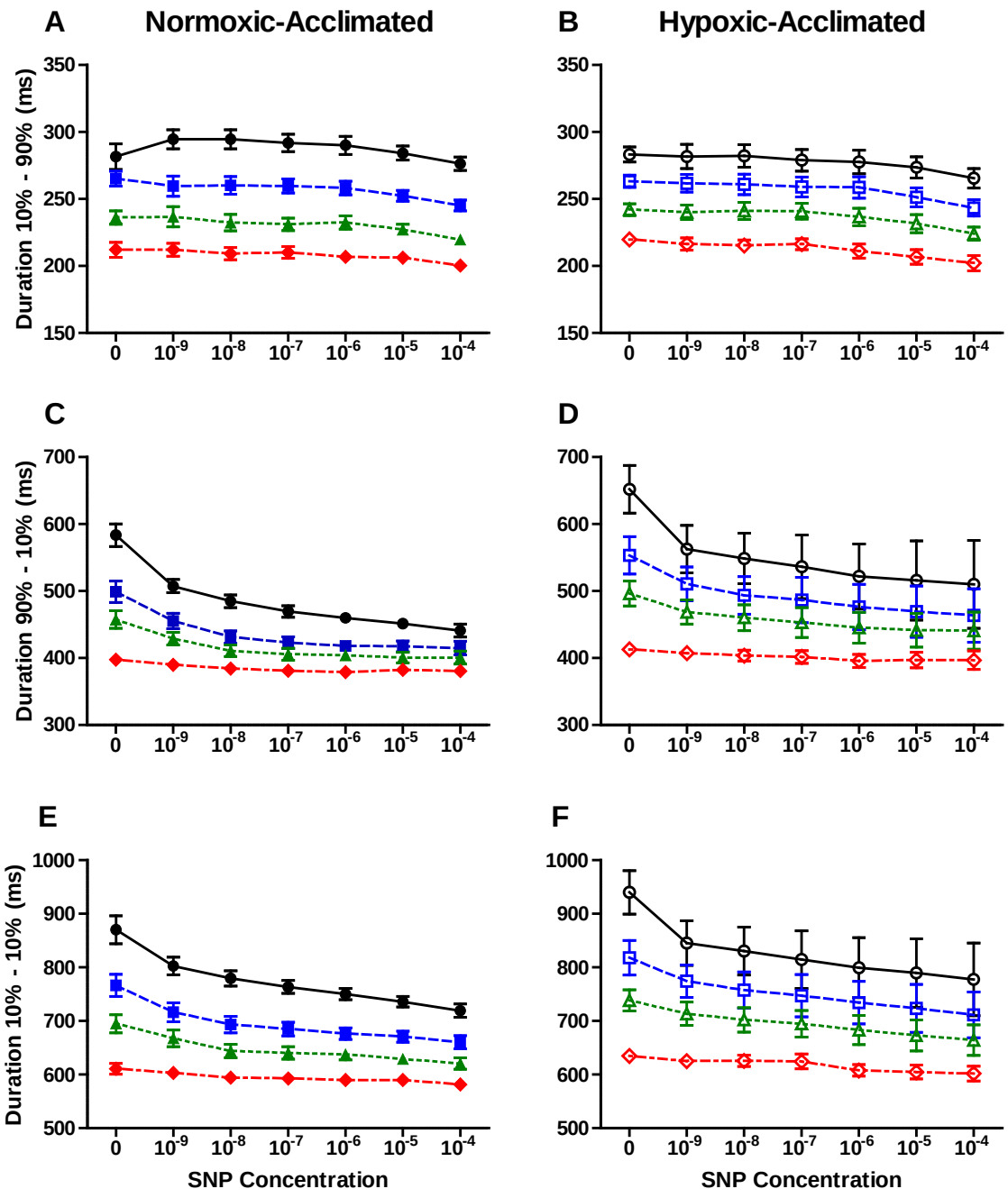

Figure 3.3. Twitch kinetics of normoxic-acclimated (solid symbols; left panels) and hypoxic-acclimated (open symbols; right panels) steelhead trout ventricular muscle strips at increasing SNP concentrations (10^{-9}-10^{-4} M) and four frequencies at 14°C. Three measurements of twitch duration are shown: time for force to rise from 10 to 90% of maximal during contraction (A and B); time for force to fall from 90 to 10% of maximal force during relaxation (C and D); and time from 10% of maximal force during contraction to 10% of maximal force during relaxation (E and F). The trabeculae were stimulated at 20 (black ● and ○,—), 40 (blue ■ and □, ---), 60 (green ▲ and △, ···) and 80 (red ◆ and ◇,-·-·) contractions min^{-1}. Values are means ± SE, *n* = 8 normoxic- and *n* = 6 hypoxic- acclimated.

Strain also had a highly significant effect, with the lengthening power required to stretch the muscle strips approximately doubling when strain was increased from 8 to 14%.

The addition of SNP decreased lengthening power in both groups, however, the influence of frequency and strain were difficult to appreciate when absolute values for power were plotted. Thus, the data were converted into relative values (Table 3.3.; Fig. 3.7). Frequency did not have a significant effect on the decrease in relative lengthening power that was seen with increasing [SNP]. However, there was a significant interaction between SNP and strain in both acclimation groups (Table 3.3.), and this resulted in a smaller decrease in relative lengthening power at 14% strain as compared to 8% strain. For example, relative lengthening power was reduced by 30% at 20 contractions min^{-1} by 10^{-4} M SNP at 8% strain, but this parameter only fell by approximately 15% at the same frequency at 14% strain.

3.4.3.3. *Net Power*

Given that net power is the product of shortening power and lengthening power, and the number of interactions between main effects (see Table 3.3.), interpretation of the data for this parameter was difficult. Nonetheless, there are several apparent trends in the data (Fig. 3.8.). First, although this parameter increased in both groups as contraction frequency was raised from 20 to 60 min^{-1}, it failed to increase at 8% strain, and actually fell at 14% strain when frequency was raised to 80 min^{-1}. Second, strips from hypoxia-acclimated trout produced ~35% less net power at each frequency when compared to normoxic-acclimated strips at both 8 and 14% strain. Third, the effect of [SNP] was frequency dependent in both groups. This can be more fully appreciated when the data are converted into relative values (Fig. 3.9.). As [SNP] increased, there was a ~30%

Table 3.2. Body and cardiac morphometrics for steelhead trout acclimated to either normoxia (water PO_2 ~20 kPa, n = 10) or hypoxia (8-9 kPa, n = 6) for 10-14 weeks, and used for SNP dose-response curve and NO inhibitor experiments. Values are means ± SE.

	Animal Mass (g)	**Fork Length (cm)**	**Condition factor (K)**	**Ventricular Mass (g)**	**Relative Ventricular Mass (RVM %)**
Normoxia	1244 ± 43	43.8 ± 0.2	1.48 ± 0.04	1.02 ± 0.06	0.082 ± 0.003
Hypoxia	1097 ± 144	42.5 ± 1.5	1.38 ± 0.05	1.05 ± 0.17	0.097 ± 0.012

Table 3.3. Results of the split plot mixed general linear model (Method 1) analysis which examined the effects of acclimation condition, frequency and [SNP] on three measures of twitch duration. Results of the split-split plot mixed general linear model (Method 2) analysis that was used to examine the effects of acclimation condition, frequency, strain and increasing concentrations of SNP on shortening, lengthening and net work and power as well as relative shortening, lengthening and net power.

Parameter	Method	Factor(s)	F	d.f.	P
Twitch Duration					
10-90%	1	Acclimation	0.0079	1	0.931
		SNP	23.606	6	$< 2x10^{-16}$
		Freq.	1374.376	3	$< 2x10^{-16}$
		Acclimation*SNP	0.548	6	0.772
		Acclimation*Freq.	18.632	3	$3.63x10^{-11}$
		SNP*Freq.	0.779	18	0.725
		Acclimation*SNP*Freq.	0.481	18	0.965
90-10%	1	Acclimation	3.343	1	0.092
		SNP	39.638	6	$< 2x10^{-16}$
		Freq.	259.358	3	$< 2x10^{-16}$
		Acclimation*SNP	0.137	6	0.991
		Acclimation*Freq.	7.685	3	$5.65x10^{-5}$
		SNP*Freq.	5.713	18	$8.20x10^{-12}$
		Acclimation*SNP*Freq.	0.191	18	1
10-10%	1	Acclimation	2.502	1	0.139
		SNP	40.149	6	$<2x10^{-16}$
		Freq.	525.827	3	$<2x10^{-16}$
		Acclimation*SNP	0.093	6	0.997
		Acclimation*Freq.	2.365	3	0.071
		SNP*Freq.	4.4	18	$1.69x10^{-8}$
		Acclimation*SNP*Freq.	0.178	18	1

sWork	2	Acclimation	1.361	1	0.266
		SNP	23.967	6	$< 2x10^{-16}$
		Strain	2765.757	1	$< 2x10^{-16}$
		Freq.	50.898	3	$< 2x10^{-16}$
		Acclimation*SNP	1.106	6	0.357
		Acclimation *Strain	46.765	1	$1.84x10^{-11}$
		SNP*Strain	0.096	6	0.996
		Acclimation *Freq.	0.316	3	0.813
		SNP*Freq.	2.943	18	$4.39x10^{-5}$
		Strain*Freq.	2.621	3	0.0499
		Acclimation *SNP*Strain	0.095	6	0.996
		Acclimation *SNP*Freq.	0.15	18	1
		Acclimation *Strain*Freq.	0.071	3	0.975
		SNP*Strain*Freq.	0.106	18	1
		Acclimation *SNP*Strain*Freq.	0.018	18	1
lWork	2	Acclimation	0.062	1	0.807
		SNP	12.731	6	$1.28x10^{-13}$
		Strain	2316.313	1	$< 2x10^{-16}$
		Freq.	80.558	3	$< 2x10^{-16}$
		Acclimation*SNP	0.36	6	0.904
		Acclimation *Strain	3.358	1	0.067
		SNP*Strain	0.098	6	0.996
		Acclimation *Freq.	1.113	3	0.343
		SNP*Freq.	0.094	18	1
		Strain*Freq.	14.746	3	$2.67x10^{-9}$
		Acclimation *SNP*Strain	0.009	6	1
		Acclimation *SNP*Freq.	0.01	18	1
		Acclimation *Strain*Freq.	0.037	3	0.990
		SNP*Strain*Freq.	0.011	18	1
		Acclimation *SNP*Strain*Freq.	0.012	18	1
nWork	2	Acclimation	1.997	1	0.183
		SNP	7.535	6	$7.93x10^{-8}$
		Strain	331.597	1	$< 2x10^{-16}$
		Freq.	295.121	3	$< 2x10^{-16}$
		Acclimation*SNP	0.761	6	0.601
		Acclimation *Strain	42.478	1	$1.43x10^{-10}$
		SNP*Strain	0.473	6	0.829
		Acclimation *Freq.	0.18	3	0.91
		SNP*Freq.	4.143	18	$2.55x10^{-8}$
		Strain*Freq.	30.343	3	$< 2x10^{-16}$
		Acclimation *SNP*Strain	0.114	6	0.995
		Acclimation *SNP*Freq.	0.303	18	0.998
		Acclimation *Strain*Freq.	0.247	3	0.863
		SNP*Strain*Freq.	0.15	18	1
		Acclimation *SNP*Strain*Freq.	0.026	18	1

sPower	2	Acclimation	1.477	1	0.247
		SNP	5.775	6	7.15×10^{-6}
		Strain	1018.442	1	$< 2 \times 10^{-16}$
		Freq.	834.593	3	$< 2 \times 10^{-16}$
		Acclimation*SNP	0.256	6	0.957
		Acclimation *Strain	19.985	1	9.2×10^{-6}
		SNP*Strain	0.052	6	0.999
		Acclimation *Freq.	16.172	3	3.77×10^{-10}
		SNP*Freq.	0.106	18	1
		Strain*Freq.	55.516	3	$< 2 \times 10^{-16}$
		Acclimation *SNP*Strain	0.029	6	1
		Acclimation *SNP*Freq.	0.022	18	1
		Acclimation *Strain*Freq.	1.423	3	0.235
		SNP*Strain*Freq.	0.029	18	1
		Acclimation *SNP*Strain*Freq.	0.004	18	1
lPower	2	Acclimation	0.6539	1	0.434
		SNP	5.969	6	4.37×10^{-6}
		Strain	1033.691	1	$< 2 \times 10^{-16}$
		Freq.	736.941	3	$< 2 \times 10^{-16}$
		Acclimation*SNP	0.125	6	0.993
		Acclimation *Strain	1.675	1	0.196
		SNP*Strain	0.085	6	0.998
		Acclimation *Freq.	0.094	3	0.963
		SNP*Freq.	0.452	18	0.976
		Strain*Freq.	109.79	3	$<2 \times 10^{-16}$
		Acclimation *SNP*Strain	0.005	6	1
		Acclimation *SNP*Freq.	0.034	18	1
		Acclimation *Strain*Freq.	0.113	3	0.953
		SNP*Strain*Freq.	0.012	18	1
		Acclimation *SNP*Strain*Freq.	0.007	18	1
nPower	2	Acclimation	2.296	1	0.155
		SNP	1.36	6	0.228
		Strain	127.221	1	$< 2 \times 10^{-16}$
		Freq.	218.209	3	$< 2 \times 10^{-16}$
		Acclimation*SNP	0.443	6	0.850
		Acclimation *Strain	26.738	1	3.10×10^{-7}
		SNP*Strain	0.448	6	0.846
		Acclimation *Freq.	38.839	3	$< 2 \times 10^{-16}$
		SNP*Freq.	1.617	18	0.0506
		Strain*Freq.	8.633	3	1.26×10^{-5}
		Acclimation *SNP*Strain	0.061	6	0.999
		Acclimation *SNP*Freq.	0.094	18	1
		Acclimation *Strain*Freq.	1.528	3	0.206
		SNP*Strain*Freq.	0.092	18	1
		Acclimation *SNP*Strain*Freq.	0.01	18	1

