EFFECTS OF CHRONIC HYPOXIA ON THE CARDIORESPIRATORY PHYSIOLOGY OF ATLANTIC COD (*Gadus morhua*)

LENE H. PETERSEN


CENTRE FOR NFLD. STUDIES
MEMORIAL UNIVERSITY
OF NEWFOUNDLAND

Effects of Chronic Hypoxia on the Cardiorespiratory Physiology of Atlantic Cod (*Gadus morhua)*

By

Lene H. Petersen

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Memorial University of Newfoundland

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Abstract

Currently, little information exists on how chronic hypoxia influences fish physiology. Thus, a comprehensive examination of how this ecologically-relevant environmental challenge affects the cardiorespiratory physiology, exercise performance and hypoxia tolerance of Atlantic cod (*Gadus morhua*) was performed.

Exposure to acute hypoxia (P_wO_2 8-9 kPa) lowered the U_{crit} of normoxic-acclimated cod by approx. 30%, and this was associated with large decreases in max. oxygen consumption (MO_2), metabolic scope ($\geq$ 50%), and maximum heart rate (f_H) and cardiac output (Q) (by 16 and 22%). Hypoxic acclimation (6-12 weeks at 10 °C; P_wO_2 8-9 kPa) elevated normoxic MO_2 (standard by 27 %; routine by 44%) compared with normoxic controls, but did not influence U_{crit}, max. MO_2 or metabolic scope under either normoxia or hypoxia. Further, although, resting and maximum values for Q were significantly diminished in hypoxic-acclimated cod due to lower values for stroke volume (S_v), increased f_H partially compensated for the latter, and hypoxic-acclimated cod were able to consume more oxygen for a given cardiac output.

This lower *in vivo* cardiac pumping capacity proved not to be a regulated decrease as hypoxic acclimation reduced *in situ* values for maximum S_v, the scope for S_v, and consequently maximum cardiac output (Q_{max}) (by 19%). However, hypoxic-acclimated fish were able to sustain Q better under hypoxia, and the recovery of Q_{max} (compared to initial Q_{max}) was significantly improved (94 vs. 83%) as compared with normoxic controls.

Although several physiological adjustments had taken place during the 6-12 weeks of hypoxic acclimation [increased f_H; elevated hematocrit (Hct) by 11 % and [Hb] by 14 %; enhanced tissue oxygen extraction efficiency by ~ 15%; and a more robust stress response (2-8 fold higher levels of plasma catecholamines at P_wO_2's of 5.3 and 2.7 kPa)], these adjustments were only successful in improving the cod's critical oxygen tension (P_{crit} of normoxic and hypoxic-acclimated cod 8.1 ± 0.5 vs. 6.6 ± 0.6 kPa, respectively), not the cod's hypoxia tolerance (H_{crit} = 4.3 ± 0.2 vs. 4.8 ± 0.3 kPa). Finally, the significance of the enhanced stress response in hypoxic-acclimated cod for cardiac function is uncertain as this species' heart is minimally responsive to adrenergic stimulation, and hypoxic-acclimation reduced the heart's adrenergic responsiveness further.

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ANOVA	Analysis of variance
ATP	Adenosine Triphosphate
B-AR	Beta-Adrenergic Receptor
BL s^{-1}	Body Length pr Second
°C	Degrees Celcius
Ca^{2+}	Calcium
$CaCl_2$	Calcium Chloride
cAMP	Cyclic Adenosine Monophosphate
CaO_2	Oxygen Content in Arterial Blood
Cm	Centimeter
CO_2	Carbon Dioxide
C_vO_2	Oxygen Content in Venous Blood
EDV	End-Diastolic Volume
EDTA	Ethtylenediaminetetraacetid acid
ESV	End-Systolic Volume
EPI	Epinephrine
EPOC	Excess Post-Exercise Oxygen Consumption
f_H	Heart Rate
g	Grams
GLUT	Glucose Transporter
G-protein	Guanine Nucleotide-Binding Regulatory Protein
GTP	Guanine Triphosphate
Hb	Haemoglobin
Hb-O_2	Haemoglobin-Oxygen Binding
HCO_3^-	Bicarbonate
H_{crit}	Critical Water Oxygen Tension Where Fish loose Equlibrium
Hct	Hematocrit
Hg	Mercury
HIF-1α	Hypoxia Inducible Factor 1-Alpha

Hr	Hour
Hz	Herz
H_2O	Water
IP_3	Inositol 1,4,5-triphosphate
K_{ATP}	ATP- Sensitive Potassium Channel
KCl	Potassium Chloride
kg	Kilogram
kPa	Kilopascal
L	Liter
LDH	Lactate Dehydrogenase
M	Molar
MCHC	Mean Corpucular Haemoglobin Concentration
mK_{ATP}	Mitochondrial ATP Sensitive Potassium Channels
Max	Maximum
Mg O_2 L^{-1}	Miligram Oxygen per Liter of Water
Mg O_2 h^{-1} kg^{-1}	Miligram Oxygen per Hour per Kilo Body Mass
$MgSO_4$	Magnesium Sulphate
min	Minute
ml	Millilitre
ml kg^{-1}	Milliter pr Kilo Body Mass
ml min^{-1} kg^{-1}	Mililiter per Minute per Kilo Body Mass
mmol	Millimolar
MO_2	Oxygen Consumption
MO_{2max}	Maximum Oxygen Consumption
MS-222	Tricaine Methane Sulphonate
mW	Milliwatt
N_2	Nitrogen
NaCl	Sodium Chloride
NE	Norepinephrine
nM	Nanomolar
NTP	Nucleotide Triphophate

O_2	Oxygen
O.D.	Outer Diameter
OMZ	Oxygen Minimum Zone
P_{50}	Partial Pressure of Oxygen Where One-Half of Haemoglobin is Saturated with Oxygen
P_aCO_2	Partial Pressure of Carbon Dioxide in Arterial Blood
P_aO_2	Partial Pressure of Oxygen in Arterial Blood
P_{crit}	Critical Oxygen Partial Pressure
PFK	Phosphofructokinase
PKC	Protein Kinase C
P_{IN}	Input Pressure
ppm	Parts Per Million
PO	Power Output
PO_{max}	Maximum Power Output
P_{OUT}	Output Pressure
P_vO_2	Partial Pressure of Oxygen in Venous Blood
P_wO_2	Partial Pressure of Oxygen in Water
Q	Cardiac Output
Q_{max1}	First Maximum Cardiac Output
Q_{max2}	Second Maximum Cardiac Output
Q_{max3}	Third Maximum Cardiac Output
RBC	Red Blood Cell
ROS	Reactive Oxygen Species
RVM	Relative Ventricular Mass
SEM	Standard Error of the Mean
sK_{ATP}	Sarcolemmal ATP-Sensitive Potassium Channel
SMR	Standard Metabolic Rate
S_v	Stroke Volume
TES acid	$C_6H_{15}NO_6S$
TES base	$C_6H_{14}NO_6SNa$

U_{crit}	Critical Swimming Speed
µl	microlitre

Chapter 1

Introduction

1.1. Hypoxia

Aquatic hypoxia is an environmental condition that occurs when dissolved oxygen levels in the water are low or deficient; deficient referring to its capacity to sustain most animal life. The amount of dissolved oxygen in freshwater and marine areas is influenced greatly by the interaction of physical, chemical and biological factors (Val, 1995; Diaz and Rosenberg, 1995; Diaz, 2001). Although water bodies in temperate zones are most prone to seasonal and daily oscillations in dissolved oxygen levels, tropical systems, such as the Amazon, also exhibit seasonal changes and extreme changes in dissolved oxygen levels that occur on a daily or even shorter timeframe (Val, 1995). This latter characteristic has rendered many fish in the Amazon extremely hypoxic tolerant (Val, 1995; Muuze et al., 1998).

Even though hypoxic and anoxic environments have existed throughout geological time (Diaz and Rosenberg, 2001; Huey and Ward, 2005), their occurrence in estuarine and coastal marine ecosystems has increased since the 1960s due to anthropogenic activities (Johannessen and Dahl, 1996; Hendriks et al., 2006). Marine hypoxia is known to exist in 146 coastal zones around the world and most of these areas are associated with major population densities or watersheds that deliver large amounts of nutrients into coastal waters (United Nations Environment Programme, Global Environment Outlook Year Book 2003). Augmented nutrient loading in estuarine and coastal areas is often associated with the increased use of fertilizers on farmland, which then washes into creeks and rivers, and ultimately into coastal waters (Wu, 2002; Dybas, 2005). In these areas, the surplus nutrient load causes increased primary production,

which increases oxygen consumption due to mineralization (Rydberg et al., 1990) and concomitantly reduces water O_2 levels. In addition to anthropogenic induced hypoxia, a reduction in dissolved oxygen can be a natural phenomenon which occurs in areas where vertical stratification of the water column causes the formation of haloclines and thermoclines (Renaud, 1985; Pihl et al., 1992; Hoback and Barnhart, 1996; Wu, 2002). This type of hypoxia may be quite serious as the oxygen concentration near the bottom normally only increases following a break in the stratification due to storms (Pihl et al., 1992). Thus, near bottom hypoxia caused by physical phenomena may last as long as 4 to 10 weeks (Pihl et al., 1991), or even longer depending on the onset of fall storms. Further, hypoxic zones associated with haloclines/thermoclines have been reported to cover very large areas, e.g. up to 18,100 km^2 in the Gulf of Mexico (Dybas, 2005), 3000 km^2 in the Kattegat between Sweden and Denmark (Smith et al., 2000) and 20,000 km^2 off the coast of Namibia (Weeks et al., 2002). Thus, the extent and persistence of hypoxia in these areas is a serious concern for marine flora and fauna.

There are many reasons why marine hypoxia is expected to become more prevalent in the coming years, including anticipated population growth near coastal areas (NOAA, 1998). However, a more pertinent concern is global warming, caused by green house gases. Global warming will increase surface water temperatures and augment the formation of thermoclines (Wu, 2002). Further, it will enhance precipitation, and the subsequent runoff will substantially increase the discharge of nutrients from rivers into coastal areas (Smith et al., 2000); with anticipated adverse effects on existing animal communities. This is because, although avoidance behaviour is a common trait in mobile invertebrates and fish in response to hypoxia (Pihl et al., 1992; Bell and Eggleston, 2005),

even these organisms may not be able to escape if large areas are affected and hypoxic conditions occur for extended periods.

1.2. Effect of Hypoxia on Fishes

1.2.1. Swimming Activity and Behaviour

Fish and invertebrates employ different strategies to survive in a hypoxic environment. Sedentary animals, such as bivalve molluscs are unable to retreat from a low oxygen environment and alter feeding habits, respiration rates, and growth rates (Shick et al., 1986; Wang and Widdows, 1991; Sobral and Widdows, 1997). Burrowing invertebrates emerge from the sediment and lie motionless on the surface, while others, such as brittle stars (class Ophiuroidea), use their arms to raise their disks off the substrate in an attempt to reach water with a higher oxygen content (Rabalais et al., 2001).