RelsPower	2	Acclimation	0.096	1	0.761
		SNP	36.235	6	$< 2x10^{-16}$
		Strain	132.332	1	$< 2x10^{-16}$
		Freq.	121.148	3	$< 2x10^{-16}$
		Acclimation*SNP	0.875	6	0.513
		Acclimation *Strain	3.083	1	0.079
		SNP*Strain	4.991	6	$5.18x10^{-5}$
		Acclimation *Freq.	1.348	3	0.257
		SNP*Freq.	4.812	18	$3.35x10^{-10}$
		Strain*Freq.	0.599	3	0.615
		Acclimation *SNP*Strain	0.133	6	0.992
		Acclimation *SNP*Freq.	0.086	18	1
		Acclimation *Strain*Freq.	1.391	3	0.244
		SNP*Strain*Freq.	0.041	18	1
		Acclimation *SNP*Strain*Freq.	0.072	18	1
RellPower	2	Acclimation	0.38	1	0.549
		SNP	29.994	6	$< 2x10^{-16}$
		Strain	109.795	1	$< 2x10^{-16}$
		Freq.	19.661	3	$3.26x10^{-12}$
		Acclimation*SNP	1.512	6	0.171
		Acclimation *Strain	1.656	1	0.198
		SNP*Strain	3.561	6	0.001
		Acclimation *Freq.	1.863	3	0.134
		SNP*Freq.	0.587	18	0.910
		Strain*Freq.	2.219	3	0.084
		Acclimation *SNP*Strain	0.056	6	0.999
		Acclimation *SNP*Freq.	0.078	18	1
		Acclimation *Strain*Freq.	0.075	3	0.973
		SNP*Strain*Freq.	0.068	18	1
		Acclimation *SNP*Strain*Freq.	0.012	18	1
RelnPower	2	Acclimation	0.989	1	0.339
		SNP	4.005	6	0.0006
		Strain	56.224	1	$2.11x10^{-13}$
		Freq.	152.342	3	$< 2x10^{-16}$
		Acclimation*SNP	1.146	6	0.333
		Acclimation *Strain	0.534	1	0.465
		SNP*Strain	3.141	6	0.004
		Acclimation *Freq.	5.659	3	0.0007
		SNP*Freq.	8.198	18	$< 2x10^{-16}$
		Strain*Freq.	7.506	3	$6.05x10^{-5}$
		Acclimation *SNP*Strain	0.087	6	0.997
		Acclimation *SNP*Freq.	0.268	18	0.999
		Acclimation *Strain*Freq.	0.172	3	0.915
		SNP*Strain*Freq.	0.37	18	0.992
		Acclimation *SNP*Strain*Freq.	0.159	18	0.999

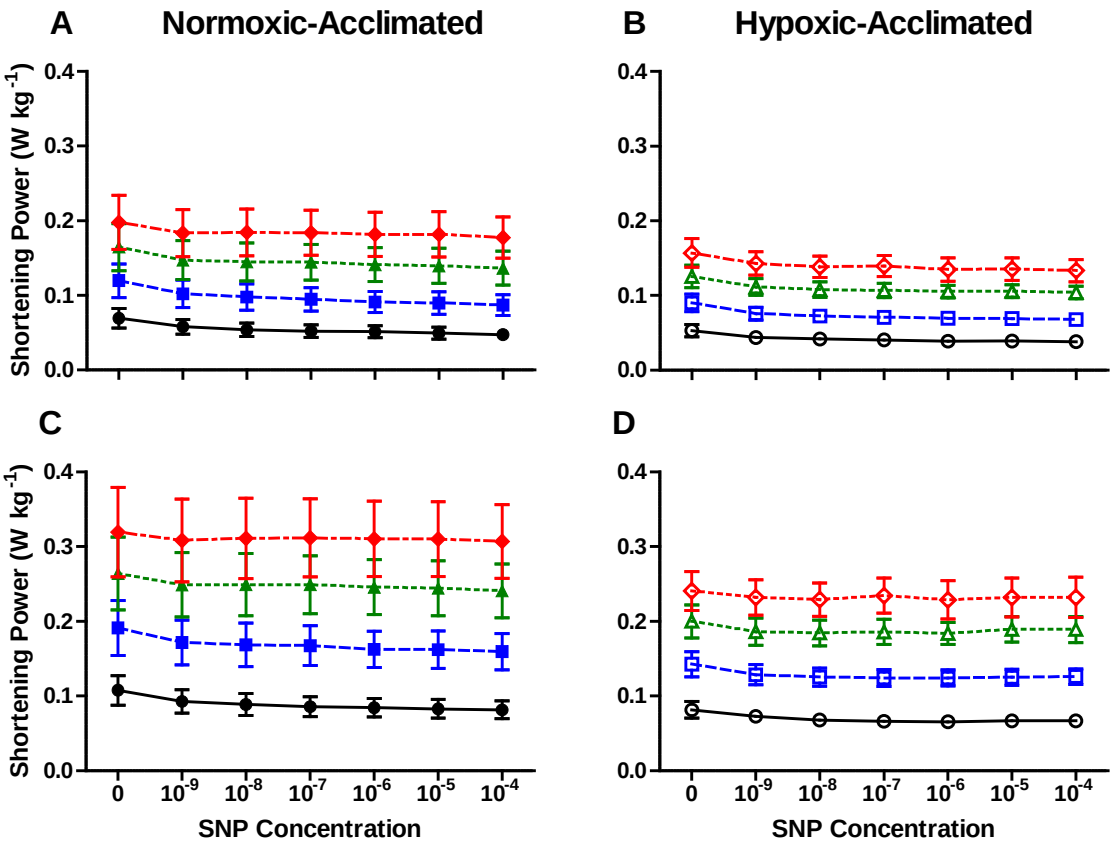

Figure 3.4. Shortening power done by ventricular trabeculae from normoxic-acclimated (solid symbols; left panels) and hypoxic-acclimated (open symbols; right panels) steelhead trout when exposed to increasing SNP concentrations (10^{-9}-10^{-4} M) at four different contraction frequencies and at 8% (A and B) and 14% (C and D) muscle strain at 14ºC. The trabeculae were cycled at 20 (black ● and ○,—), 40 (blue ■ and □, ---), 60 (green ▲ and △, ···) and 80 (red ◆ and ◇,– – –) contractions min^{-1}. Values are means ± SE; *n* = 8 normoxic and *n* = 6 hypoxic acclimated.

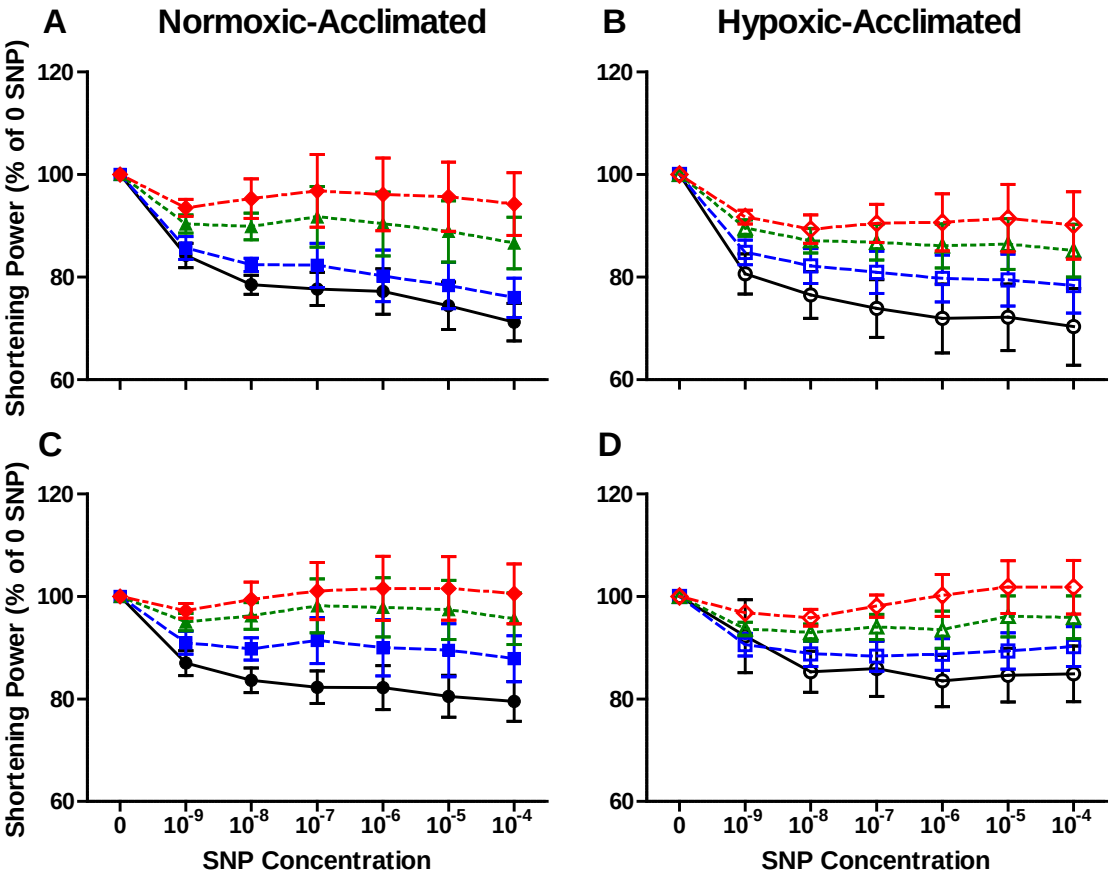

Figure 3.5. Relative shortening power done by ventricular trabeculae from normoxic-acclimated (solid symbols; left panels) and hypoxic-acclimated (open symbols; right panels) steelhead trout when exposed to increasing SNP concentrations (10^{-9}-10^{-4} M) at four different contraction frequencies and at 8% (A and B) and 14% (C and D) muscle strain at 14ºC. The trabeculae were cycled at 20 (black ● and ○,—), 40 (blue ■ and □, ---), 60 (green ▲ and △, ···) and 80 (red ◆ and ◇,-·-·) contractions min^{-1}. Values are expressed as % change relative to the power output measured before SNP addition using values from individual strips. Values are means ± SE; *n* = 8 normoxic and *n* = 6 hypoxic acclimated.

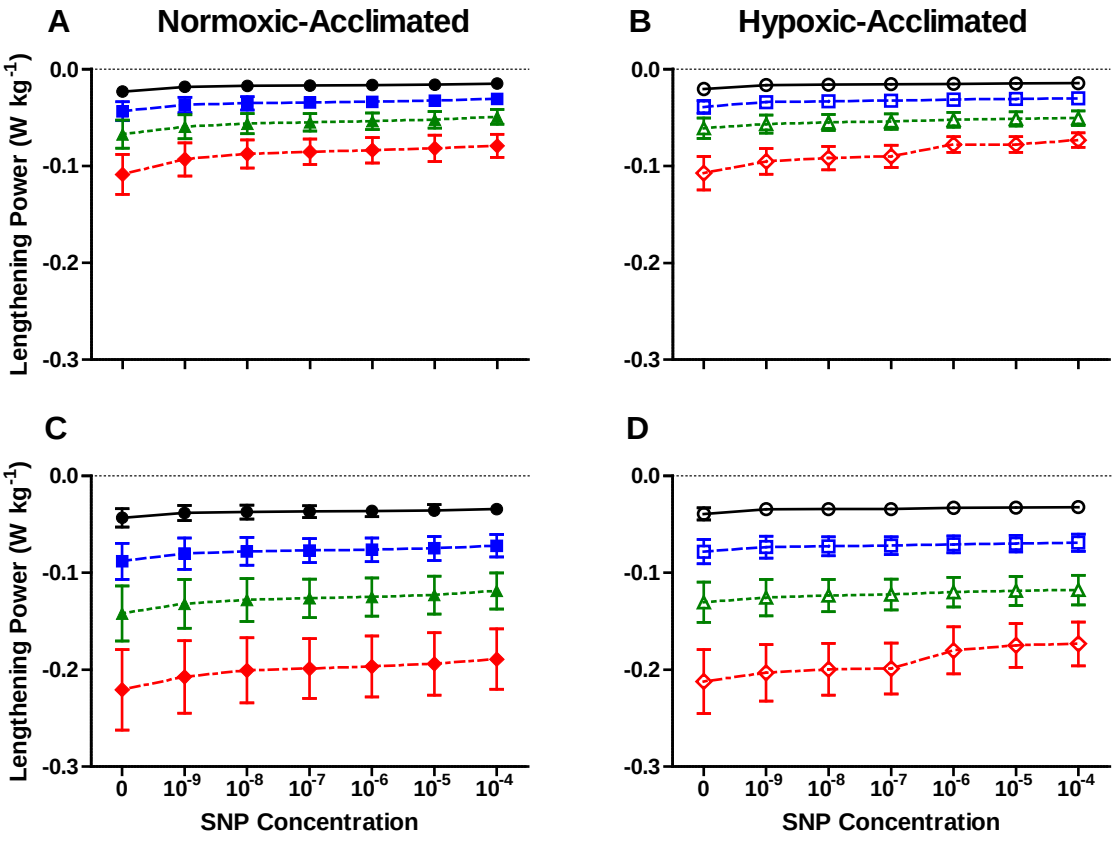

Figure 3.6. Lengthening power required to stretch ventricular trabeculae from normoxic-acclimated (solid symbols; left panels) and hypoxic-acclimated (open symbols; right panels) steelhead trout when exposed to increasing SNP concentrations (10^{-9}-10^{-4} M) at four different contraction frequencies and at 8% (A and B) and 14% (C and D) muscle strain at 14ºC. The trabeculae were cycled at 20 (black ● and ○,—), 40 (blue ■ and □, ---), 60 (green ▲ and △, ···) and 80 (red ◆ and ◇,-·-·) contractions min^{-1}. Lengthening work is shown as a negative value to reflect the power that was done by the servomotor to stretch the muscle. Values are means ± SE; *n* = 8 normoxic and *n* = 6 hypoxic acclimated.