When fish are exposed to hypoxia a decrease in swimming activity is commonly observed (Jones, 1971; Bushnell et al., 1984; Shurmann and Steffensen, 1994; Chabot and Dutil, 1999; Herbert and Steffensen, 2005). This reduction in activity decreases the fish's oxygen requirement, but also reduces the chance of the fish reaching a more favourable environment (Shurmann and Steffensen, 1994; Chabot and Dutil, 1999). As discussed by Herbert and Steffensen (2005), previous studies (Fischer et al., 1992; Nilsson, et al., 1993; Schurmann and Steffensen, 1994; Dalla Via et al., 1998; Sneddon and Yerbury, 2004) have suggested that the reduced activity observed for various fish species upon hypoxic exposure is an active regulation which enables fish to conserve energy (Herbert and Steffensen, 2005), and ensures that metabolic scope is left for other important

functions (e.g. feeding, digestion, growth etc.) (Claireaux et al., 2000; Chabot and Claireaux, 2008).

An alternative strategy when faced with hypoxia is to increase activity level, which enhances the probability of encountering better oxygen conditions, but also increases the risk of depleting important energy stores. Avoidance of hypoxic waters is an important behaviour that some fish use to minimize the adverse effects of low oxygen on their fitness (Davis, 1975; Kramer, 1987). Burleson and co-workers (2001) suggested that the level of dissolved oxygen avoided increases with increasing temperature, which makes sense given that elevated metabolic rates are associated with higher temperature. These authors studied the selection of water oxygen levels in largemouth bass (*Micropterus salmoides*), and also reported that hypoxic avoidance behaviour was size and age dependent; small fish tending to be in water with lower oxygen levels compared to larger adult fish. Burleson et al. (2001) argued that this was most likely associated with a reduced predation threat from larger fish.

1.2.2. Metabolism

Although fish that remain in hypoxic waters increase ventilation rate/ventilatory effort (Kerstens et al., 1979; Steffensen et al., 1982; Glass et al., 1990, 1991; Pihl et al., 1991; Stecyk and Farrell, 2002), they can employ several metabolic strategies when exposed to hypoxia. The suppression of metabolism is an obligate survival strategy in many hypoxic/anoxic adapted animals, as an overall reduction in metabolic rate will delay the depletion of glycogen stores, and post-pone the onset of anaerobic glycolysis and the acidosis that is associated with the accumulation of lactate (Dalla via et al., 1994;

Muusze et al., 1998). For instance, the sole (*Solea solea*) reduces metabolic rate by 27 and 48 % during hypoxia (P_wO_2 2.4 kPa and 1.2 kPa, respectively), indicating that metabolic depression represents a more effective survival strategy than the induction of anaerobic metabolism in this species (Dalla Via et al., 1994). On the contrary, common carp (*Cyprinus carpio*) exposed to a P_wO_2 decline from 17.4 kPa to 0.3 kPa over a 3 hour period, show a large increase in plasma lactate indicating a significant reliance upon anaerobic glycolysis (Van Raaij et al., 1996). A shift from aerobic and anaerobic contributions to total metabolism is common in several species during hypoxia (Cooper et al., 2002), and this shift is often dependent on the oxygen partial pressure of the ambient water (P_wO_2) and whether the fish is an oxygen (oxy) conformer or an oxygen regulator. In oxyconformers, oxygen consumption falls continuously with decreasing P_wO_2. On the contrary, oxyregulators maintain oxygen consumption relatively constant until a critical oxygen partial pressure (P_{crit}; or S_{crit}, oxygen saturation) is reached; oxygen consumption declining in direct proportion to P_wO_2 thereafter. The majority of fish tend to be oxygen regulators (Berschick et al., 1987; Waller, 1989; Virani and Rees, 2000; Behrens and Steffensen, 2007). However, some are oxygen conformers (Wu and Woo, 1984), and in others, it appears that swimming alters the response to hypoxia. For example, the Adriatic sturgeon (*Acipenser naccarii*) behaves as an oxyconformer in static water, but as an oxyregulator when allowed to swim at a low sustained speed (McKenzie et al., 2007).

1.2.3. Ventilation

The transport of oxygen from the environment to the tissue/cells involves four steps, the first one being ventilation of the gills which elevates the PO_2 and oxygen

content of the blood (Jensen et al., 1993). Exposure to both acute hypoxia and graded hypoxia increases ventilation in carp (Glass et al., 1990, 1991; Stecyk and Farrell, 2002) and this is mainly due to an increase in tidal volume, and to a lesser extent, an elevated breathing frequency (Glass et al., 1990). This increase in ventilation has been reported in numerous species such as the rainbow trout (*Onchorhynchus mykiss*) (Holeton and Randall, 1967), largemouth and smallmouth bass (*Micropterus dolomieu*) (Furimsky et al., 2003), flounder (*Platichthys flesus*), the plaice (*Pleuronectes platessa*) (Steffensen et al., 1982), and Amazonian species such as the traira (*Hoplias malabaricus*), jeju (*Hoplerythrinus uniaeniatus*) and pacu (*Piaractus mesopotamicus*) (Sakuraui et al., 2003; Perry et al., 2004; Soares et al., 2006). Thus, it appears that increased ventilation is a general response of fish to hypoxia regardless of their habitat and activity level.

Ventilatory adjustments during chronic hypoxia have also been reported in various fish species. For instance, 6 weeks of hypoxia at 2.7 kPa increased ventilation frequency in the sailfin molly (*Poecilia latipinna*) (Timmerman and Chapman, 2004a). Kerstens et al. (1979) showed that a minimum of 3 weeks acclimation to a P_wO_2 of ~ 4 kPa increased ventilatory volume in flounder (*Platichthys flesus*), and that this increase was the result of increases in both stroke volume and breathing frequency. Finally, in channel catfish (*Ictarulus punctatus*) acclimated to moderate hypoxia (P_wO_2 10 kPa) for 7 days or severe hypoxia (P_wO_2 2.4 kPa) for 27 days, ventilatory sensitivity to hypoxia increased and this was associated with a large increase in opercular pressure amplitude, and to a lesser extent, ventilatory rate (Johnston et al., 1983; Burleson et al., 2002).

In addition to increased ventilation, hypoxic fish are also known to increase oxygen transfer across the respiratory epithelium via the recruitment of secondary

lamellae (Randall, 1982). This recruitment is generally believed to occur when filamental perfusion pressure rises due to a constriction in the proximal efferent filamental artery (Sundin and Nilsson, 1997), as seen in the perfused gill preparation of cod (*Gadus morhua*) (Pettersson, 1983) and the cod *in vivo* (Sundin, 1995). Thus, both improved ventilation and gill oxygen transfer are used to limit the decrease in arterial PO_2 (P_aO_2) and content during hypoxia (Claireaux and Dutil, 1992). Increased ventilation, however, comes at a cost, and at some point the ventilatory muscles are no longer able to sustain the heightened ventilatory effort and ventilation collapses (Randall and Shelton, 1963; Marvin and Heath, 1968; Gehrke and Fielder, 1988; Mckenzie et al., 2009).

1.2.4. Cardiovascular Function

Bradycardia is a commonly observed response to hypoxia in teleost fish and has been considered a reflex compensatory mechanism (Farrell, 1984). Oxygen chemoreceptors located in the first gill arch are stimulated by hypoxia and trigger this reflex through the efferent limb of the cardiac vagus, which consists of cholinergic fibres (Rantin et al., 1995). In the rainbow trout (Wood and Shelton, 1980) and Atlantic cod (Fritsche and Nilsson, 1989) bradycardia is typically compensated for by increased stroke volume so that cardiac output is relatively constant or increases. However, the maintenance of cardiac output during hypoxia is not universal in teleosts as fish such as the lingcod (*Ophidon elongates*) (Farrell, 1982), common carp (Stecyk and Farrell, 2002), smallmouth bass (Furimsky et al., 2003), European eel (*Anguilla anguilla*) (Peyraud-Waitzenegger and Soulier, 1989) and rainbow trout (Perry et al., 1999) all exhibit depressed cardiac output during acute hypoxic exposure. It therefore appears that

stroke volume does not always compensate for bradycardia, and the degree and onset of hypoxia is likely to influence the overall cardiac response.

A recent review (Farrell, 2007) discusses the physiological benefits of hypoxia-induced bradycardia in teleosts. These include: 1) increasing diastolic residence time of blood in the lumen of the heart; 2) increased coronary blood flow (which mainly occurs during diastole); 3) an improvement of cardiac contractility due to the negative force-frequency effect; and 4) a reduction of cardiac oxygen demand by reducing cardiac power output (Farrell, 2007), just to mention a few. Some studies (Glass et al. 1991; Rantin et al., 1995) have also reported the development of a cardio-respiratory synchronization (Glass et al., 1991) that has added benefits for branchial gas exchange. However, the majority of data (e.g. Desforges and Perry, 1998; McKenzie et al., 2009) now suggests that bradycardia has little benefit for gas exchange across the gill epithelium.

1.2.5. Blood Oxygen Carrying Capacity and Affinity

Acute hypoxia results in a decrease in water oxygen content, and therefore a concomitant decrease in arterial PO_2 in fish. If severe hypoxia is encountered, a decrease in arterial oxygen content will also occur (Jensen et al., 1993), as metabolic extracelullar acidosis results in a Root induced reduction in Hb-O_2 binding affinity (Perry and Gilmour, 1999). In order to maintain P_aO_2, fish respond by hyperventilating to increase oxygen transfer across the gills and hence limit the decrease in P_aO_2 (Claireaux and Dutil, 1992; Jensen et al., 1993; Perry and Gilmour, 1999). Hyperventilation not only enhances oxygen transfer across the gills, and thus defends arterial PO_2, but also results in a respiratory alkalosis due to an increase in CO_2 excretion (Claireaux and Dutil, 1992;

Thomas et al., 1992; Perry and Gilmour, 1996). Respiratory alkalosis causes a left shift of the Hb-O_2 dissociation curve through the Bohr effect, and thus an increase in Hb-O_2 binding affinity (Tetens and Lykkeboe, 1985; Jensen, 1991; Jensen et al., 1993; Perry and Gilmour, 1996, 1999).

Severe hypoxia normally results in a mobilization of catecholamines in response to low blood oxygen content. The hormones epinephrine and norepinephrine stimulate the release of erythocytes from the spleen (Tetens and Lykkeboe, 1985; Wells and Weber, 1990), and thus an increase in blood O_2 carrying capacity. In addition, the stimulation of RBC β-adrenergic receptors further enhances Hb-O_2 binding affinity by increasing Na^+/H^+ exchange across the erythrocyte membrane, which promotes RBC alkalinisation, RBC swelling and a decrease in RBC organic phosphates. Collectively, these changes augment Hb-O_2 binding affinity (Tetens and Lykkeboe, 1985, Tetens and Christansen, 1987), and serve to counteract the effect of reduced P_aO_2 and blood acidosis on haemoglobin-oxygen binding affinity until the hypoxic episode is over.

In contrast to acute hypoxia, hypoxic-acclimated fish generally show adaptations in blood respiratory properties that result in improved oxygen loading and carrying capacity (Wood and Johansen, 1972; Driedzic et al., 1985; Rees et al., 2001). For instance, hypoxia can result in the production of Hb isomorphs with a higher Hb-O_2 binding affinity (Tun and Houston, 1986; Weber and Jensen, 1988; Marinsky et al., 1990), and oxygen carrying capacity can be further enhanced by decreasing RBC levels of ATP and GTP as these allosteric effectors provide a way of rapidly adapting Hb function to meet tissue oxygen demand (Weber and Jensen, 1988). However, the response of blood haematological parameters to chronic hypoxia does show variation amongst

fishes. For example, forty days of hypoxia (at either P_wO_2 12.4, 8.7 or 5.3 kPa) did not noticeably influence blood oxygen carrying capacity in sea bass (*Dicentrarchus labrax*) and turbot (*Scophthalmus maximus*) as there were no changes in the concentration of allosteric co-factors (ATP, GTP) or blood [Hb] and Hct (Pichavant et al., 2001, 2003). Similarly, Atlantic cod acclimated to a P_wO_2 of 9 kPa for 12 weeks did not have elevated levels of [Hb] and Hct as compared with normoxic controls (Chabot and Dutil, 1999).

1.3. Catecholamines

Like other vertebrates, there are also two major groups of adrenergic cells in fish: the adrenergic neurons and chromaffin cells (Nilsson, 1984). Fish lack adrenal glands and clusters of chromaffin cells can be found in different organs depending on species (Laurent et al., 1983). The catecholamines, epinephrine (EPI) and norepinephrine (NE), are released into the circulation from chromaffin cells which most often line the posterior cardinal vein within the head kidney (Nandi, 1961). Once in the circulation they bind to cell-surface receptors (α- and β-adrenergic receptors; Ask, 1983) on target tissues and initiate a series of responses (many of them detailed above) aimed at alleviating the disruptive effects of stressors on physiological and metabolic functions (Perry and Wood, 1989; Thomas and Perry, 1992; Randall and Perry, 1992; Fabbri et al., 1998). For example, EPI and NE both act as vasodilators of the branchial vasculature, and EPI concurrently elicits a constrictory effect on the systemic vasculature (Pettersson and Nilsson, 1980). These combined effects promote lamellar recruitment (Farrell et al., 1979), and result in the enhanced diffusion of oxygen across the respiratory surface

(Pettersson and Nilsson, 1980; Wahlqvist and Nilsson, 1980; Wahlqvist, 1980; Perry and Wood, 1989). Catecholamines increase heart rate (f_H) (Wahlqvist and Nilsson, 1977; Cameron and Brown 1981; Fox et al., 1992) and myocardial contractility (stroke volume) (Keen et al., 1993), and thus support cardiac function. Finally, these hormones result in an increase in Hb-O_2 binding affinity and capacity via mechanisms mentioned previously (section 1.2.5.).

Hypoxia is a powerful stimulus for the release of catecholamines into the blood stream (Tetens and Christensen, 1987; Perry et al., 1991; Kinkead et al., 1991), and these hormones are likely to have an especially significant effect on blood oxygen carrying capacity during chronic hypoxia as this condition increases the number of β-adrenergic receptors on the erythrocyte membrane (Marttila and Nikinmaa, 1988; Reid and Perry 1991). No data exists on the effects of chronic hypoxia on fish cardiac ß-adrenoreceptor density/binding characteristics. However, unlike mammals (Voelkel et al., 1981; Rocha-Singh et al., 1991), hours of exposure of moderate to severe hypoxia does not result in alterations in trout myocardial ß-adrenoreceptor density or affinity (Gamperl et al., 1998). This latter result suggests that the cardiac function of hypoxic-acclimated fish should respond appropriately to the release of catecholamines into the circulation.

1.4. The Atlantic cod (*Gadus morhua*)

The Atlantic cod is a demersal North Atlantic species of considerable economic and cultural importance. However, population numbers have declined dramatically over the past several decades, especially in areas prone to chronic hypoxia, such as the Baltic

Sea (Gerlach, 1988) and the Gulf of St. Lawrence (GSL) (D'Amours, 1993; Kiceniuk and Colbourne, 1997). In these regions, they can encounter hypoxia during either part of, or throughout their life history and this may strongly affect their survival and distribution (Plante et al., 1998). Previous studies in the GSL showed that the distribution of cod is sensitive to hypoxia (D'Amours, 1993) and that cod completely avoided regions with oxygen levels below a threshold oxygen partial pressure (P_wO_2) of ~ 6.6 kPa (Kiceniuk and Colbourne, 1997). In the Baltic Sea, cod avoid areas with a P_wO_2 lower than 7.5 kPa (Tomkiewicz et al., 1998), and thus, there are areas in both the GSL and the Baltic Sea that are unsuitable habitat for cod (Plante et al., 1998).

1.5. Objectives

Although the Atlantic cod has been reported to be moderately tolerant of hypoxia (Claireaux and Dutil, 1992; Schurmann and Steffensen, 1992; Plante et al., 1998 Chabot and Dutil, 1999), we have a poor understanding of what effect chronic hypoxia has on cod physiology and if cod can adapt to chronic hypoxic conditions. Thus, in this Ph.D. thesis, I used a multi-level approach (*in vivo*, *in situ* and *in vitro*) to perform a comprehensive examination of how chronic hypoxia (6-12 weeks; P_wO_2 8-9 kPa) affects the swimming performance, metabolism, cardiorespiratory physiology, and hypoxia tolerance of Atlantic cod.

At the beginning of my thesis, only two studies (Kutty, 1968; Bushnell et al., 1984) had investigated how chronic hypoxia affects fish swimming performance and metabolism, and no studies had examined the effect(s) of chronic hypoxia on fish *in vivo*

cardiovascular function. Further, this lack of information was surprising given that: 1) acute exposure to reduced oxygen levels decreases metabolic scope (Claireaux et al., 2000; Evans et al., 2007) and swimming performance (Dahlberg et al., 1968; Kutty, 1968; Bushnell et al., 1984; Dutil et al., 2007); 2) exercise/swimming capacity is determined by the interaction of many organ systems and is thus considered as an integrated measure of an animal's physiological capacity for a particular environment (Nelson, 1989); and 3) chronic hypoxia has been reported to damage the fish heart (e.g. see Lennard and Huddart, 1992). Based on the above, the first objective of my thesis was to determine the effects of chronic hypoxia on the: 1) swimming performance, 2) *in vivo* cardiac function, and 3) metabolism (resting and maximum oxygen consumption) of Atlantic cod.

This initial study showed that cod acclimated to hypoxia had significantly lower values for resting and maximum stroke volume and cardiac output, and a significantly lower scope for stroke volume as compared with normoxic-acclimated fish. While this work provided novel insights into how fish cardiorespiratory physiology is impacted by prolonged exposure to hypoxia, the reason(s) for the diminished cardiac function in hypoxia-acclimated cod was not clear. Thus, the second part of my research used *in situ* heart preparations to investigate whether hypoxic acclimation has a direct negative effect on the normoxic and hypoxic performance of the Atlantic cod heart.

Environmental hypoxia is among several stressors that cause the release of the catecholamines, epinephrine and norepinephrine, in fish (Butler et al., 1978; Fritsche and Nilsson, 1990; Perry et al., 1991; Kinkead et al., 1991). Interestingly, however, *in situ* and *in vitro* studies (Axelsson, 1988; Lurman et al., unpubl.) suggest that these hormones do not play a major role in supporting cod cardiac function. Given that this finding is

contrary to that reported for most other teleost species (Graham and Farrell, 1989; Farrell et al., 1996; Claireaux et al., 2005; Hanson et al., 2006), the third part of my thesis focused on the ability of catecholamines to stimulate *in vivo* cardiac function, and whether the heart's responsiveness to these hormones is affected by hypoxic acclimation. To perform this research, I fitted normoxic- and hypoxic-acclimated cod with Transonic® flow probes and afferent branchial artery cannulae, and measured cardiac variables following the sequential injection of increasing doses of epinephrine.

Finally, the hypoxic-acclimated cod in Chapter 2 (see below) were able to consume more oxygen for a given Q than their normoxic-acclimated counterparts at rest and during exercise. This result strongly suggested that important adjustments (adaptations) had taken place in other aspects of the cod's physiology when exposed to prolonged hypoxia. However, there was little indication what these changes might be, and whether these alterations might also improve the cod's hypoxia tolerance. Thus, for the final part of my thesis, I exposed normoxic and hypoxic-acclimated cod to a graded hypoxic challenge until loss of equilibrium, while continuously recording numerous cardiorespiratory variables and periodically sampling the cod's blood for the analysis of plasma catecholamines. Further, I collected blood from normoxic and hypoxic-acclimated cod so that the effects of chronic hypoxia on *in vitro* haemoblogin-oxygen binding characteristics could be determined.

1.6. Co-Authorship Statement

The design and identification of the research proposal was solely carried out by the first author in collaboration with Dr. Kurt Gamperl. The practical aspects of this Ph.D. research: 1) fish husbandry; 2) surgery; and 3) experimental work was exclusively carried out by the first author in conformity with techniques taught to her by Dr. Kurt Gamperl and the staff at the Joe Brown Aquatic Research Building (JBARB; formerly the Aquaculture Research and Development Facility, ARDF). Finally, data analysis and manuscript preparation were performed by myself under the helpful supervision of Dr. Kurt Gamperl.

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Chapter 2

Effects of Acute and Chronic Hypoxic on the Swimming Performance, Metabolic Capacity and Cardiac Function of Atlantic Cod (*Gadus morhua*).