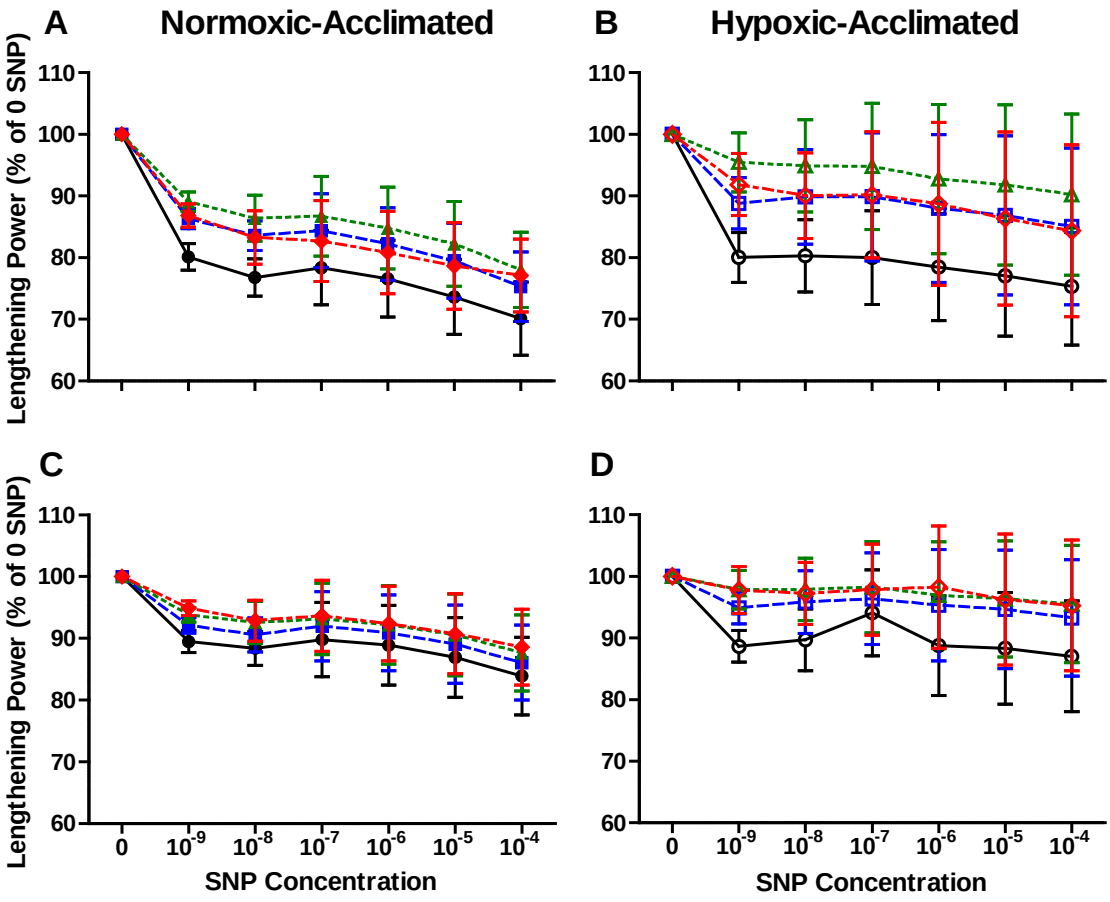

Figure 3.7. Relative lengthening power required to stretch ventricular trabeculae from normoxic-acclimated (solid symbols; left panels) and hypoxic-acclimated (open symbols; right panels) steelhead trout when exposed to increasing SNP concentrations (10^{-9}-10^{-4} M) at four different contraction frequencies and at 8% (A and B) and 14% (C and D) muscle strain at 14ºC. The trabeculae were cycled at 20 (black ● and ○,—), 40 (blue ■ and □, ---), 60 (green ▲ and △, ···) and 80 (red ◆ and ◇,-·-·-) contractions min^{-1}. Values are expressed as % change relative to the power output measured before SNP addition using values from individual strips. Values are means ± SE; *n* = 8 normoxic and *n* = 6 hypoxic acclimated.

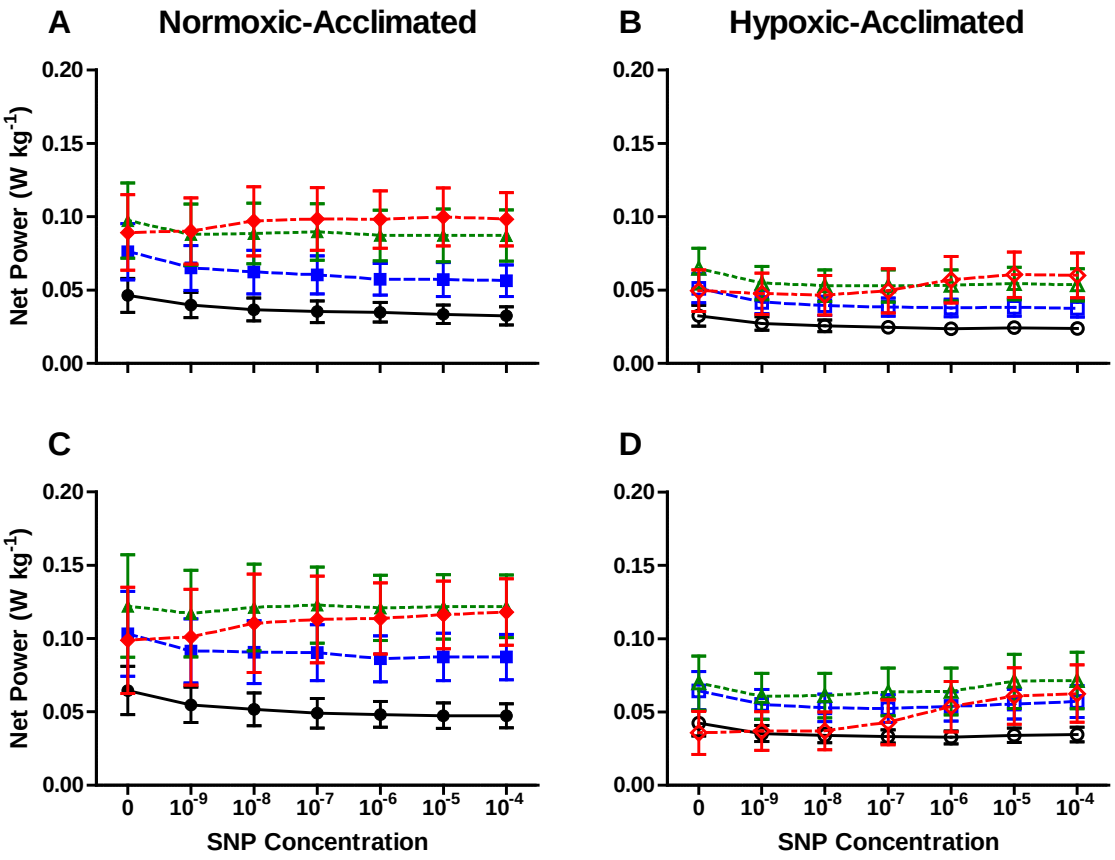

Figure 3.8. Net power done by ventricular trabeculae from normoxic-acclimated (solid symbols; left panels) and hypoxic-acclimated (open symbols; right panels) steelhead trout when exposed to increasing SNP concentrations (10^{-9}-10^{-4} M) at four different contraction frequencies and at 8% (A and B) and 14% (C and D) muscle strain at 14ºC. The trabeculae were cycled at 20 (black ● and ○,—), 40 (blue ■ and □, ---), 60 (green ▲ and △, ···) and 80 (red ◆ and ◇,-·-·) contractions min^{-1}. Values are means ± SE; *n* = 8 normoxic and *n* = 6 hypoxic acclimated.

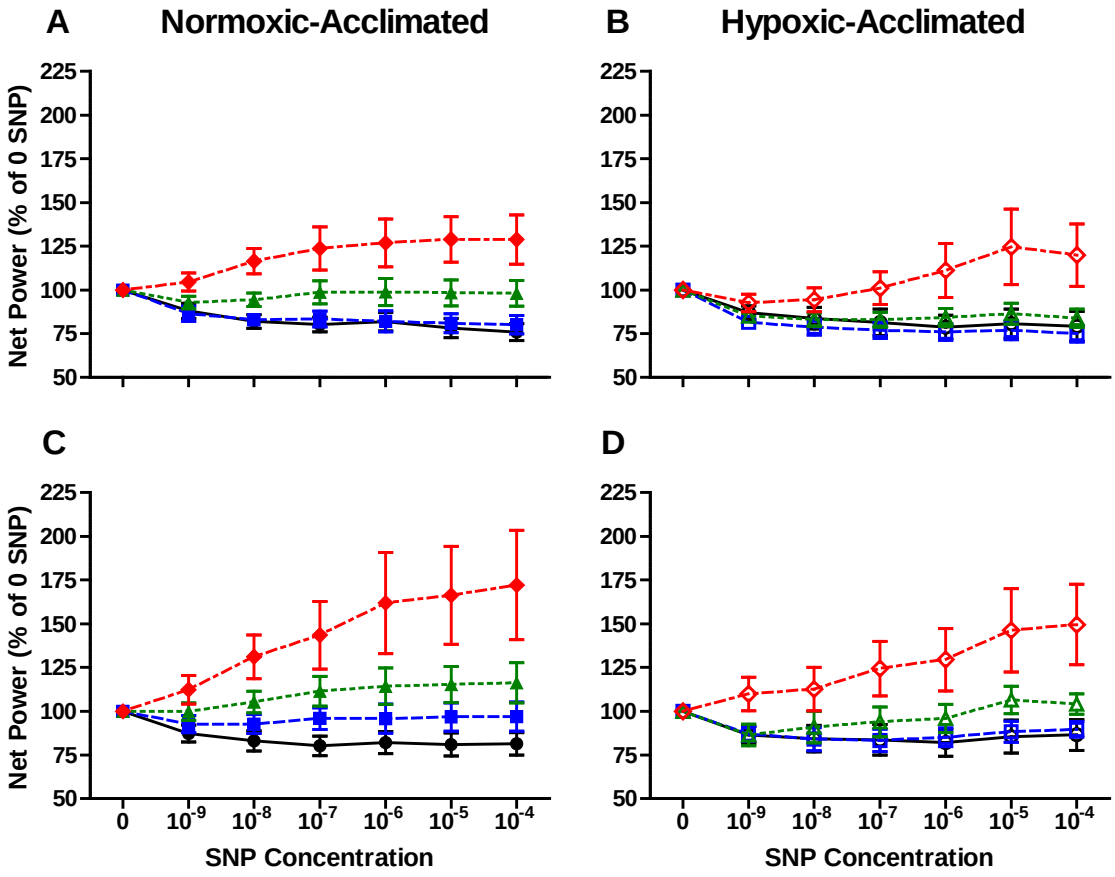

Figure 3.9. Relative net power done by ventricular trabeculae from normoxic-acclimated (solid symbols; left panels) and hypoxic-acclimated (open symbols; right panels) steelhead trout when exposed to increasing SNP concentrations (10^{-9}-10^{-4} M) at four different contraction frequencies and at 8% (A and B) and 14% (C and D) muscle strain at 14ºC. The trabeculae were cycled at 20 (black ● and ○,—), 40 (blue ■ and □, ---), 60 (green ▲ and △, ···) and 80 (red ◆ and ◇,-·-·-) contractions min^{-1}. Values are expressed as % change relative to the power output measured before SNP addition using values from individual strips. Values are means ± SE; *n* = 8 normoxic and *n* = 6 hypoxic acclimated.

reduction in relative net power in both groups at 20 and 40 contractions min^{-1}. At 60 contractions min^{-1}, the addition of SNP had little to no effect on relative net power. Finally, in all strain/group combinations, increasing the [SNP] had a positive effect on relative net power at 80 contractions min^{-1}; this increase ranged from 25% in both acclimation groups at 8% strain, to 70% in strips from normoxic-acclimated fish when tested at 14% strain.