2.1 Abstract

Low water oxygen content (hypoxia) is a common feature of many freshwater and marine environments. However, we have a poor understanding of the degree to which diminished cardiac function contributes to the reduction in fish swimming performance concomitant with acute hypoxic exposure, or how fish cardiorespiratory physiology is altered by, or adapts to, chronic hypoxia. Thus, we acclimated adult Atlantic cod (*Gadus morhua*) to either ~ 8-9 kPa O_2 (40-45% oxygen saturation) or 21 kPa O_2 (100% oxygen saturation) (normoxia) for 6-12 weeks at 10°C, and subsequently measured metabolic parameters [routine oxygen consumption (MO_2), maximum MO_2, metabolic scope] and cardiac function (Q, f_H and S_V) in these fish during critical swimming speed (U_{crit}) tests performed at both levels of water oxygenation. Although surgery (flow probe implantation) reduced the U_{crit} of normoxic-acclimated cod by 14% (from 1.74 to 1.50 BL s^{-1}) under normoxic conditions, exposure to acute hypoxia lowered the U_{crit} of both groups (surgery and non-surgery) by approx. 30% (to 1.23 and 1.02 BL s^{-1}, respectively). This reduction in swimming performance was associated with large decreases in maximum MO_2 and metabolic scope ($\geq$50%), and maximum f_H and Q (by 16 and 22%), but not S_V. Long-term acclimation to hypoxia resulted in a significant elevation in normoxic standard and routine metabolic rates as compared with normoxic-acclimated fish (by 27 and 44%, respectively) but did not influence normoxic or hypoxic values for U_{crit}, maximum MO_2 or metabolic scope. This was surprising given that resting and maximum values for Q were significantly lower in hypoxic-acclimated cod at both levels of oxygenation, because of lower values for S_V. However, hypoxic-acclimated cod apparently compensated for

diminished myocardial function/contractility by consuming more oxygen for a given cardiac output. These results provide important insights into how fish cardiorespiratory physiology is impacted by short-term and prolonged exposure to hypoxia and further highlight the tremendous capacity of the fish cardiorespiratory system to deal with environmental challenges.

2.2 Introduction

Hypoxia is a frequently occurring environmental phenomenon in many freshwater and coastal systems, and can be caused by either anthropogenic input, or naturally occurring biological and physical factors (Rosenberg et al, 1991; Pihl et al., 1992; Hoback and Barnhart, 1996; Wu, 1999). Recent studies show that hypoxia in marine waters is not restricted to localized areas, but is more extensive and longer-lasting (weeks to months) than previously thought (Diaz and Rosenberg, 1995; Wu, 1999; Weeks et al, 2002; Bell and Eggleston, 2005; Gilbert et al., 2005). This can create inhospitable habitats for fish and sedentary animals (Wu, 2002), and severe oxygen depletion of coastal waters has significant negative consequences for economically important fisheries, ecosystems and biodiversity (UN Global Environment Outlook Year Book, 2003). This problem becomes even more serious if large areas are affected by hypoxia for an extended time, as fish may not be able to leave these areas; avoidance being the predominant reaction to hypoxia (Pihl et al., 1991; Claireaux et al. 1995).

Locomotor performance is determined by the interaction of many organ systems, and is considered to be an integrated measure of an animal's physiological capacity in a

particular environment (Nelson, 1989). Thus, measuring the locomotor performance of fishes could provide valuable information on their physiological response to hypoxia. Furthermore, circulatory adjustments are prerequisite for vital functions such as locomotion, feeding/digestion and for appropriate responses to environmental changes (Farrell et al., 2001; Claireaux et al., 2005; Gollock et al., 2006). An understanding of how chronic hypoxia affects both swimming performance and cardiovascular function could therefore reveal important insights about whether fish will survive, and how well they adapt to, hypoxic environments. At present, studies on the effects of chronic (weeks of) hypoxia have been conducted on a limited number of teleost species, and focused on a range of aspects such as food intake (Chabot and Dutil, 1999; Pichavant et al., 2000; Zhou et al., 2001; Pichavant et al., 2001), reproduction (Wu et al., 2003), oxygen carrying capacity (Greaney et al., 1980; Taylor and Miller, 2001; Pichavant et al., 2003); cardiomyocyte physiology (Lennard and Huddart, 1992; Paajanen and Vornanen, 2003) and circulating catecholamine levels (Butler et al., 1979; Montpetit and Perry, 1998). However, to our knowledge only two studies (Kutty, 1968; Bushnell et al., 1984) have investigated how chronic hypoxia affects fish swimming performance and metabolism, and only one study has examined the effect of chronic hypoxia on fish cardiovascular function (Burleson et al., 2002). Further, this lack of information is surprising given that acute exposure to reduced oxygen levels decreases metabolic scope (Claireaux et al., 2000; Evans et al., 2007) and swimming performance (Dahlberg et al., 1968; Kutty, 1968; Bushnell et al., 1984; Dutil et al., 2007), and that although hypoxia induces bradycardia in many species, this reduction in heart rate is often compensated for by an increase in

stroke volume that maintains cardiac output relatively constant (Wood and Shelton, 1980; Fritsche and Nilsson, 1989; Gamperl et al., 1994; Sandblom and Axelsson, 2005, 2006).

The Atlantic cod (*Gadus morhua*) is a demersal North Atlantic species of significant economic and cultural importance that has experienced dramatic population declines over the past several decades (Myers et al., 1996; Rose et al., 2000; Svedäng and Bardon, 2003; Hutchings and Reynolds, 2004). Further, this species has traditionally inhabited areas, such as the Baltic Sea (Gerlach, 1988) and the Gulf of St. Lawrence (GSL) (D'Amours, 1993; Kiceniuk and Colbourne, 1997; Gilbert et al., 2005), where they are now likely to encounter environmental oxygen levels, at least during part of their life history, that may strongly affect their distribution, growth, and reproduction. For example, GSL cod are sensitive to hypoxia (D'Amours, 1993), completely avoid regions of water oxygen partial pressures (P_wO_2) below a threshold of ~6.6 kPa (Kiceniuk and Colbourne, 1997), and there are thus areas in the GSL that are below the threshold for survival (Plante et al., 1998; Gilbert et al., 2005)

Given the expanding threat of hypoxia to marine organisms including cod (Wu et al., 2002; Breitburg et al., 2002; Neuenfeldt et al., 2002; Gilbert et al., 2005), and our incomplete understanding of fish physiology when exposed to low oxygen conditions, the main goal of the present study was to determine whether Atlantic cod are able to adapt to chronic hypoxia. To accomplish this, I acclimated adult Newfoundland cod to water oxygen levels (P_wO_2) of 8-9 kPa (hypoxia) and 21 kPa (normoxia) for 6-12 weeks, fitted them with Transonic® flow probes around their ventral aorta, and measured cardiac function and oxygen consumption during critical swimming speed (U_{crit}) tests conducted at both water oxygen levels.

2.3. Material and Methods

These studies were conducted in accordance with the guidelines of the Canadian Council on Animal Care, and approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (Protocol # 05-03-KG).

2.3.1. Experimental Animals

Experiments were performed on adult (0.62 ± 0.03 kg; range = 0.43-0.78 kg) Atlantic cod (*Gadus morhua*) at the Ocean Sciences Centre (OSC; Memorial University, St. John's, Newfoundland, Canada). Cod were obtained from stocks reared at the OSC's Aquaculture Research and Development Facility (ARDF), and subsequently held in sea-cages at Hermitage Bay (Newfoundland, Canada) for approximately 18 months before being transported back to the OSC. At the OSC, the cod were originally held in a 12,000 litre tank supplied with aerated seawater at 10°C for at least 2 months prior to being moved to acclimation tanks. During this period, the fish were fed a commercial cod diet (EWOS, Canada) to satiation three times a week, and photoperiod was maintained at 12h light: 12 h dark.

2.3.2. Experimental Conditions

2.3.2.1. Normoxic Acclimation

Prior to experiments, 40 fish from the holding tank were acclimated at a P_wO_2 of 21 kPa for 6-12 weeks at 10 ± 0.1°C in two ~1300 litre tanks, each supplied with aerated seawater (10 ± 0.1 °C) from a header tank at a flow-rate of ~6-8 L min^{-1}. The header tank

was fitted with two submersible heaters (Process Technology, Ohio, USA; model NA15E-2) and connected to a separate heater/chiller (custom built by Technical Services, Memorial University of Newfoundland). Furthermore, a wooden lid was placed on the tank to reduce stress from external stimuli (presence of people, noise etc.) and to reduce fluctuations in water temperature. The normoxic acclimation tank was fully aerated to ensure normoxic conditions (> 19 kPa), and fish were fed three times a week with commercial cod pellets at a ration equal to that consumed by the hypoxic group.

2.3.2.2. Hypoxic Acclimation

The hypoxic acclimation tank was supplied with ambient seawater from its own header tank, and fitted with a wooden lid to reduce noise and fluctuations in water temperature, and to reduce the exchange of oxygen with the atmosphere. Temperature in the hypoxic tank was controlled by a small submersible rod-type heater (Process Technology, Ohio, USA; model DRAE15-1) fitted on the lid, and by circulating water in the tank through a custom built heater/chiller unit (Technical Services, Memorial University of Newfoundland). These two systems were sufficient to maintain water temperature at $10 \pm 0.1^{\circ}C$.

Twenty fish were transferred from the holding tank to the hypoxic acclimation tank and held under normoxic conditions (> 19 kPa) for one week before the oxygen level was reduced. A hypoxia level of approx. 8 kPa (~ 40% O_2 saturation) was achieved over the time course of 1 week by: 1) slowly reducing the flow rate to the tank to 1-2 L min^{-1} (i.e. fish metabolism partially reduced the water O_2 content); and 2) using a custom designed solenoid valve system (Electronics Workshop, Memorial University of

Newfoundland). This system continuously monitored the oxygen level in the tank by pumping water through an external circuit of tubing (Tygon Food, ser. 6-419, Cole Parmer) that contained a galvanic oxygen electrode (model CellOx 325, WTW) housed in a D201 flow cell (WTW: Weilheim, Germany). Further, the oxygen probe was connected to an oxygen meter (model Oxi 340, WTW), which was subsequently connected to two solenoid valves; one which bubbled pure N_2 into the tank when O_2 reached an upper limit of 9 kPa, and the other bubbling air into the tank when oxygen levels reached 7 kPa. This design allowed the oxygen level in the hypoxic tank to be kept within a narrow O_2 range (± 1 kPa), and together with the reduced water flow, proved to be highly efficient in maintaining appropriate O_2 levels; average O_2 level 8.56 ± 0.17 kPa over the 6-12 week acclimation period. As the experiments could only be conducted on 3 fish per week, experiments were alternated between a normoxic and hypoxic-acclimated fish, and the acclimation time ranged from a minimum of 6 weeks to a maximum of 12 weeks. This approach was subsequentially used in chaps 3, 4 and 5.

Fish were fed three times a week with commercial cod pellets, and most fish were feeding from the first day of hypoxia. Average food consumption over the period of acclimation was 1.4% body mass day^{-1} (= 17.14 g day^{-1}). To avoid build up of carbon dioxide and nitrogen that may have affected the hypoxic fish, the water quality was tested (total nitrogen, un-ionized ammonia, pCO_2 and pH) in the hypoxic tank once a week throughout acclimation.