3.4.4. *Effects of NOS and sGC Inhibition on Myocardial Performance at Varying Frequencies, Strains and Acclimation Conditions*

There were numerous acclimation, strain, frequency and 'drug' effects, as well as interactions between these fixed factors (see Table 3.4.). For clarity of presentation, graphs of shortening, lengthening and net work were omitted, and results for these parameters are limited to presentation of statistical tests (Table 3.4.). When cycled at 80 contractions min^{-1} at both strains, only 50% of the muscle strips from normoxic-acclimated fish were able to maintain regular muscle contractions when ODQ was applied. Thus, the data for this frequency were not included in the analysis / graphs.

3.4.4.1. *Effects of NOS and sGC Inhibition on Twitch Duration*

The effect of NOS and sGC inhibition on twitch duration was assessed by comparing the duration of the isometric contractions before and after the addition of L-NMMA, ODQ and SNP (Fig. 3.10.). Although the various measures of twitch duration were inversely related to contraction frequency prior to the addition of various agents (i.e., the various intervals decreased by 15 - 20% as contraction frequency was increased from

20 – 60 min^{-1}), acclimation condition had no significant effect ($P < 0.05$) (Table 3.4.). Inhibition of NOS by L-NMMA resulted in small (i.e., ~5%) decreases in the time for force to go from 10 to 90% of maximal at 40 and 60 min^{-1} in both groups, but a slight increase or no change at 20 min^{-1}. In contrast, L-NMMA application decreased the duration of the 90 – 10% force interval at all frequencies (by 5 to 15%), and this resulted a decrease in the duration of the entire contraction cycle (10 – 10% of maximum force). Application of ODQ resulted in similar effects on the period of contraction (i.e., 10 – 90% maximum force). However, application of this drug resulted in an increase in both the 10 – 90% maximum force and 10 – 10% maximum force intervals at all frequencies; with many of these values returning to levels observed prior to the application of drugs. SNP did not have any significant effect on the various measures of twitch duration. This is in clear contrast to the substantial decrease in the duration of relaxation (90 – 10% force) and of the contraction cycle (10 – 10% force) when 10^{-4} SNP was applied alone (see Figure 3.3.), and suggests that 10^{-5} M ODQ was effective at largely blocking the effects on SNP on myocardial function (also see Figs. 3.9.- 3.11.).

3.4.4.2. *Effects of NOS and sGC Inhibition on Relative Myocardial Power*

3.4.4.2.1 Shortening Power

While there was no apparent effect of strain on the effects of the blockers on relative shortening power, the response of the strips to L-NMMA (NOS blocker) and ODQ (inhibitor of sGC) was greatly influenced by both frequency and acclimation condition. For example, in the normoxia-acclimated group there was a frequency-dependent reduction in

this parameter (the effect more pronounced at low frequencies) after L-NMMA addition followed by an ODQ-mediated increase to values equal to, or slightly higher, than those measured with saline alone (Fig. 3.11. A and C). In contrast, in the hypoxia-acclimated group, L-NMMA resulted in a decrease in relative shortening power at 20 contractions min^{-1} (by ~12%), but almost no change at 60 contractions min^{-1} (~1% increase). Further, the increase in relative shortening power was much greater in hypoxia vs. normoxia acclimated fish (e.g. ~20% vs. 10% at 60 contractions min^{-1}, respectively) following the addition of ODQ.

3.4.4.2.2. Lengthening Power

As with shortening power (Fig. 3.11.), strain (8 vs. 14%) did not have a significant effect on the effects of the drugs on lengthening power. However, the response to the drugs was different between the two acclimation groups (Acclimation*DrugApp $P = 9.82 \times 10^{-14}$). Relative lengthening power decreased slightly following the addition of both L-NMMA and ODQ in normoxia-acclimated trout, with the effect being greater at the lowest frequencies [25% at 20 vs. 15% at 60 contractions min^{-1} (Fig. 3.12. A and C)]. In strips from hypoxic-acclimated trout, L-NMMA addition had the opposite effect, with relative lengthening power increasing at both muscle strains by ~10 – 15% at all frequencies. (Fig. 3.12. B and D). This difference in response pattern between acclimation groups continued with respect to the effects of ODQ. Instead of relative lengthening power consistently decreasing following the addition of ODQ into the bath, the application of ODQ in the hypoxic-acclimated group resulted in an ~10% reduction in relative lengthening power at lower frequencies, but no effect (or even a slight decrease) at 60 contractions min^{-1}. This

difference in response pattern ultimately resulted in the relative lengthening power after L-NMMA and ODQ addition being not different at 20 contractions min^{-1} to as much as 15% greater in strips cycled at 60 contractions min^{-1}.

3.4.4.2.3. Net Power

The effects of the applied drugs on relative net power were not influenced by acclimation condition. However, there were clear frequency and strain effects (Fig. 3.13.). For example: 1) relative net power at 8% strain decreased by approximately 20% and 10% following the addition of L-NMMA at 20 and 40 contractions min^{-1}, respectively, but was unchanged or increased slightly at 60 contractions min^{-1}; 2) the increase in relative net power after the addition of ODQ at 8% strain was positively related to frequency and this, combined with the effect of frequency on L-NMMA mediated changes in relative net power, meant that this parameter was elevated by approx. 40% vs. 25% at 60 vs. 20 contractions min^{-1}; and 3) these effects were more pronounced when the strips were tested at 14% strain, and thus, values for relative net power after ODQ were approx. 2-fold higher as compared to those measured at 8% strain.

3.5. Discussion

3.5.1. *Effects of Chronic Hypoxia on Fish Size and Cardiac Morphometrics*

Acclimation to chronic hypoxia had no significant effect on fish mass, length, condition factor, ventricular mass or RVM in the present study. However, fish mass tended to be lower in hypoxia-acclimated trout, and RVM tended to be higher in this group, in

Table 3.4. Results of the split plot mixed general linear model (Method 1) analysis which examined the effects of acclimation treatment, frequency and drug application on three measures of twitch duration. Results of the split-split plot mixed general linear model (Method 2) analysis that was used to examine the effects of acclimation treatment, frequency, strain and drug application on shortening, lengthening and net work and power as well as relative shortening, lengthening and net power.

Parameter	Method	Factor(s)	F	d.f.	P
Twitch Duration					
10-90%	1	Acclimation	0.653	1	0.436
		DrugApp	9.163	3	1.64×10^{-5}
		Freq.	439.424	2	$< 2 \times 10^{-16}$
		Acclimation*DrugApp	0.429	3	0.733
		Acclimation*Freq.	1.386	2	0.254
		DrugApp*Freq.	8.445	6	1.17×10^{-7}
		Acclimation*DrugApp*Freq.	0.359	6	0.903
90-10%	1	Acclimation	0.705	1	0.419
		DrugApp	104.769	3	$< 2 \times 10^{-16}$
		Freq.	103.083	2	$< 2 \times 10^{-16}$
		Acclimation*DrugApp	0.729	3	0.536
		Acclimation*Freq.	2.486	2	0.088
		DrugApp*Freq.	7.654	6	5.57×10^{-7}
		Acclimation*DrugApp*Freq.	0.741	6	0.618
10-10%	1	Acclimation	0.902	1	0.363
		DrugApp	56.721	3	$< 2 \times 10^{-16}$
		Freq.	233.853	2	$< 2 \times 10^{-16}$
		Acclimation*DrugApp	0.553	3	0.647
		Acclimation*Freq.	2.74	2	0.0686
		DrugApp*Freq.	1.836	6	0.098
		Acclimation*DrugApp*Freq.	0.253	6	0.957

sWork	2	Acclimation	0.605	1	0.453
		DrugApp	14.935	3	5.56×10^{-9}
		Strain	1098.279	1	$< 2 \times 10^{-16}$
		Freq.	17.041	2	1.14×10^{-7}
		Acclimation*DrugApp	4.684	3	0.003
		Acclimation*Strain	3.911	1	0.049
		DrugApp*Strain	1.823	3	0.144
		Acclimation*Freq.	1.059	2	0.348
		DrugApp*Freq.	1.141	6	0.339
		Strain*Freq.	0.444	2	0.641
		Acclimation*DrugApp*Strain	0.350	3	0.789
		Acclimation*DrugApp*Freq.	0.136	6	0.991
		Acclimation*Strain*Freq.	0.128	2	0.880
		DrugApp*Strain*Freq.	0.050	6	0.999
		Acclimation*DrugApp*Strain*Freq.	0.047	6	0.999
lWork	2	Acclimation	0.008	1	0.931
		DrugApp	11.829	3	2.82×10^{-7}
		Strain	867.474	1	$<2 \times 10^{-16}$
		Freq.	5.818	2	0.003
		Acclimation*DrugApp	3.915	3	0.009
		Acclimation*Strain	0.361	1	0.548
		DrugApp*Strain	0.558	3	0.643
		Acclimation*Freq.	0.094	2	0.910
		DrugApp*Freq.	0.558	6	0.764
		Strain*Freq.	2.047	2	0.131
		Acclimation*DrugApp*Strain	0.430	3	0.732
		Acclimation*DrugApp*Freq.	0.070	6	0.999
		Acclimation*Strain*Freq.	0.055	2	0.946
		DrugApp*Strain*Freq.	0.113	6	0.995
		Acclimation*DrugApp*Strain*Freq.	0.067	6	0.999
nWork	2	Acclimation	1.204	1	0.296
		DrugApp	53.538	3	$<2 \times 10^{-16}$
		Strain	267.42	1	$<2 \times 10^{-16}$
		Freq.	58.949	2	$<2 \times 10^{-16}$
		Acclimation*DrugApp	2.751	3	0.043
		Acclimation*Strain	10.356	1	0.001
		DrugApp*Strain	6.112	3	4.98×10^{-4}
		Acclimation*Freq.	2.258	2	0.107
		DrugApp*Freq.	0.688	6	0.659
		Strain*Freq.	4.23	2	0.016
		Acclimation*DrugApp*Strain	0.125	3	0.945
		Acclimation*DrugApp*Freq.	0.13	6	0.992
		Acclimation*Strain*Freq.	0.388	2	0.679
		DrugApp*Strain*Freq.	0.064	6	0.999
		Acclimation*DrugApp*Strain*Freq.	0.012	6	1

sPower	2	Acclimation	0.702	1	0.42
		DrugApp	7.582	3	7.10×10^{-5}
		Strain	502.269	1	$<2 \times 10^{-16}$
		Freq.	548.673	2	$<2 \times 10^{-16}$
		Acclimation*DrugApp	2.508	3	0.059
		Acclimation*Strain	2.219	1	0.138
		DrugApp*Strain	0.903	3	0.44
		Acclimation*Freq.	8.699	2	2.22×10^{-4}
		DrugApp*Freq.	1.512	6	0.174
		Strain*Freq.	38.243	2	3.08×10^{-15}
		Acclimation*DrugApp*Strain	0.196	3	0.899
		Acclimation*DrugApp*Freq.	0.381	6	0.891
		Acclimation*Strain*Freq.	0.428	2	0.653
		DrugApp*Strain*Freq.	0.144	6	0.990
		Acclimation*DrugApp*Strain*Freq.	0.037	6	1
lPower	2	Acclimation	0.006	1	0.939
		DrugApp	4.212	3	0.006
		Strain	420.53	1	$<2 \times 10^{-16}$
		Freq.	326.594	2	$<2 \times 10^{-16}$
		Acclimation*DrugApp	1.904	3	0.129
		Acclimation*Strain	0.218	1	0.641
		DrugApp*Strain	0.249	3	0.862
		Acclimation*Freq.	0.027	2	0.973
		DrugApp*Freq.	0.106	6	0.996
		Strain*Freq.	45.784	2	$<2 \times 10^{-16}$
		Acclimation*DrugApp*Strain	0.209	3	0.890
		Acclimation*DrugApp*Freq.	0.201	6	0.976
		Acclimation*Strain*Freq.	0.043	2	0.958
		DrugApp*Strain*Freq.	0.058	6	0.999
		Acclimation*DrugApp*Strain*Freq.	0.038	6	1
nPower	2	Acclimation	1.489	1	0.668
		DrugApp	34.421	3	$<2 \times 10^{-16}$
		Strain	141.028	1	2.26×10^{-9}
		Freq.	260.843	2	4.13×10^{-11}
		Acclimation*DrugApp	1.753	3	0.164
		Acclimation*Strain	7.815	1	0.309
		DrugApp*Strain	3.937	3	4.61×10^{-4}
		Acclimation*Freq.	19.286	2	0.606
		DrugApp*Freq.	4.048	6	4.92×10^{-4}
		Strain*Freq.	4.894	2	0.056
		Acclimation*DrugApp*Strain	0.093	3	0.925
		Acclimation*DrugApp*Freq.	0.265	6	0.997
		Acclimation*Strain*Freq.	1.426	2	0.902
		DrugApp*Strain*Freq.	0.408	6	0.824
		Acclimation*DrugApp*Strain*Freq.	0.022	6	1

RelsPower	2	Acclimation	3.096	1	0.106
		DrugApp	49.313	3	$<2x10^{-16}$
		Strain	26.41	1	0.52
		Freq.	35.119	2	$3.47x10^{-14}$
		Acclimation*DrugApp	15.49	3	$2.79x10^{-9}$
		Acclimation*Strain	0.252	1	0.616
		DrugApp*Strain	2.978	3	0.032
		Acclimation*Freq.	4.536	2	0.011
		DrugApp*Freq.	4.651	6	$1.66x10^{-4}$
		Strain*Freq.	0.022	2	0.978
		Acclimation*DrugApp*Strain	0.03	3	0.993
		Acclimation*DrugApp*Freq.	0.576	6	0.749
		Acclimation*Strain*Freq.	0.283	2	0.753
		DrugApp*Strain*Freq.	0.023	6	0.999
		Acclimation*DrugApp*Strain*Freq.	0.046	6	0.999
RellPower	2	Acclimation	4.327	1	0.061
		DrugApp	8.572	3	$1.93x10^{-5}$
		Strain	3.185	1	0.076
		Freq.	10.755	2	$3.29x10^{-5}$
		Acclimation*DrugApp	24.065	3	$9.82x10^{-14}$
		Acclimation*Strain	1.425	1	0.234
		DrugApp*Strain	0.438	3	0.726
		Acclimation*Freq.	0.154	2	0.857
		DrugApp*Freq.	2.012	6	0.065
		Strain*Freq.	1.546	2	0.2151
		Acclimation*DrugApp*Strain	0.203	3	0.894
		Acclimation*DrugApp*Freq.	0.126	6	0.993
		Acclimation*Strain*Freq.	0.076	2	0.927
		DrugApp*Strain*Freq.	0.252	6	0.958
		Acclimation*DrugApp*Strain*Freq.	0.038	6	0.999
RelnPower	2	Acclimation	0.1553	1	0.668
		DrugApp	80.333	3	$<2x10^{-16}$
		Strain	38.13	1	$2.62x10^{-9}$
		Freq.	26.32	2	$4.13x10^{-11}$
		Acclimation*DrugApp	1.717	3	0.164
		Acclimation*Strain	1.037	1	0.309
		DrugApp*Strain	6.171	3	$4.61x10^{-4}$
		Acclimation*Freq.	0.501	2	0.606
		DrugApp*Freq.	4.187	6	$4.92x10^{-4}$
		Strain*Freq.	2.917	2	0.056
		Acclimation*DrugApp*Strain	0.157	3	0.925
		Acclimation*DrugApp*Freq.	0.093	6	0.997
		Acclimation*Strain*Freq.	0.103	2	0.902
		DrugApp*Strain*Freq.	0.478	6	0.824
		Acclimation*DrugApp*Strain*Freq.	0.016	6	0.999

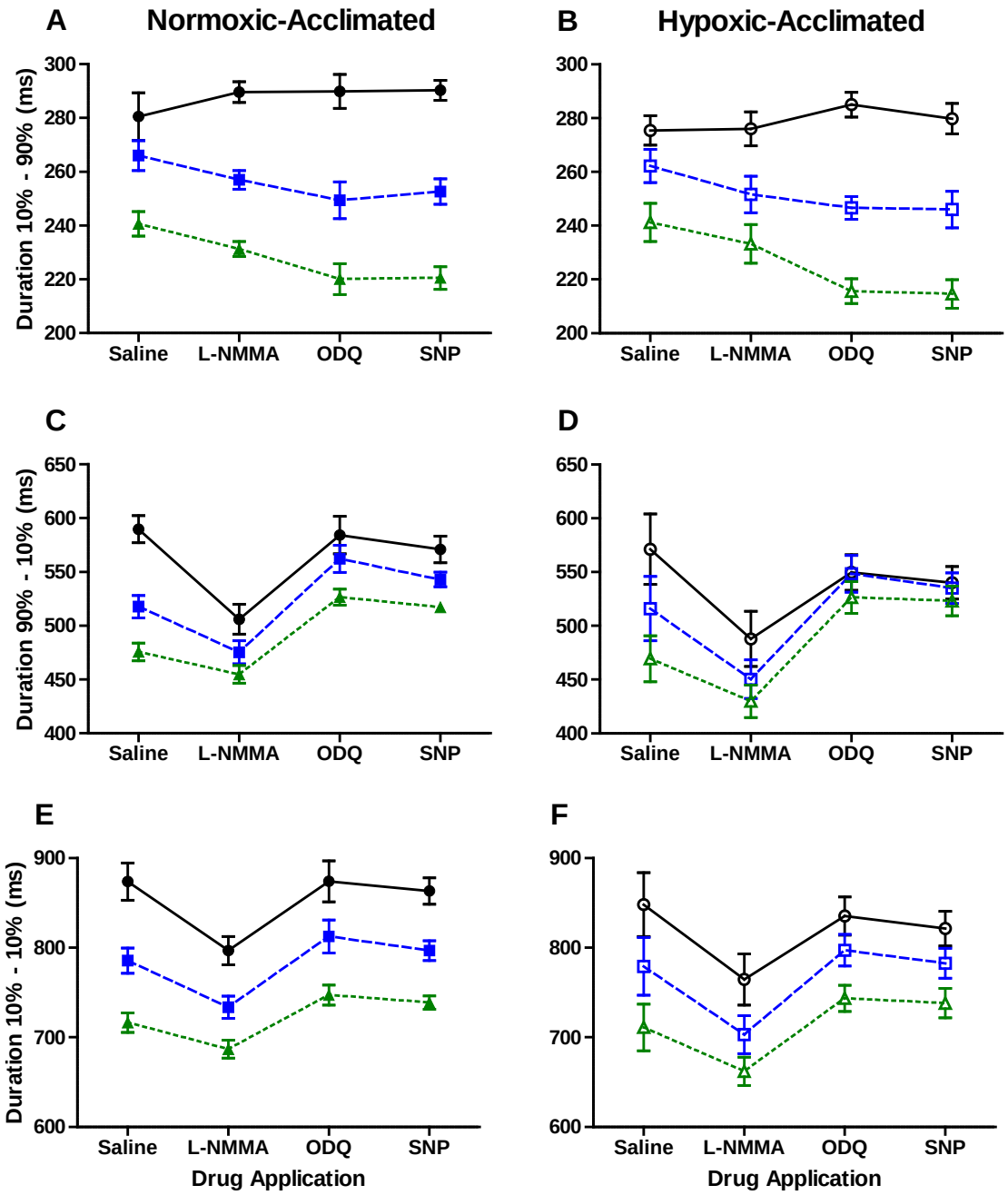

Figure 3.10. Twitch kinetics of normoxic-acclimated (solid symbols; left panels) and hypoxic-acclimated (open symbols; right panels) steelhead trout ventricular muscle strips in saline, when exposed 10^{-4} L-NMMA, 10^{-5} M ODQ and 10^{-4} SNP at four different contraction frequencies at 14ºC. Three measurements of twitch duration are shown: time for force to rise from 10 to 90% of maximal during contraction (A and B), time for force to fall from 90 to 10% of maximal force during relaxation (C and D), and time from 10% of maximal force during contraction to 10% of maximal force during relaxation (E and F). The trabeculae were stimulated (black ● and ○,—), 40 (blue ■ and □, ---) and 60 (green ▲ and △, ···) contractions min^{-1}. Values are means ± SE, n = 8 normoxic and n = 5 hypoxic acclimated.

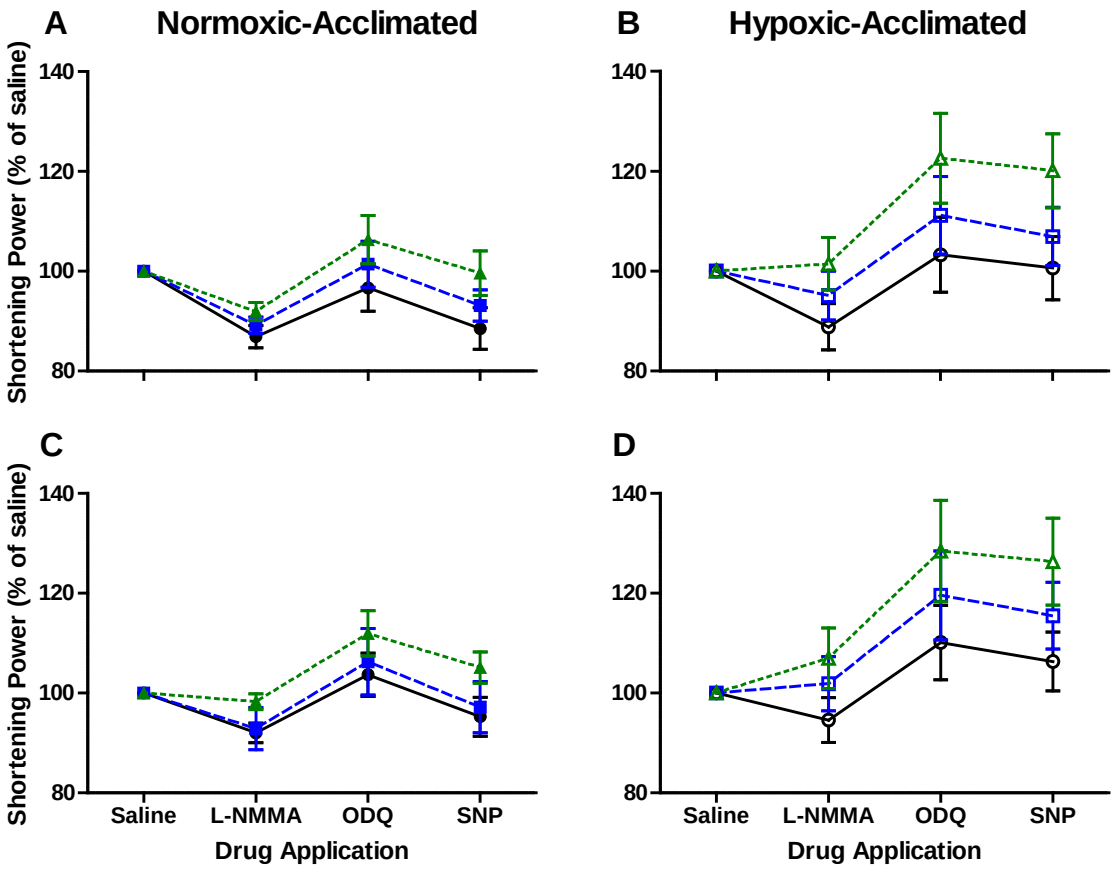

Figure 3.11. Relative shortening power done by ventricular trabeculae from normoxic-acclimated (solid symbols; left panels) and hypoxic-acclimated (open symbols; right panels) steelhead trout in saline, when exposed 10^{-4} L-NMMA, 10^{-5} M ODQ and 10^{-4} SNP at four different contraction frequencies and at 8% (A and B) and 14% (C and D) muscle strain at 14ºC. The trabeculae were cycled at 20 (black ● and ○,—), 40 (blue ■ and □, ---) and 60 (green ▲ and △, ···) contractions min^{-1}. Values are expressed as % change relative to the power output measured under saline using values from individual strips. Values are means ± SE; *n* = 8 normoxic and *n* = 5 hypoxic acclimated.