2.3.3. Surgical Procedures

The fish were netted and anaesthetized in seawater containing tricaine methane sulphonate (MS-222, 0.1 g L^{-1}) until ventilatory movements ceased. Then fish were weighed and measured before being transferred to an operating table where chilled (4°C) oxygenated seawater, containing a lower dose of MS-222 (0.05 g L^{-1}), was continuously pumped over the fish's gills.

To allow for the direct measurement of cardiac function (cardiac output, Q; heart rate f_H ; and stroke volume, S_V), a 2S or 2.5S Transonic® flow probe was fitted around the ventral aorta of each cod as previously described by Gollock et al. (2006) (Plate 2.1 A). After the flow probe was carefully placed around the vessel, it was connected to a flow meter (Transonic Systems Inc., Ithaca, NY; Model TS-420) to test for correct placement of the probe, and the flow probe lead was secured to the cod's skin at 3 locations using silk suture (3-0, American Cyanamid Company, Pearl River, NY): one location close to the incision, a second under the pectoral fin, and a third close to the dorsal fin.

Once surgery had been completed, the fish were transferred to the swim-tunnel, and all fish commenced ventilation within < 2 minutes. The water velocity in the swim-tunnel was set at 0.25 body length per second (BL s^{-1}; a velocity where the fish did not swim actively, but had no trouble orienting themselves), and all fish were allowed at least 18 hours of recovery in normoxic water prior to the first swim trial.

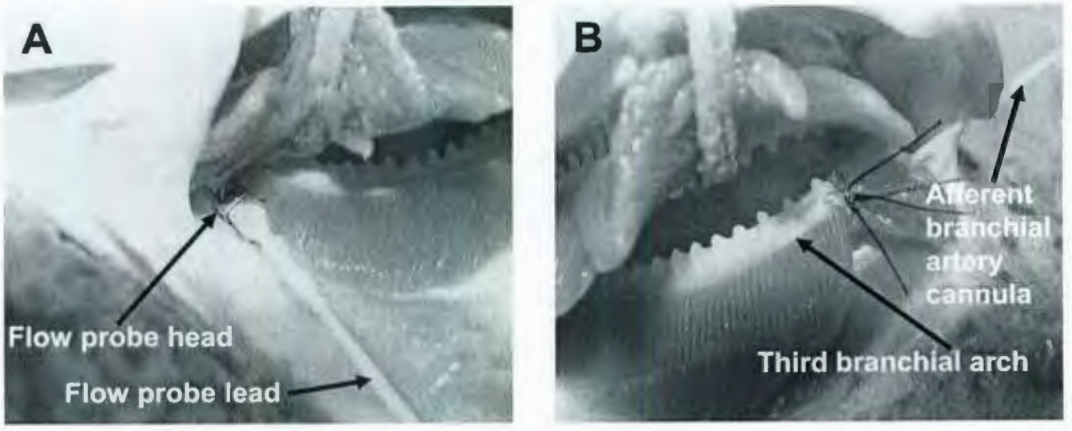

Plate 2.1. Surgical procedures used to determine *in vivo* cardiovascular function in Atlantic cod. Surgery involved placed an ultrasonic flow probe around the ventral aorta (A) and a afferent branchial artery cannula (B). Procedure shown in (A) was used in chaps 2,4 and 5 with the addition of an afferent branchial artery cannula in chaps 4 and 5.

2.3.4. Critical Swimming Speed Tests

Critical swimming speed (U_{crit}) tests were performed in a 81 litre Blazka-type swim-tunnel respirometer (University of Waterloo, Biotelemetry Institute, Waterloo, ON). The front of the respirometer was fitted with a plastic grid, which created uniform water flow in the swimming section of the respirometer (Taylor and McPhil, 1985), and the rear of the tunnel was fitted with a stainless steel grid connected to an external electrical circuit. This stainless steel grid could be electrified with a small current (< 5 V, ~ 0.2 A) to discourage the fish from resting on the grid during swimming trials. Further, the tunnel was covered with black plastic to provide the fish with a dark refuge, and to minimize stress from external stimuli (i.e. investigators presence).

Water (PO_2 level of 21 or 8-9 kPa) was supplied to the swim-tunnel from a temperature-controlled 270 L water reservoir that was maintained at 10 ± 0.1°C using an external, custom built, ¾ horse-power heater/chiller (Memorial University of Newfoundland, Technical Services, St. John's, NL). Further, the O_2 content of the water

was controlled by bubbling pure N_2 into the reservoir at rates predetermined to achieve the desired O_2 level.

2.3.5. Experimental Protocol

Resting and active oxygen consumption and cardiac function, and swimming performance, of individual fish were initially measured under normoxic conditions using a critical swimming speed (U_{crit}) test (Brett, 1964). After measuring cardiac function and oxygen consumption (see below) at the baseline speed of 0.25 BL s^{-1}, swimming speed was increased in 0.125 BL s^{-1} increments every 20 min. until the fish were exhausted; exhaustion determined as the inability of the fish to move away from the electric grid after three successive mild (5 V) shocks. Thereafter, water velocity was returned to 0.25 BLs^{-1} and the fish left overnight to recover. During the morning of the second day, the oxygen level in the tunnel was reduced over a period of 3 hours by bubbling pure N_2 into the reservoir to reduce the oxygen level in the tunnel to 16 kPa in the first hour, 12 kPa in the second hour, and 8-9 kPa by the end of the third hour. The oxygen level in the swim-tunnel was then maintained at 8-9 kPa for 1 hour before the hypoxic U_{crit} trial was performed. The hypoxic U_{crit} trial was identical to that performed during normoxia, and fish swum under hypoxic conditions were also allowed to recover under normoxic conditions; water P_wO_2 increased from 8-9 kPa to ~ 19 kPa during the first 20 min. of recovery. Fish swum under hypoxic conditions (P_wO_2 8-9 kPa) were not recovered at this oxygen level because preliminary experiments showed that some fish had difficulty recovering/righting themselves during the first 2 hours post-exercise.

For both normoxic and hypoxic swim trials, U_{crit} was calculated as:

$$U_{crit} = (V + ((T_f) \times V_i))/ T_i$$

where V = velocity at which the fish swam for the entire time increment; V_i = velocity increment; T_f = time elapsed from the last change in current velocity to fatigue; and T_i = time increment, the time between step increases in velocity (20 min.).

2.3.6. Measurement of Cardiac Function and Metabolism

Cardiac output (Q) was continuously measured during the U_{crit} trial and for approx. 2 hours after the fish became exhausted [i.e. measurements were taken immediately after the fish stopped swimming (0 min.), and at 25 min., 50 min., 75 min., 100 min. and 125 min. of recovery]. Q was measured by connecting the flow meter to a MP100A-CE data acquisition system (BIOPAC Systems Inc., Santa Barbara, CA) and a laptop running AcqKnowledge software (BIOPAC Systems Inc. Santa Barbara, CA). Data were recorded at a frequency of 20 Hz, and values of cardiac output were obtained during the last 5 min at each swimming speed and during the first 5 min. of each 25 min. period during recovery. Cardiac output (Q, in ml min^{-1} kg^{-1}) was calculated by dividing the raw data (ml min^{-1}) by the body mass of the fish (kg). Heart rate (f_H, beats min^{-1}) was calculated by dividing 60 (sec/min) by the time (sec.) required for 20 systolic peaks. Stroke volume (S_V, ml kg^{-1}) was calculated as Q/f_H. Maximum values of Q, S_V and f_H were measured as the highest value that each individual fish achieved. Finally, the absolute scope for cardiac variables (Q, S_V and f_H) was calculated by subtracting routine (resting) values from maximum values. Routine metabolic rate was determined at 0.25

BL s^{-1}; a speed at which the fish did not swim actively in the tunnel but had no trouble orientating themselves against the current.

Water temperature and oxygen concentration (mg O_2 L^{-1}) in the swim tunnel were continuously measured via an external circuit containing an oxygen probe housed in a D201 flow through cell (see description for the hypoxic acclimation tank). Oxygen consumption (MO_2) of the cod was measured over 10 min. intervals at rest, at each swimming speed, and at 0, 25, 50, 75, 100 and 125 min. of recovery by stopping the flow of water into the swim-tunnel, recording the drop in water-oxygen concentration in the swim-tunnel, and using the following equation (Cech, 1990):

$$MO_2 \text{ (mg } O_2 \text{ } h^{-1} \text{ } kg^{-1}) = \frac{((C_i - C_f) \times V_c) \times 60}{M \times T}$$

Where C_i = water oxygen concentration (mg O_2 L^{-1}) at the start of MO_2 measurement; C_f = oxygen concentration (mg O_2 L^{-1}) at the end of MO_2 measurement; V_c = volume of the respirometer and external circuit (81 L); M = fish mass (kg); and T = time required to make MO_2 measurement (10 min.).

Standard oxygen consumption was obtained from a semi-log plot of swimming speed (BL s^{-1}) versus log MO_2, and using the derived linear regression to extrapolate back to 0 BL s^{-1}. Maximum oxygen consumption (MO_{2max}) was measured as the highest oxygen consumption that each individual fish achieved, and absolute metabolic scope was then calculated by subtracting routine (resting) MO_2 from MO_{2max}. Finally, each fish's total Excess Post-Exercise Oxygen Consumption (EPOC, in mg O_2 kg^{-1}; a measure of the

non-aerobic cost of exercise) was obtained by integrating the area underneath the MO_2 – time curve until MO_2 returned to routine MO_2 (see Lee et al., 2003).

It is well established that the routine metabolic rate of fishes, including cod, scales allometrically with body mass with a slope of ~ 0.8 – 0.85 (Saunders, 1963; Post and Lee, 1996; Killen et al., 2007). However, isometrically scaled metabolic rate data (i.e. mg O_2 hr^{-1} kg^{-1}) are reported, because to my knowledge no scaling exponents have been reported for fish cardiovascular function, and I wanted to report all the parameters using common units. However, I do provide allometrically scaled metabolic rates when comparing my data with the literature (see discussion).

2.3.7. Effect of Surgery on Swimming Performance and Metabolism

In this experiment, I did not want to give the hypoxic fish an extended period of exposure to normoxic water prior to the initial U_{crit} test. However, I was also cognisant of the fact that surgery/the post-surgical recovery period can potentially affect swimming and cardiovascular performance (e.g. Butler et al., 1989; see Fig. 7 in Webber et al., 1998). Thus, normoxic and hypoxic swim trials were also performed on 9 (0.55 ± 0.03 kg) normoxic acclimated cod that did not undergo surgery or anaesthesia. These fish were taken from the same acclimation tanks as the `surgery` fish, and were placed directly in the swim tunnel after being netted from their acclimation tank. These fish were also allowed approx. 18 hours to recover.