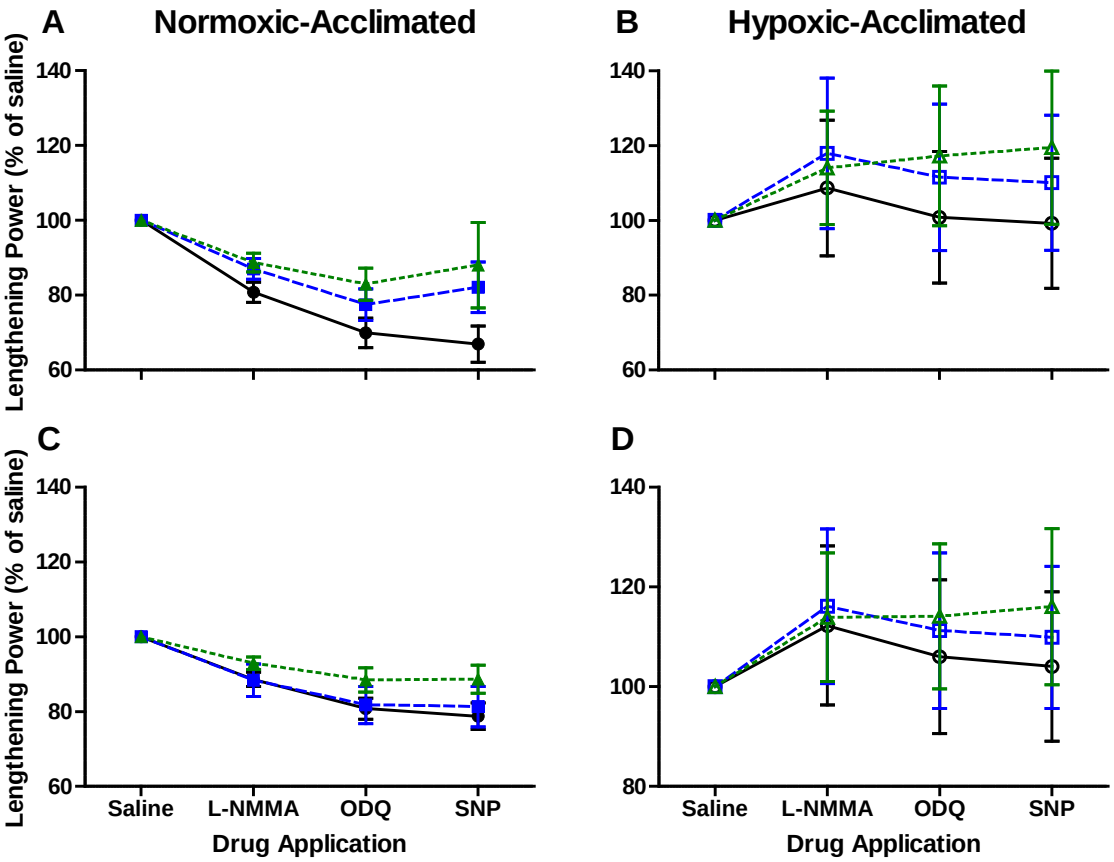

Figure 3.12. Relative lengthening power required to stretch ventricular trabeculae from normoxic-acclimated (solid symbols; left panels) and hypoxic-acclimated (open symbols; right panels) steelhead trout in saline, when exposed 10^{-4} L-NMMA, 10^{-5} M ODQ and 10^{-4} SNP at four different contraction frequencies and at 8% (A and B) and 14% (C and D) muscle strain at 14ºC. The trabeculae were cycled at 20 (black ● and ○,—), 40 (blue ■ and □, ---) and 60 (green ▲ and △, ···) contractions min^{-1}. Values are expressed as % change relative to the power output measured under saline using values from individual strips. Values are means ± SE; *n* = 8 normoxic and *n* = 5 hypoxic acclimated.

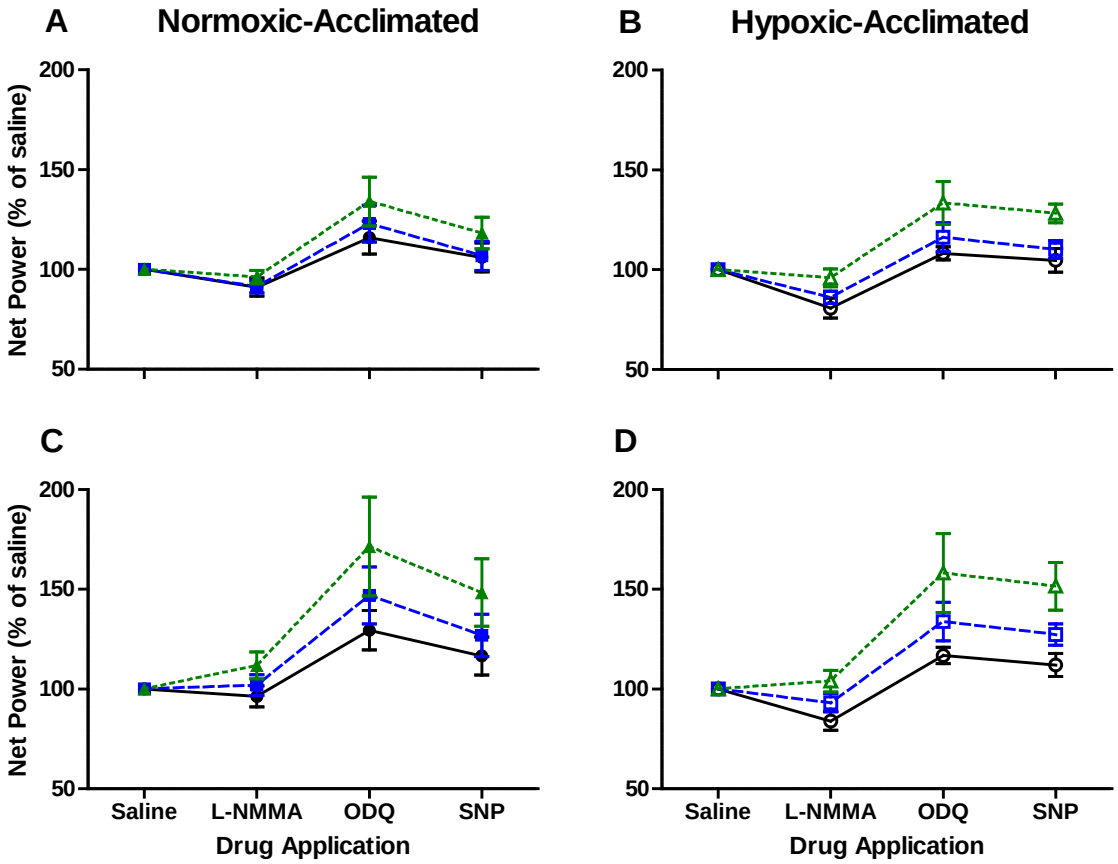

Figure 3.13. Relative net power done by ventricular trabeculae from normoxic-acclimated (solid symbols; left panels) and hypoxic-acclimated (open symbols; right panels) steelhead trout in saline, when exposed 10^{-4} L-NMMA, 10^{-5} M ODQ and 10^{-4} SNP at four different contraction frequencies and at 8% (A and B) and 14% (C and D) muscle strain at 14ºC. The trabeculae were cycled at 20 (black ● and ○,—), 40 (blue ■ and □, ---) and 60 (green ▲ and △, ···) contractions min^{-1}. Values are expressed as % change relative to the power output measured under saline using values from individual strips. Values are means ± SE; *n* = 8 normoxic and *n* = 5 hypoxic acclimated.

both experiments (Table 2.1.). This trend agrees with the data from Carnevale *et al.* (submitted Chapter 2) where hypoxic-acclimated fish had a significantly lower body mass and higher RVM. It is not surprising that the hypoxia-acclimated trout were smaller, despite our attempt to provide equal amounts of food, as fish exposed to chronic hypoxia have been shown to have reduced size and growth (Chabot and Dutil 1999; Wu, 2002; Glencross, 2009; Moytka *et al.*, 2017). The higher RVM in both this study and Carnevale *et al.* (submitted; Chapter 2) is most likely due to a direct effect of chronic hypoxia, and not the result of the difference in body mass between the two groups. The relationship between ventricular mass and body mass in rainbow has a slope very close to 1 (exponent 0.968; Farrell *et al.*, 1988). In both studies, a higher temperature during acclimation (14 vs 10^{o}C) was used in comparison with previous studies (Petersen and Gamperl 2010; 2011; Moytka *et al.*, 2017), and the increase in cardiac workload associated with rearing at 14^{o}C (i.e., increased metabolic demand, decreased oxygen availability) might have required the ventricle to be larger relative to body mass to maintain oxygen delivery under hypoxic conditions. Indeed, the data of Antilla *et al.* (2015) suggest that repeated intermittent exposure to hypoxia (65% oxygen saturation for 4 weeks), in combination with increased temperatures (~15^{o}C), can increase ventricle size in salmonids.

3.5.2. *Effects of Increasing Muscle Strain and Frequency on Myocardial Performance*

Prior to the addition of SNP, the net work and power produced by the ventricular trabeculae of normoxic-acclimated steelhead trout were 0.105 J kg^{-1} and 0.077 W kg^{-1} at 8% strain and 0.137 J kg^{-1} and 0.097 W kg^{-1} at 14% strain, averaged across the four

frequencies employed. These values fall within the reported range for teleosts (Harwood *et al.*, 1998; 2002; Shiels *et al.*, 1998; Syme *et al.*, 2013), and our values at 8% strain are only slightly lower than that reported for cycling isolated trabeculae from the lumen of the ventricle of rainbow trout (0.13 J kg^{-1} and 0.12 W kg^{-1} at 12^{o}C and 3% strain; Shiels *et al.*, 1998) and Atlantic cod (0.15 J kg^{-1}, 0.09 W kg^{-1} at 10^{o}C and 8% strain; Syme *et al.*, 2013). Previously, Carnevale *et al.* (submitted; Chapter 2) reported net work and power values of 0.300 J kg^{-1} and 0.280 W kg^{-1} when averaged across the four frequencies used in their study (30, 50, 70 and 90 contractions min^{-1}). However, their protocol was specifically intended to maximize power output, whereas, the protocol utilized in this study was designed to reflect cardiac pumping under physiological conditions; i.e., the distortion and phase values were customized for each individual strip. This likely explains the lower values reported in this study vs. Carnevale *et al.* (submitted; Chapter 2).

When strain was increased from 8 to 14%, there was no significant increase in net power (Figure 3.8.) because increases in shortening power (Figure 3.2.) were offset by increases in lengthening power (Figure 3.6.). This was particularly noticeable at 80 contractions min^{-1} where lengthening power doubled in both acclimation groups when muscle strain was increased to 14%, and this resulted in 20 and 50% reductions in net power when contraction frequency was increased from 60 to 80 contractions min^{-1} in the normoxic and hypoxic-acclimated strips, respectively (Figs. 3.6. and 3.8.). This latter result is not that surprising as Harwood *et al.* (1998) showed that the lengthening work required to stretch compact muscle strips often increases more than shortening work at increased muscle strains, and that the strain that achieved maximum work output decreased as

frequency increased. The decrease in net power with increasing strain at high frequencies is likely due to several interacting factors. Fish cardiac muscle displays a "negative-staircase" relationship, where the isometric force decreases with increasing frequency (Driedzic and Gesser, 1988). Fish cardiomyocytes also have higher titin-based passive tension that results in massive increases in this parameter when muscle length increases above 100% L_{opt} (length of muscle at which developed twitch force is maximal; refer to Chapter 2) (Shiels *et al.*, 2006; Patrick *et al.* 2010). Importantly, as muscle strain has been suggested to be analogous to cardiac stroke volume (S_V) (Syme, 1993; Syme and Josephson, 1995), this relationship likely explains the significant reduction in maximum S_V when frequency increases *in situ* (Graham and Farrell, 1989; Farrell *et al.*, 1992). Further, these results may partially explain why S_V does not normally increase when fish are exposed to acute increases in temperature (Steinhausen *et al.*, 2008; Farrell *et al.*, 2009; Petersen *et al.*, 2010; Keen and Gamperl, 2012; Penney *et al.*, 2014; Motyka et a., 2017). In salmonids, heart rate (f_H) plateaus at 110 – 130 beats min^{-1}, ~2-3^oC before the fish's critical thermal maximum, and it would be very difficult to increase cardiac muscle strain, and thus S_V, due to the negative effects of strain on both lengthening and net power at high contraction frequencies.

3.5.3. *Myocardial Function and Power Output Following Hypoxic Acclimation*

There were several differences between the acclimation groups in terms of muscle performance, some which support those reported previously and other novel ones which expand on these results (e.g., Carnevale *et al.*, submitted.; Chapter 2). Similar to Carnevale

et al., (submitted; Chapter 2): 1) shortening power was 20 – 25% lower in strips from hypoxic-acclimated fish as compared to those from normoxic-acclimated individuals, whereas there was no difference in lengthening power (Figs. 3.2. and 3.4.); and 2) these changes resulted in net power values that were ~35% lower in strips from hypoxia-acclimated fish. A high degree of myocardial stunning is likely the reason for this significant reduction in myocardial performance (Carnevale *et al.*, submitted.; Chapter 2). Stunning can be defined as mechanical dysfunction that persists after re-oxygenation and/or reperfusion, and can occur without permanent damage to the myofibrils (Bolli and Marbán 1999). In addition, myocardial strips from hypoxic-acclimated fish took longer to relax when contracting isometrically (i.e., the time required for strips to go from 90 – 10% force and 10 – 10% force were 10 and 8% longer, respectively) (Figure 3.1.). While these results were not confirmed in the NOS inhibitor experiment (likely due to the small sample size; $n = 5$) (see Figure 3.10.), they suggest that there may be less time available to fill the heart of hypoxia-acclimated fishes. This decrease in the time available for cardiac filling, when combined with a reduced capacity of the myocardium to produce work and power (this study; Carnevale *et al.*, submitted.) may largely explain why *in situ* and *in vivo* cardiac function is compromised in hypoxic-acclimated fish (Petersen and Gamperl, 2010a, b; Petersen *et al.*, 2011; Moytka *et al.*, 2017).

3.5.4. *Effects of SNP (an Exogenous Source of NO) on Myocardial Performance in Strips from Normoxic-Acclimated Fish*

Sodium nitroprusside had a number of significant effects on the performance of the trout myocardial strips, and these are discussed in the following sections.

3.5.4.1. *Effects on the Duration of Contraction and Relaxation*

The duration of contraction (10 - 90% maximal twitch force) decreased slightly at all frequencies (by < 10%) as [SNP] was increased from 10^{-9} to 10^{-4} M. Further, there were substantial, but frequency-dependent (see below), decreases in the duration of relaxation (90-10% maximal twitch force) and total duration of muscle contraction (10-10% maximal twitch force). NO has previously been shown to enhance the rate of relaxation in the hearts of mammals and fish (Shah and Lewis, 1993; Joa *et al.*, 2000; Tota *et al.*, 2005), an effect which my data supports. This result can be partially explained by a decrease in action potential duration (APD) due to the NO-induced enhancement of sarcolemmal K_{ATP} channel activity (Cameron *et al.*, 2003), and activation of phosphodiesterase 2 (PDE2) which leads to the local depletion of cAMP within the microenvironment around L-type calcium channels and reduces Ca^{2+} trans-sarcolemmal influx (Dittrich *et al.*, 2001; Omori and Kotera, 2017). This reduction in relaxation duration, however, is likely also to include a cGMP-independent mechanism as NO mediates the S-nitrosylation of PLB which results in increased Ca^{2+} reuptake by the SERCA2a pumps, and this enhances the rate of myocyte relaxation (Imbrogno and Cerra, 2017; Imbrogno *et al.*, 2018). In our study, the effect of SNP on relaxation duration was concentration dependent for both acclimation groups. The effect of SNP (or exogenous NO donors in general) on twitch duration has not been extensively investigated in fish. However, a reduction in the total contraction period, due to the significant shortening of relaxation time, would benefit the teleost heart under conditions of high strain and/or frequency when ventricular filling time is shortened (Altimiras and Axelsson, 2004; Sandblom and Grans, 2017).

3.5.4.2. *The Effect of SNP (NO) on Myocardial Contractility*

NO is an important regulator of ventricular performance in fish hearts (Imbrogno, *et al.*, 2001; Gattuso *et al.*, 2002; Tota *et al.*, 2005; Garofalo *et al.*, 2009; Imbrogno *et al.*, 2014; Imbrogno and Cerra, 2017; Imbrogno *et al.*, 2018), and its effects are dependent upon the working conditions of the heart. Basally, NO acts as a negative inotrope, often resulting in reductions in S_V and stroke work [S_W; S_V x mean arterial pressure (MAP)] in species such as the eel (*Anguilla anguilla*), goldfish and salmon (*Salmo salar*) (Imbrogno, *et al.*, 2001; Garofalo *et al.*, 2009; Imbrogno *et al.*, 2014). Using various NO donors, previous studies have found a ~20% reduction in S_V at the highest concentration of donor used at 50 – 60 beats min^{-1} (Imbrogno, *et al.*, 2001; Garofalo *et al.*, 2009; Imbrogno *et al.*, 2014). To compare my data to these studies, the data collected from strips from normoxic-acclimated fish at 60 contractions min^{-1} and 8% muscle strain can be utilized. Under these conditions, we found a 10% reduction in net power in the current study. Multiple mechanisms are likely at play due to the experimental design which "flooded" the cell with NO. First, cGMP-mediated phosphorylation, and possibly S-nitrosylation, of the sarcolemmal K_{ATP} channel, results in shortening of the duration of the cardiac action potential (AP), and less Ca^{2+} entry and a weakened contraction of the myocardium (Imbrogno and Cerra, 2017; Imbrogno *et al.*, 2018). Second, a loss of myofilament Ca^{2+} sensitivity due to cGMP-mediated phosphorylation of troponin I and cMyBPC, and S-nitrosylation of other myofibrillar proteins (e.g. TNC-Cys35, -Cys84), resulting in negative inotropy (Imbrogno and Cerra, 2017; Zhang *et al.*, 2017; Imbrogno *et al.*, 2018). Third, S-nitrosylation of PLB results in increased Ca^{2+} re-uptake by the SERCA pump, enhanced

myocardial relaxation and diminished contractility (Imbrogno and Cerra, 2017; Imbrogno *et al.*, 2018). Finally, and in contrast to the above effects, enhanced Ca^{2+} release via the ryanodine receptor (RyR), due to the S-nitrosylation of thiol residues on these channels, may have had a positive influence on myocardial contraction (Imbrogno and Cerra, 2017; Imbrogno *et al.*, 2018) (for a summary of all these effects, see Figures 3.1. and 3.2.). Since lengthening work and power required to stretch the muscle decreased along with contractile force, it appears that the lusitropic effects (i.e., effects on the rate of relaxation) of NO dominated over positive inotropic effects when the strips were exposed the NO donor SNP.

In previous studies, the S_V of hypoxia-tolerant eel hearts was decreased in a dose-dependent manner in response to increasing concentrations of the NO donors SIN-1 (10^{-12} to 10^{-7} M) and SNAP (10^{-12} to 10^{-8} M) (Imbrogno, *et al.*, 2001; Garofalo *et al.*, 2009). However, we do not report a typical dose-dependent response of myocardial contractility to SNP in steelhead trout, and this may due to a few interacting factors. The use of exogenous NO donors (particularly at high concentrations) may not replicate the *in vivo* effects of constitutively expressed NOSs which produce NO in a spatial and temporal manner as well as in response to specific stimuli (Barouch *et al.*, 2002; Garofalo *et al.*, 2009; Imbrogno *et al.*, 2018). The different NO donors used in these experiments make comparisons difficult due to varying modes of NO delivery, and the downstream effectors activated. Finally, the effect of exogenous and endogenous NO on cardiomyocyte contractility can be concentration dependent, where positive effects are seen at low NO concentrations and negative effects at high concentrations (Shah and MacCarthy, 2000). In contrast to the study of Shah and MacCarthy (2000) on rats, 10^{-9} M SNP in our study

initially produced negative inotropy and this effect plateaued as [SNP] increased to 10^{-4} M in both groups of trout trabeculae. Further, other studies have reported dose-dependent decreases in contractility, S_V volume and Q (Imbrogno, *et al.*, 2001; Gattuso *et al.*, 2002; Garofalo *et al.,* 2009). It is possible that the enhanced negative inotropy at higher SNP concentrations which was expected (Imbrogno, *et al.*, 2001; Garofalo *et al.*, 2009) may have been off-set by enhanced RyR-dependent Ca^{2+} release (Imbrogno and Cerra, 2017; Imbrogno *et al.*, 2018), or that flooding the cell with a high [SNP] may have activated other NO autocrine or paracrine pathways that impacted the shape of the concentration-dependent curve.

3.5.5. Strain and Frequency Effects on SNP-Dependent Cardiac Function

The effect of exogenous NO on isometric twitch duration and muscle contractility was highly frequency dependent in both acclimation groups. The duration of relaxation was reduced by 25% at 20 contractions min^{-1}, but only by 5% at 80 contractions min^{-1}, in both treatment groups when exposed to 10^{-9} SNP (Figure 3.3.). This resulted in SNP's effect on total contraction duration being frequency dependent, despite no frequency effect on contraction duration. Frequency-dependent effects of SNP were also seen when the muscle was actively cycled (Figs. 3.3. – 3.7.). SNP had a large negative inotropic effect on shortening power at slower contraction frequencies and a weaker effect at higher frequencies, an effect mirrored by the response of lengthening power (the influence of SNP on lengthening power decreasing with frequency). The combined effect of reductions in both of these parameters was a 30% reduction in net power at 10^{-4} M SNP at 20 and 40

contractions min^{-1}, while this parameter increased by 25% at the same [SNP] when the frequency was raised to 80 contractions min^{-1} in strips from normoxic-acclimated fish (Figure 3.7. A). This frequency-dependent effect appeared to be magnified at 14% muscle strain, as net power increased by 75% as SNP concentration reached 10^{-4} M in this acclimation group (Figure 3.7. B). This highly frequency-dependent effect of exogenous SNP on myocardial function in fish has not been previously reported, and is one of the most important findings of this study. At present, we do not have an explanation for why exposure to SNP increased net power at high contractile rates. However, it is likely that the decreased negative effects of SNP on shortening and lengthening power were at least partially related to the stretched-induced S-nitrosylation of RyR thiol groups and increased SR Ca^{2+} release (see Figure 3.2.). Overall, these results suggest that while exogenous NO usually acts as a negative inotrope, it can improve myocardial performance under conditions of high heart rate and high strain.

3.5.6. *Effect of NOS and sGC Inhibition on Myocardial Contractility*

The second experiment investigated the effects of paracrine and autocrine produced NO on myocardial function, and the extent to which the effects of NO and NO_2^- are dependent on sGC mediated pathways. This is a much different experiment as compared to Experiment $^{\#}1$ and was likely to reveal different results given the spatial compartmentalization of NOS enzymes and their downstream effectors (Barouch *et al.*, 2002; Imbrogno *et al.*, 2017; Imbrogno and Cerra, 2017; Zhang *et al.*, 2017; Imbrogno *et al.*, 2018). When exposed to 10^{-4} M L-NMMA twitch duration decreased (Figure 3.10.),

and at 8% strain and a low contraction frequency both relative shortening and lengthening power decreased, and this resulted in a small (10-15%) decrease in relative net power (Figures 3.11.- 3.13.). These data suggest that L-NMMA blocked an autocrine or paracrine (i.e., from the endocardial endothelium) NOS-dependent NO signal that activates intracellular targets to elicit positive inotropic effects. This finding is hard to reconcile with studies on the frog and hypoxia-tolerant fishes (eel and goldfish) which have reported that NOS inhibition using various pharmacological agonists (e.g., L-NMMA, L-NAME, L-NIO) improves basal mechanical performance and increases S_V by ~10% in working heart preparations (Sys *et al.*, 1997; Imbrogno *et al.*, 2001; Imbrogno *et al.*, 2014), and with the fact that blockade of NOS-dependent NO generation by L-NMMA would be expected to increase twitch duration by removing the effect of this gasotransmitter on SERCA function. However, NO can have a positive inotropic effect mediated through the S-nitrosylation of the RyR (Figure 3.2.), and it is possible that this effect predominated over the negative effects of NOS-generated NO on myocardial contractility. This may be particularly relevant as our strips were being constantly stretched and L-NMMA has been shown to decrease the maximum, Frank-Starling-mediated, S_V achieved by perfused goldfish hearts (Imbrogno *et al.*, 2014).

Clearly, the effects of ODQ after L-NMMA suggest that there was a substantial NOS-independent, sGC-mediated, negative effect on myocardial contractility. However, this effect was somewhat surprising as L-NMMA would have blocked the formation of NO_2^- through the oxidation of NOS-generated NO, and these strips should not have been hypoxic during testing (i.e., it is unlikely that myoglobin was contributing significantly to

NO generation; Dungel *et al.*, 2017). While we cannot rule out that the observed response (e.g., increased net power following sGC blockade; Fig. 3.13.) was due to the cumulative / interactive effects of these two antagonists, it has recently been reported in mammalian cardiomyocytes that mitochondrial nitrite reductase operates at physiological oxygen partial pressures, and that it is mitochondria-derived NO regulated cGMP that mediates cardiomyocyte contractility (Dungel *et al.*, 2017). This raises the possibility that mitochondrial-derived NO plays a major role in the regulation of contractility in salmonids (fishes), a hypothesis that is somewhat supported by the results of Pedersen *et al.* (2010). These authors showed that exposure of trout myocardial strips to nitrite (13 μM) after NOS activity was blocked by asymmetric dimethylamine (ADMA, 150 μM) increased myocardial oxygen consumption and twitch force per O_2 consumed, and increased twitch force by 20% (although not significantly). This enhancement of contractility is quantitatively similar to what I found for strips from normoxic-acclimated trout following the application of ODQ at 8% strain and paced at 40-60 min^{-1} (Fig. 3.13.). SNP (10^{-4} M) only resulted in minor alterations in myocardial function after ODQ application (Figs. 3.8. – 3.11.) as compared to that seen in the first experiment. For example, relative shortening power only decreased by 5-10% with 10^{-4} SNP after L-NMMA/ODQ application (Fig. 3.9.), whereas this effect was as much as 30% in the first experiment (Fig. 3.3.). These data strongly suggest that ODQ was largely effective at blocking sCG/cGMP effects on myocardial function, and that the main effects of NO on the trout myocardium are mediated via this pathway. This hypothesis agrees with Imbrogno *et al.* (2001) who showed that ODQ blocked L-arginine's effects on basal cardiac function in the eel, and with Imbrogno

et al. (2014) who showed that P-TIO (a NO scavenger) and ODQ resulted in similar reductions in the Frank Starling response of goldfish hearts under hypoxia. Further, this finding agrees with data collected on other tissues. For example, Jensen *et al.* (2016) showed that pre-incubation with ODQ reduced NO's effect on epithelial short-circuit current by ~85% in killifish (*Fundulus heteroclitus*).

The effect of L-NMMA on relative net power in normoxic-acclimated fish was slightly negative at 20 min^{-1} and 8% strain, whereas it was positive at 60 min^{-1} and 14% strain, and the effect of ODQ was much greater in the latter (Fig. 3.13. A and C). These results are in agreement with the data for Experiment $^{\#}1$, and provide further evidence that contraction frequency and strain have major effects on NO-mediated myocardial contractility (Figs. 3.11. – 3.13.).