2.3.8. Statistical Analyses

A one-way ANOVA was used to examine whether fish mass, length and condition factor were affected by chronic acclimation to hypoxic conditions (Table 2.1). Two-way ANOVAs with repeated measures were used to determine at which swimming speeds or times during recovery MO_2 and cardiovascular variables were different between: 1) surgery vs. non-surgery groups (Figure 2.1); and 2) normoxic- vs. hypoxia-acclimated cod (Figure 2.2). This analysis was also used to determine at which values of water O_2 saturation variables were different between normoxic- and hypoxic-acclimated cod during the stepdown period (Figure 2.3). Further, Dunnett's post-hoc tests were performed to examine if/when variables became different from values at 100% air saturation (Figure 2.3). Two-way ANOVAs with repeated measures, followed by paired (normoxic vs. hypoxic swim) or unpaired (normoxic- vs. hypoxic-acclimated or surgery vs. non-surgery) t-tests were used to identify differences in metabolic and cardiovascular variables (see Tables 2.2 and 2.3). Finally Pearson's correlation analysis was carried out to define the strength of the relationship between oxygen consumption and cardiac output during the normoxic and hypoxic swims and graded hypoxia (Figure 2.4). All data presented in figures, tables and the text are means ± standard errors (SEM). Statistical analyses were carried out using SPSS (v.13.0; SPSS, Chicago, IL, USA) and a difference was considered significant when $P < 0.05$.

2.4. Results

Water quality in the hypoxic tank did not deteriorate during the 6-12 weeks of hypoxic acclimation. Mean values for total nitrogen, un-ionized ammonia, carbon dioxide and pH were 0.03 ± 0.01 ppm, 0.0003 ± 0.0 ppm, 2.3 ± 0.6 ppm and 7.8 ± 0.1, respectively. Further, although the mass of hypoxia-acclimated cod, following acclimation, was approx. 15% lower as compared to those held under normoxic conditions, neither this parameter or condition factor were significantly different between the two groups (Table 2.1).

2.4.1. Effects of Anaesthesia/Surgery

Surgery resulted in a 14% decrease in the normoxic U_{crit} value (from 1.74 to 1.50 BL s^{-1}). However, the effect of surgery was similar when the fish were swum under hypoxia (17% decrease), and as a result, the reduction in U_{crit} between normoxic and hypoxic conditions was similar for both groups (non-surgery, 29%; surgery, 32%) (Table 2.2). This pattern of change in swimming performance was not reflected by all metabolic parameters. For example, cod fitted with flow probes had significantly lower routine and standard metabolic rates (by ~ 25%) when measured under normoxic conditions but surgery had no significant effect on normoxic values of MO_{2max} or metabolic scope. MO_{2max} and metabolic scope decreased to a much greater extent in the surgery fish as compared with non-surgery fish when they were swum at 8-9 kPa (57 vs. 43% and 80% vs. 55%, respectively; Table 2.2, Figure 2.1).

Table 2.1. *Physical characteristics of the Atlantic cod used in the various experiments.*

	Treatment		
	Non-Surgery	**Normoxic Acclimated**	**Hypoxic Acclimated**
Mass (kg)	0.55* (0.03)	0.68 (0.03)	0.57 (0.04)
Length (cm)	39.7 (0.8)	41.9 (0.9)	40.4 (0.8)
Condition factor (K)	0.90 (0.04)	0.94 (0.03)	0.86 (0.03)

*Indicates significant difference between non-surgery and normoxic acclimated groups. N=9-12 for all groups. Values are means ± SEM. Mass was determined in all three groups prior to surgery.

Table 2.2: *The effect of surgery and anaesthesia on swimming performance and oxygen consumption of Atlantic cod measured under normoxia (P_wO_2~21kPa) and hypoxia (P_wO_2~8-9 kPa).*

	Non-surgery		*Surgery (normoxic-acclimated)*	
	Normoxic Swim	**Hypoxic Swim**	**Normoxic Swim**	**Hypoxic Swim**
SMR	64.26a[a]* (8.76)	53.55 (7.42)	47.41 (2.02)	55.26 (4.14)
Routine MO_2	82.54[a]* (7.65)	65.25 (5.43)	63.45 (2.43)	63.58 (3.22)
Max MO_2	234.58[a] (21.16)	133.08* (8.75)	214.05[a] (10.58)	92.72 (3.36)
Scope	152.09[a] (20.66)	67.83* (8.03)	150.60[a] (9.97)	29.15 (4.14)
EPOC	73.09[a]* (11.94)	47.66* (3.37)	42.60 (4.29)	28.01 (7.71)
U_{crit}	1.74* (0.06)	1.23* (0.05)	1.50[a] (0.04)	1.02 (0.03)

*Indicates a significant difference between groups (non-surgery vs surgery) within a particular test condition.
Values are means ± SEM, N= 9-10 for both groups
[a]Indicates a significant difference between normoxic and hypoxic swims within each acclimation condtion.

Interestingly, EPOC was significantly ($P < 0.05$) higher in the non-surgery group when swum under normoxia and hypoxia (by 42 and 46%, respectively), as compared with the cod that underwent anaesthesia/surgery (Table 2.2).

2.4.2. Normoxic U_{crit} Test

In normoxic water, routine f_H, S_V, Q and MO_2 were 32.9 ± 2.2 beats min^{-1}, 0.73 ± 0.07 ml kg^{-1}, 23.1 ± 1.8 ml min^{-1} kg^{-1} and 63.5 ± 2.4 mg O_2 h^{-1} kg^{-1}, respectively in the normoxic-acclimated group (Figure 2.2, Table 2.3). Although resting f_H was similar in hypoxic-acclimated cod, MO_2 was significantly higher (by 40%) in this group, despite the fact that both Q and S_V were significantly lower (by 26% and 30%, respectively).

During the normoxic U_{crit} test, MO_2 and all cardiovascular variables increased with swimming speed, and differences between normoxic- and hypoxic-acclimated cod were generally retained (Figure 2.3, Table 2.3). For example, standard metabolic rate (SMR) was 30% higher in the hypoxic-acclimated group (68 vs. 47 mg O_2 h^{-1} kg^{-1}), and there were no differences in the scope for MO_2 (~ 150 mg O_2 h^{-1} kg^{-1}) or any of the cardiac parameters (f_H, ~ 14 beats min^{-1}; S_V, ~ 0.25 ml kg^{-1}; Q, 17 vs 21 ml min^{-1} kg^{-1}). However, there were some notable differences. First, f_H became significant elevated in the hypoxic-acclimated group, as compared with normoxic-acclimated fish, at swimming speeds between 1.0 and 1.375 BL s^{-1}. Second, although MO_2, f_H, S_V and Q generally increased in normoxic-acclimated fish until exhaustion, these parameters either plateaud or decreased slightly in hypoxic-acclimated fish after 1.25 BL s^{-1}. Given that there were no differences in metabolic scope or the scope for cardiac parameters, it was not surprising that U_{crit} (~ 1.5 BL s^{-1}) was identical between the two groups.

During recovery from the U_{crit} test, MO_2 fell rapidly in both groups, and there was no significant difference in EPOC (P = 0.31; Table 2.3), although it was 30% higher in the normoxia-acclimated group. Interestingly, the post-exercise pattern of change for f_H was different than for Q and S_V. The former parameter fell slowly after the cod were exhausted, whereas both Q and S_V decreased rapidly (ie. within 25 min.) to values comparable to, or below, routine levels and then rebounded (Figure 2.2).

2.4.3. Graded hypoxia and the hypoxic U_{crit} test

At the start of the 2nd day (i.e. at P_wO_2 21 kPa; Figure 2.3) MO_2 and values for cardiac function were very similar to those measured at the beginning of day 1 (i.e. prior to the normoxic U_{crit} test, Figure 2.2), and the differences between groups were maintained. For example, routine MO_2 was slightly higher (by ~ 10%, P= 0.22), and Q and S_V were again significantly lower (by ~ 22 and 30% respectively), in the hypoxic-acclimated group (Figure 2.3). There were very few changes in MO_2 or cardiovascular parameters as water O_2 partial pressure was lowered from 21 kPa to 8-9 kPa. However, f_H and Q did increase slightly, and significantly, after one hour of exposure to water of 8-9 kPa O_2 in the normoxic-acclimated cod.

The pattern of change in MO_2 and cardiac parameters during the hypoxic U_{crit} test was qualitatively similar to that seen during the normoxic swim (Figure 2.2). However, U_{crit} for both groups (1.02 BL s^{-1}) was only approx. two-thirds of that measured during normoxia (1.5 BL s^{-1}), and this diminished swimming performance was associated with important differences in how cardiorespiratory parameters in normoxic- and hypoxic-acclimated cod responded to the exercise regimen. First, as compared with the normoxic

swim, maximum MO_2 was greatly reduced in both groups (214 and 231 mg O_2 h^{-1} kg^{-1} vs. 93 and 101 mg O_2 h^{-1} kg^{-1}), and this resulted in a dramatically reduced metabolic scope (to ~ 30 mg O_2 h^{-1} kg^{-1}). Second, although the scope for Q was reduced to a similar degree in both groups (to ~ 11.5 ml min^{-1} kg^{-1}) when the cod were swum under hypoxic conditions as compared with normoxia, the reason for the reduced scope for Q was different. In the normoxic acclimated group, the scope for Q was diminished because the scope for f_H was reduced by 50% (normoxia 13.4 beats min^{-1}; hypoxia 5.8 beats min^{-1}). Whereas, the scope for S_V fell from 0.25 to 0.15 ml kg^{-1} in the hypoxia acclimated group when the cod were swum at an oxygen level of 8-9 kPa (Table 2.3; Figure 2.2).

During recovery from the hypoxic U_{crit} test, the pattern of change in MO_2, Q and S_V was similar to that observed after the normoxic swim. However, the pattern of change in f_H was quite different. Heart rate increased in both groups between 0 and 25 min. post-exercise, before declining to pre-swim levels (Figure 2.2). This was likely due to the fact that these fish were recovered in normoxic, not hypoxic water. As with the normoxic swim, there was no difference in EPOC values between the two groups. However, for both groups, EPOC was approx. 35% lower as compared to values for cod swum under normoxic conditions (Table 2.3).

2.4.4. Relationship Between Oxygen Consumption and Cardiac Output

During the normoxic swim (Figure 2.4A) there was a strong linear relationship between swimming speed and oxygen consumption in both the normoxic- (r^2 = 0.97, $P<0.0001$) and hypoxic-acclimated groups (r^2 = 0.95, $P<0.0001$). However, the relationship for the hypoxic-acclimated fish was shifted decidedly upwards, and this

resulted in a substantially greater MO_2 for a given Q in hypoxic- as compared with normoxic-acclimated fish. There was no clear relationship between MO_2 and Q when the fish were exposed to graded hypoxia. However, the MO_2 of hypoxia-acclimated fish was generally above that of the normoxia-acclimated fish, and this elevated level of MO_2 was achieved at reduced levels of Q (Figure 2.4B). Finally, although the relationship between MO_2 and Q was not as strong during the hypoxic swim ($r^2 = 0.83$ and 0.84, $P<0.001$), and the data for the hypoxia-acclimated group were much more variable, it was again apparent that the hypoxia-acclimated fish could consume more O_2 for a given cardiac output (Figure 2.4C).

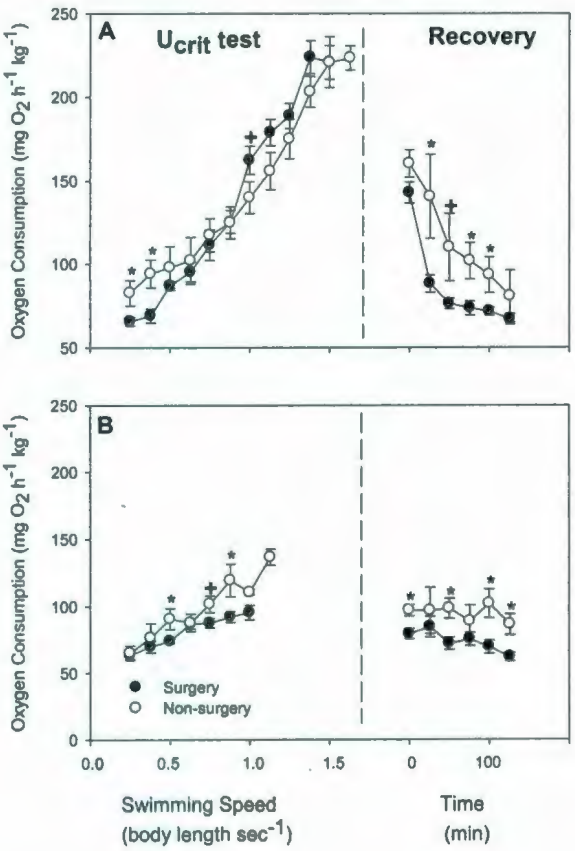

Figure 2.1. The effect of anaesthesia and surgery (Transonic® flow probe placement around ventral aorta) on the oxygen consumption of cod during normoxic (A) and hypoxic (B, P_wO_2 8-9 kPa) critical swimming speed tests, and during post-exercise recovery. Non-surgery fish were netted from their holding tank, and placed directly into the swim-tunnel respirometer. All fish were swum in normoxic water on day 1 and hypoxic water on day 2, but recovery was performed in normoxic water for all swims. N = 9 for non-surgery fish, and N = 10 for fish that underwent surgery. *Indicates significant differences between non-surgery and surgery groups at $P < 0.05$ while $^{+}$indicates differences at ($P < 0.10$). See Table 2.2 for a statistical analysis of differences in maximum swimming speed (U_{crit}) and metabolic parameters between groups.

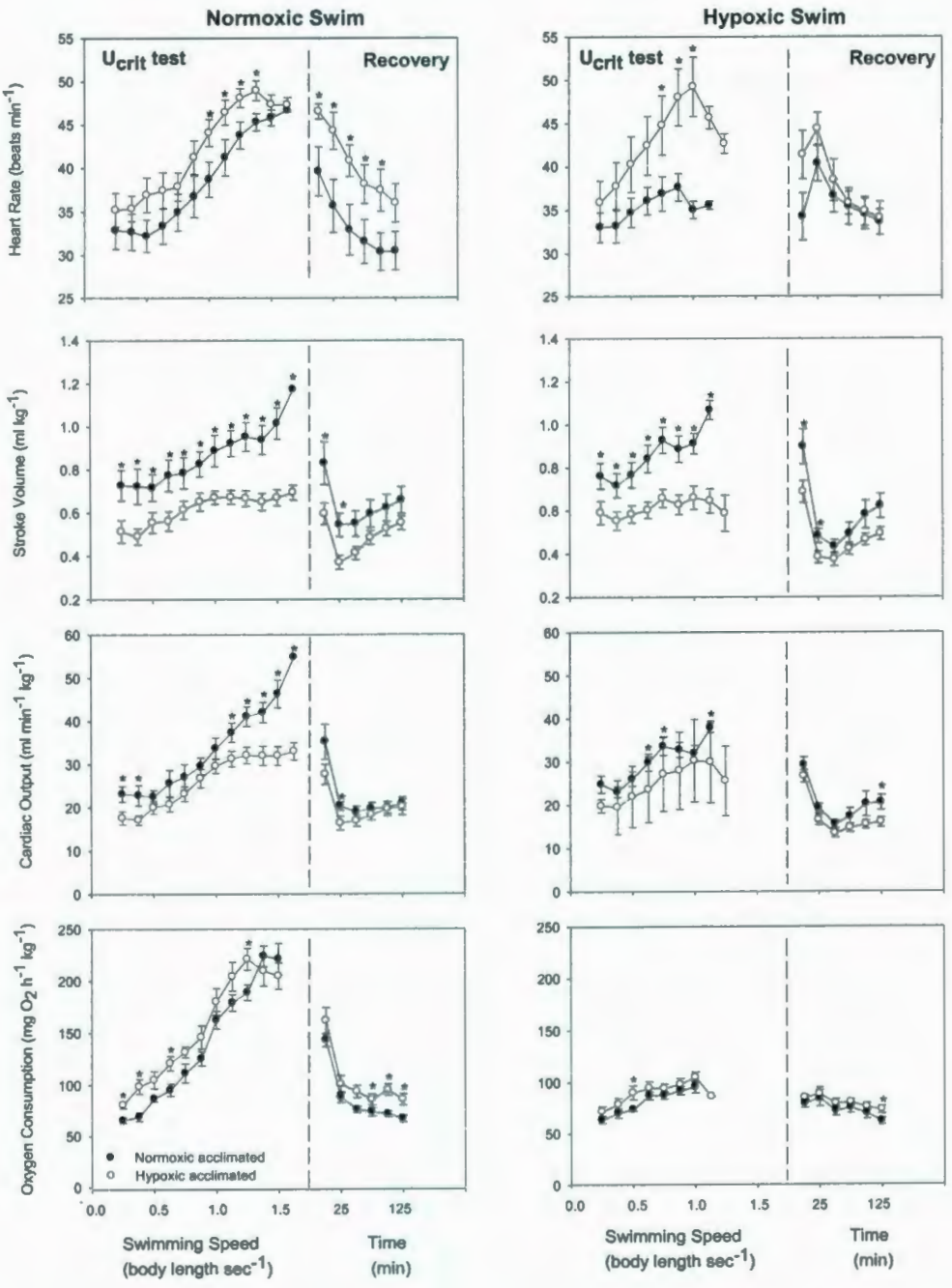

Figure 2.2. Heart rate (f_H), stroke volume (S_V), cardiac output (Q) and oxygen consumption (MO_2) in normoxia- (N = 10) and hypoxia-acclimated (N = 12) cod during critical swimming speed (U_{crit}) tests, and during post-exercise recovery. All fish were swum in normoxic water on day 1 and hypoxic water on day 2, but recovery was performed in normoxic water for all swims. *Indicates a significant difference ($P < 0.05$) between the normoxia- and hypoxia-acclimated groups at a particular swimming speed.

Table 2.3: *Metabolic and cardiac parameters, and swimming performance, in normoxic and hypoxic acclimated Atlantic cod subjected to critical swimming speed (U_{crit}) tests under both normoxic (PwO_2~21kPa) and hypoxic (PwO_2~8-9 kPa) conditions.*

	Normoxic Acclimated								Hypoxic Acclimated							
	Normoxic Swim				Hypoxic Swim				Normoxic Swim				Hypoxic Swim			
	MO_2	f_H	S_V	Q	MO_2	f_H	S_V	Q	MO_2	f_H	S_V	Q	MO_2	f_H	S_V	Q
Routine	63.45* (2.43)	32.88 (2.16)	0.73* (0.07)	23.07* (1.82)	63.58 (3.22)	33.01 (1.70)	0.73* (0.04)	23.24 (0.88)	80.88[a] (3.40)	35.2 (1.93)	0.49[a] (0.04)	16.96 (1.09)	71.97 (4.29)	35.90 (2.35)	0.56 (0.04)	19.79 (1.54)
Max	214.05[a] (10.58)	46.25[a] (0.94)	0.99* (0.07)	44.54[a]* (2.70)	92.72 (3.36)	38.84* (1.48)	0.99* (0.06)	34.62 (1.74)	231.44[a] (12.32)	50.08 (0.80)	0.74 (0.04)	34.23 (2.16)	100.58 (5.14)	48.48 (2.99)	0.71 (0.04)	31.30 (1.78)
Scope	150.60[a] (9.97)	13.37[a] (1.93)	0.26 (0.04)	21.47[a] (2.16)	29.15 (4.14)	5.83* (1.01)	0.27* (0.04)	11.37 (1.49)	150.56[a] (12.50)	14.88 (1.97)	0.25[a] (0.03)	17.28[a] (1.93)	28.60 (2.84)	12.59 (1.25)	0.15 (0.03)	11.51 (1.77)
SMR	47.41* (2.02)	-	-	-	55.26 (4.14)	-	-	-	68.32 (4.04)	-	-	-	63.21 (4.07)	-	-	-
EPOC	42.60 (4.29)	-	-	-	28.01 (7.71)	-	-	-	32.96 (6.79)	-	-	-	22.27 (3.85)	-	-	-
U_{crit}	1.50[a] (0.04)				1.02 (0.03)				1.51[a] (0.07)				1.02 (0.05)			

Oxygen consumption (MO_2, mg O_2 h^{-1} kg^{-1}), heart rate (f_H, beats min^{-1}), stroke volume (S_V, ml kg^{-1}), cardiac output (Q, ml min^{-1} kg^{-1}) and U_{crit} (BLs^{-1}). Routine refers to values measured at rest (0.25 BLs^{-1}). SMR (standard metabolic rate) was obtained by plotting log MO_2 of individual fish against swimming speed and extrapolating to 0 BLs^{-1}. EPOC (Excessive Post-Exercise Oxygen Consumption). Values are means ± SEM, N= 10 for both groups.
[a]Indicates a significant difference between normoxic and hypoxic swims with each acclimation condition.
*Indicates a significant difference between groups (normoxic vs hypoxic acclimation) with a particular test condition.