3.5.7. *Hypoxic Acclimation and NO-Mediated Myocardial Effects*

This is the first study to examine the effects of chronic hypoxic exposure on NO-mediated myocardial function in fishes. I was able to show that hypoxic acclimation increased the duration of relaxation (i.e., 90% to 10% of maximal force) by ~10% (Fig. 3.3.). However, the effects of exogenous NO (SNP) on myocardial power were not significantly different in strips from hypoxic- vs. normoxic-acclimated fish (e.g. see Figs. 3.5.-3.9.) Further, although hypoxic acclimation prevented the decrease in relative lengthening power and resulted in a more pronounced frequency effect on shortening power following L-NMMA and ODQ application (Figs. 3.11. and 3.12.), relative net power was not affected (Fig. 3.13.). Collectively, these results suggest that hypoxic acclimation

does not have / has little influence on NO-mediated myocardial function in rainbow (steelhead) trout. There are no previous studies which have measured the effect of exogenous NO, or sGC and ODQ inhibition, on fish cardiac performance after hypoxic acclimation, but there are relevant studies on mammals. Hypoxic acclimation (14 days at 10.5% O_2) in pregnant guinea pig mothers resulted in a 5-fold increase in iNOS mRNA, a 23% increase in iNOS protein, as well as a 2.5-fold increase in nitrite/nitrate levels in fetal hearts, but no change in nNOS levels / activity (Thompson *et al.*, 2009). Further, iNOS activity was increased in enzymatically isolated rat cardiomyocytes following chronic hypoxia (21 days at 10% O_2; Rouet-Benzineb *et al.*, 1999). The increase in nitrite levels reported by Thompson *et al.* (2009) is consistent with studies which showed that the concentration of nitrite was significantly higher in the hearts of carp exposed to anoxia for 1-5 days or 24 hours of deep hypoxia (~0.25 kPa O_2) (Sandvik *et al.*, 2012; Hansen *et al.*, 2016). These authors suggested that nitrite was acting as a NO donor for cytoprotection under anoxia / re-oxygenation. Our failure to identify such effects in the trout may be due to species-specific differences in how the myocardium of hypoxia-tolerant vs. -intolerant species responds to long-term oxygen limitations. For example, 24 hours of hypoxia (at 9.5 kPa O_2) did not alter myocardial nitrite levels in brown trout (*Salmo trutta*)(Jensen *et al.*, 2015). However, it is also possible that we would have identified hypoxia-mediated differences in NO-mediated contractile function if we had also examined the performance of myocardial strips under hypoxic conditions.

3.5.8. Perspectives

This study showed that acclimating trout to 40% air saturation had relatively minor effects on NO-mediated myocardial function. This result was somewhat surprising, but may be due to differences in how hypoxia-tolerant vs. intolerant species respond to limitations in O_2 or that the strips were only tested under normoxic conditions (e.g. see Imbrogno *et al.*, 2014). Nonetheless, our results provide a number of insights, and important findings, that contribute significantly to our understanding of fish cardiovascular physiology. First, our results confirm the results of Carnevale *et al.* (submitted, Chapter 2) that hypoxic acclimation decreases the capacity of the trout myocardium to perform work / produce power, and show that it increases the duration of myocardial relaxation (which could affect ventricular diastolic distensibility and reduce cardiac filling). These results strongly suggest that cardiac function is depressed in hypoxia-acclimated fishes, like cod and trout, due largely to compromised myocardial function. Second, the disparate effects on NO vs. the NO blockers on myocardial contractility (e.g. SNP-mediated decrease in relative net power vs. the same effect of L-NMMA; Figs. 3.9. vs. 3.13.), and the strong frequency-dependent effect on NO-mediated changes in myocardial contractility support the principle that cellular effects of NO are highly spatio-temporally controlled / dependent (Balligand *et al.*, 2009; Imbrogno *et al.*, 2017). In fact, to my knowledge, this study is the first to examine the effect of contraction frequency on NO-mediated cardiac function, and shows that the effects on NO can be diminished, or even opposite, at low vs. high contraction frequencies (e.g., see Fig. 3.9.). These data suggest that the benefits of NO on the hypoxic heart may be diminished, or reversed, if the fish's heart rate is elevated. Such

a scenario could occur if hypoxic fish are exposed to acute or chronic increases in temperature, and the control of cardiac function (including that by NO) should be investigated under such environmental challenges.

Chapter Four: Summary and Future Research

4.1. Methodological Considerations

The exclusion of catecholamines from all experiments is a potential limitation of these studies, as circulating levels of noradrenaline and adrenaline (< 5 nM; Gamperl *et al.*, 1994b) provide tonic support for cardiac function (Farrell *et al.*, 1986; Graham and Farrell, 1989). Catecholamines were not added to the saline for a number of reasons. First, it is difficult to maintain catecholamine levels constant in a system where the saline is recirculated; i.e., adrenaline and/or nordarenaline would have to be periodically added and their effect would have waned (due to catecholamine photooxidation / degradation) between additions. This would have increased variability in the data. Catecholamines are more important for supporting cardiac function at cold temperatures in trout (Graham and Farrell, 1989). Finally, most previous studies on the effect of NO on myocardial function have not included adrenaline in the perfusate [including Pedersen *et al.* (2010) who used paced myocardial rings in their studies), and Pedersen *et al.* (2010) showed that high levels of adrenaline abolished NO's effects on myocardial performance. I was able to achieve high levels of work and power without sympathetic stimulation in these experiments, and the recovery of shortening work was 85-90% (depending on stimulation frequency) after an experiment of 2 h in duration, and where PO_2 was lowered to 1.5 kPa (e.g., see Chapter 2, Fig. 2.4.). This suggests that the collected data is robust, and that the results provide important insights into the impacts of chronic hypoxia and NO-mediated / -stimulated pathways on heart function. The only concern with regards to excluding adrenaline from

the perfusate is how this might have affected how chronic hypoxia impacted myocardial performance. This is addressed in Chapter 2.

Most previous studies on aspects of NO-mediated effects on fish cardiac performance have used spontaneously beating hearts, where the pacemaker cells provide the stimulation for myocardial contraction (Sys *et al.*, 1997; Imbrogno *et al.*, 2001; Gattuso *et al.*, 2002; Garofalo *et al.*, 2009; Imbrogno *et al.*, 2014). In these studies, I used myocardial strips where stimulation electrodes placed on either side of (but not touching) the heart muscle delivered electrical stimulations (~10 v) that elicited contraction. I cannot exclude the possibility that this influenced the results, and may explain why some of our results differ from those obtained with hypoxia-tolerant species. Nonetheless, Pedersen *et al.* (2010) used stimulated ventricular rings in their studies and showed that trout ventricular rings increased their contractile force by ~60% following adrenergic stimulation (150 μM adrenaline). These data show that myocardial preparations receiving extraneous stimulation still have significant scope left to respond to physiologically relevant stimuli. In particular, since adrenaline's main effects are on Ca^{2+} influx and the rate of relaxation (Ca^{2+} sequestration into the SR) (Shiels and Farrell 1997; Shiels *et al.*, 1998; Shiels *et al.*, 2003), and these pathways are common with many of the effects on NO on the cardiomyocyte (e.g. see Figure 3.1), it appears that my preparations were suitable for testing the effects of NO donors and antagonists on myocardial function in hypoxia- and normoxia-acclimated trout.

In the NO blocker experiment (Chapter 3), I inhibited NO synthase activity using L-NMMA and then sGC activity using ODQ. This was done given the limited number of

fish available from the two groups (hypoxia- and normoxia-acclimated). However, even though 30 minutes was allowed between drug applications, I cannot exclude the possibility that there were some cumulative (i.e., interactive) effects that influenced the results. In addition, the SNP and NO inhibitor experiments were performed at 21 kPa (100% air saturation), whereas the PO_2 of the venous blood supplying the spongy myocardium is approximately 5 kPa (Gamperl *et al.*, 1994a Thomas *et al.*, 1994, Steffensen and Farrell, 1998; Farrell and Clutterham, 2003). This means that the data may not be directly comparable to *in vivo* conditions, particularly as the contribution of NOS vs. the conversion of NO_2^- to NO by various cellular proteins (e.g., myoglobin) is altered by oxygen level (Imbrogno et al., 2014).

Finally, in this study I acclimated the fish to ~14ºC as compared to 10-11ºC in previous experiments (Peterson and Gamperl 2010a; 2010b; Moytka *et al.*, 2017). Given that the hypoxic acclimated fish suffered significant mortality early on during acclimation (~30%), it is apparent that at this temperature 40% air saturation should be considered a severe (not moderate) hypoxic challenge for this species. Thus, it may not be surprising that I found differences between this study (e.g., an increase in RVM; Table 2.1.) as compared to previous work (e.g., Motyka *et al.*, 2017). Collectively, these data suggest that experiments should be performed to examine how the severity of chronic hypoxia impacts cardiac performance and morphology / ultrastructure.

4.2. Significance of Study

This thesis examined the effects of hypoxic acclimation on cardiac function in steelhead trout, and the role of NO in the cardiac response to chronic low oxygen exposure. The myocardium from hypoxic-acclimated fish produced less work / power output under normoxic conditions (by ~35 %), performed similarly during graded hypoxic exposure, and recovered less net power (by ~20%) following this exposure when compared with the myocardium of normoxic-acclimated individuals. The diminished work output by the myocardium of hypoxic-acclimated fish was largely due to a significant decrease in shortening work, as lengthening work was unaffected. Previous studies on hypoxia-intolerant fish have found diminished S_V following acclimation to low O_2 (Petersen and Gamperl 2010a; 2010b; Moytka *et al.*, 2017), and my results suggest that this is at least partially related to an increase in end-systolic volume, due to the inability of the ventricle to eject all of the blood it contains into the circulation (i.e., a decrease in ejection fraction). This would be difficult to test / confirm *in vivo*, however echocardiography may prove a useful technique for examining this question further. I also report that the hearts of these hypoxic-acclimated trout were not more tolerant of acute hypoxic exposure, no evidence of cardiac pre-conditioning, and that these hearts did not recover as well following graded hypoxia. The reason(s) for the diminished myocardial performance following chronic hypoxia is not known, but I feel it is more likely related to myocardial stunning, rather than myocardial remodeling or necrosis.

The myocardium of hypoxic-acclimated fish displayed an inverse frequency-dependent decrease in shortening work when initially exposed to hypoxia (a PO_2 of 13.5

kPa). This is a novel finding that is theorized to be due to an 'oxygen sensing' mechanism such as H_2S or NO (e.g. see Syme *et al.*, 2013; Gesser and Rodnick, 2019). Under hypoxic conditions, concentrations of NO and nitrite have been found to increase in the hearts of crucian carp (Hansen *et al.*, 2016). However, exogenous NO only produced modest (10-20%) decreases in ventricular shortening power in these steelhead trout, this effect was not different between normoxic- and hypoxic-acclimated fish (Chapter 3, Carnevale *et al.*, submitted), and myocardial nitrite levels are not higher in this species following 24 h of hypoxia (Jensen *et al.*, 2016). My results suggest that hypoxia-sensitive and tolerant fish may respond differently to NO, and future studies should directly compare the response of hearts from these two groups to this gasotransmitter.

In this thesis, I also show that acclimating trout to chronic hypoxia had limited effects on NO-mediated myocardial function (i.e., largely limited to an increase in the duration of relaxation), and that exogenous NO (i.e., SNP) and sGC and NOS inhibitors produced conflicting results. The lack of major differences in myocardial function following NO addition or blockade indicates that this gasotransmitter is unlikely to play a prominent role in the myocardial dysfunction that is observed in the heart / myocardium of hypoxia-acclimated fish. Net power did not increase when NOS was inhibited by L-NMMA, a result seen in previous studies (Sys *et al.*, 1997; Imbrogno *et al.*, 2001; Imbrogno *et al.*, 2014). However, inhibition of sGC did produce significant increases in relative shortening and net power in both acclimation groups, which supports previous research (Imbrogno *et al.*, 2014). The first finding may be due to the non-selective nature of L-NMMA (i.e., it blocks all forms of NOS), and thus, a disruption in the finely-tuned spatio-

temporal influence of autocrine NO on myocardial function (Tota *et al.*, 2005; Imbrogno and Cerra, 2017; Imbrogno *et al.*, 2018). The results following sGC inhibition suggest that nitrite plays a major role in the trout myocardial NO/NOS system, a finding that is at least partially supported by the data of Pedersen *et al.* (2010).

The most important finding of Chapter 3 was that contraction frequency had a major effect on the myocardium's response to SNP, L-NMMA and sGC. For example, NO had a depressive effect on myocardial performance at low contraction frequencies, but either no effect or a stimulatory effect at higher contraction frequencies. This is a very novel, and significant, finding which suggests that the effects of NO on myocardial function are highly frequency dependent. Future studies should expand on these results, and potentially investigate the effect of both contraction frequency and temperature on NO-mediated cardiac / myocardial function. Heart rate increases with temperature, and it is important to separate out frequency vs. temperature effects. For example, Amelio *et al.* (2012) examined the effect of winter (5^{o}C) and spring (20^{o}C) acclimation on temperature-dependent heart function in eel (*Anguilla anguilla*), and only reported NO effects when acclimation and test temperature (5, 10 and 20^{o}C) were equivalent. However, all preparations were paced at 50 beats min^{-1}, and the magnitude and /or interpretation of their results might have been affected greatly if the hearts had been paced at physiological (i.e., temperature relevant) values.

5.0. References

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