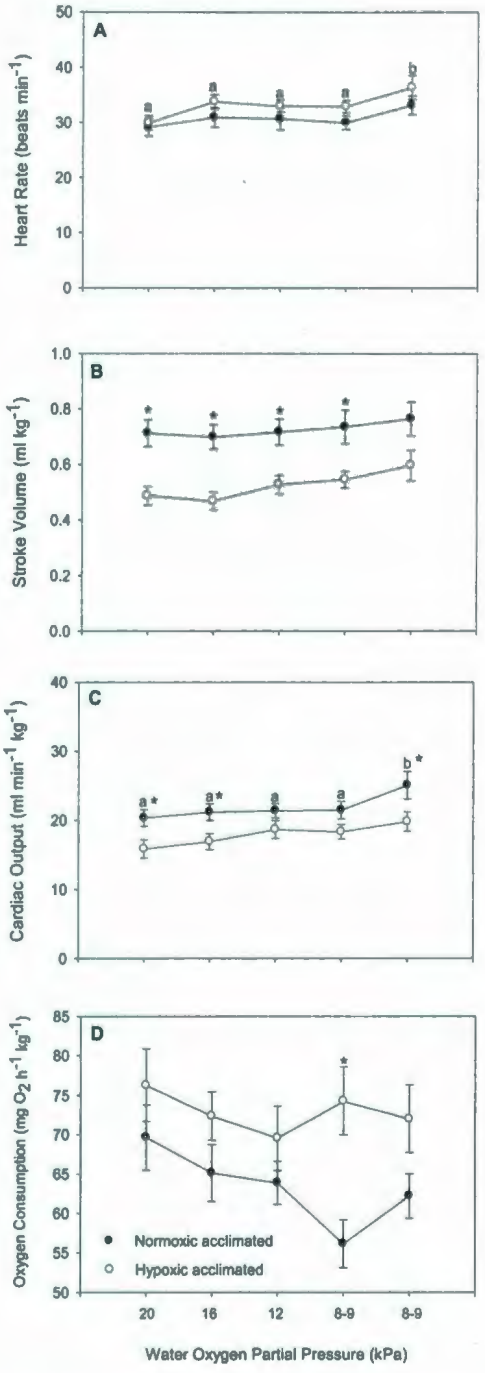

Figure 2.3. Cardiac function (f_H, S_V, Q) and metabolism when normoxia- and hypoxia-acclimated cod were exposed to graded hypoxia. Oxygen levels were dropped from a P_wO_2 of 21 kPa to 16 kPa in the first hour, from 16 to 12 kPa in the second hour and finally from 12 to 8-9 kPa in the third hour. Dissimilar letters indicate significant ($P < 0.05$) differences from 21 kPa and other oxygen levels within the normoxic group. *Indicates values significantly ($P<0.05$) different between normoxia- and hypoxia-acclimated fish at a particular P_wO_2.

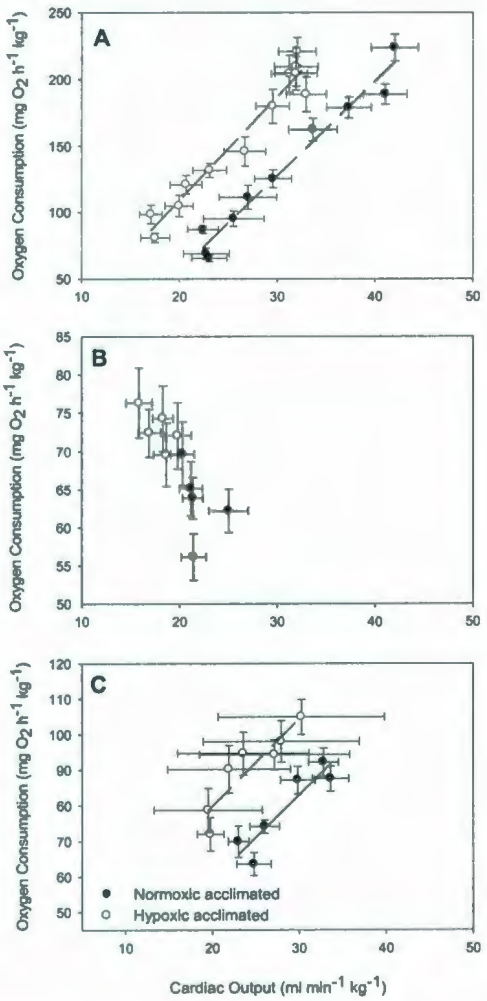

Figure 2.4. Relationship between oxygen consumption and cardiac output in the normoxia- and hypoxia-acclimated cod during a normoxic U_{crit} test (A), exposure to graded hypoxia (B), and finally a hypoxic U_{crit} test (C). Dashed lines define the linear regressions that were fitted to the data. Normoxic swim: normoxia-acclimated group ($y = 7.1x-85.48$, $r^2 = 0.97$, $P< 0.0001$), hypoxia-acclimated group ($y = 7.79x-46.76$, $r^2=0.95$, $P<0.0001$). Hypoxic swim: normoxia-acclimated group ($y =2.40x+11.14$, $r^2= 0.84$, $P<0.0001$), hypoxia-acclimated group ($y = 2.46x+30.62$, $r^2 = 0.83$, $P<0.0001$).

2.5. Discussion

The cod in this study were swum to exhaustion, first under normoxia, and then at a P_wO_2 of ~ 8-9 kPa after being allowed to recover from the initial U_{crit} test for approx. 24 hours. This experimental design has some limitations. Most importantly the possibility that different degrees of recovery from surgery (18 vs. approx. 42 hours) or effects related to the initial U_{crit}, might have obscured some effects of acute hypoxia on swimming performance, cardiac function and metabolic capacity. We believe that these were minimal given how closely our data fit with the literature, data showing that fish recover quickly (within 2 hours) from exhaustive exercise under normoxic conditions (Jain and Farrell, 2003; Jain et al., 1998), and that the reduction in U_{crit} with acute hypoxia was similar in fish that were simply placed into the swim-tunnel (non-surgery) vs. those that were implanted with flow probes (see below). Further, it is unlikely that the experimental design significantly affected our major findings with respect to the effects of hypoxic acclimation on cod cardiorespiratory function. This is because our results are very similar to those obtained in a later study where cod were given a graded hypoxic challenge approx. 24 hours after recovering from surgery (L.H. Petersen and A.K. Gamperl, in review b) and to those recently reported by Lamarche et al. (Lamarche et al., 2009).

In this study I made the first measurements of fish cardiorespiratory function during exercise under hypoxia (P_wO_2 8-9 kPa), and of how acclimation of cod to this same level of hypoxia for 6-12 weeks influenced resting and exercise-induced cardiac function under both hypoxic and normoxic conditions. These experiments revealed that: 1) when cod are swum under hypoxic conditions cardiac function is diminished, and this

is associated with reduced aerobic scope, and an ~30% lower U_{crit}; 2) hypoxic acclimation does not improve the cod's swimming capacity, maximum metabolic rate or scope for activity when swum to U_{crit} under either normoxic or hypoxic conditions; and 3) although the resting and maximal cardiac output of hypoxia acclimated cod are diminished due to a reduced S_V, this does not significantly affect the swimming speed-O_2 consumption relationship, maximum MO_2 or aerobic scope because hypoxic-acclimated cod can consume more oxygen for a given Q. These results offer novel insights into how fish cardiorespiratory physiology is impacted by short-term and prolonged exposure to environmental hypoxia, and another important example of how incredibly flexible cardiac function and its control is in fishes.

2.5.1. Fish Husbandry

In this study, Atlantic cod were acclimated to normoxic (P_wO_2 ~21 kPa) and hypoxic (P_wO_2 ~8 kPa) conditions for 6-12 weeks to assess the effects of chronic hypoxia on cardiorespiratory and swimming performance. However, chronic exposure to hypoxia also reduces appetite and growth rate (Jobling, 1994; Chabot and Dutil., 1999; Pichavant et al. 2001), and growth rate can influence metabolism and swimming performance (Kolok and Oris, 1995; Gregory and Wood, 1998). Thus, the normoxic-acclimated group was fed identical amounts as the chronic hypoxic group. This manipulation resulted in condition factors (means ranging from 0.86 – 0.94) that were not significantly different between groups, and surprisingly similar to those reported for Atlantic cod in good nutritional status (~ 0.8 – 0.95: Lambert and Dutil, 1997b; Martinez et al. 2003, 2004;

Alkanani et al., 2005; Lapointe et al., 2006). This latter finding was likely because of the reduced activity levels of the cod in the holding tanks, and the fact that the hypoxic fish began feeding (albeit at reduced levels) shortly after the final P_wO_2 of ~ 8 kPa was reached. In addition, water quality in the hypoxic tank was within the recommended range for fish in aquaculture conditions (Alken-Murray, 2003) throughout the 12 weeks of acclimation. Thus, based on the above, we are confident that the chronically hypoxic cod were not affected by factors other than reduced oxygen levels.

2.5.2. Normoxic Resting Parameters and the Effects of Surgery

The resting heart rate (f_H) of normoxic-acclimated cod (~ 33 beats min^{-1}) was in the middle of the range of values (24 – 43 beats min^{-1}) previously reported for normoxic 10°C cod that were outfitted for cardiovascular measurements (Jones et al., 1974; Pettersson and Nilsson, 1980; Wahlqvist and Nilsson, 1980; Smith et al., 1985; Axelsson and Nilsson, 1986; Axelsson, 1988; Butler et al., 1989; Fritsche and Nilsson, 1989; Webber et al., 1998; Gollock et al., 2006), and only slightly higher than those recorded in free-swimming cod using ultrasonic telemetry (Wardle and Kanwisher, 1974; Claireaux et al., 1995). Further, the standard metabolic rate (SMR) of the normoxic cod that underwent surgery (47 mg O_2 hr^{-1} kg^{-1}; 42 mg O_2 hr^{-1} $kg^{-0.8}$) was lower than that measured for non-surgery fish, and the majority of values reported by other authors for cod at 10°C (Soofiani and Priede, 1985, 98 mg O_2 hr^{-1} $kg^{-0.8}$; Webber et al., 1998, 75 mg O_2 hr^{-1} $kg^{-0.8}$; Schurmann and Steffensen, 1997, 57 mg O_2 hr^{-1} $kg^{-0.8}$; Claireaux et al., 2000, 67 mg O_2 hr^{-1} $kg^{-0.8}$); with the exception of recent studies by Lurman et al. (2007, 36 mg O_2 hr^{-1} $kg^{-0.8}$) and Sylvestre et al. (2007, ~ 37 mg O_2 hr^{-1} $kg^{-0.8}$). While the f_H and SMR data suggests

that these cod had recovered fully from surgery/anaesthesia before the initial swim trial in normoxia, a different interpretation results when the routine values of stroke volume (0.73 ml kg^{-1}) and Q (23 ml min^{-1} kg^{-1}) in our normoxic-acclimated cod are compared with the literature. These values were 10-20% above the values reported by other authors (ranges ~ 0.4 – 0.6 ml kg^{-1} and 19 – 21 ml min^{-1} kg^{-1}, respectively: Jones et al., 1974; Axelsson and Nilsson, 1986; Axelsson, 1988; Fritsche and Nilsson, 1989; Gollock et al., 2006). Why these basal measurements provide disparate assessments of the recovery state of the cod used in these experiments is not known. However, the 14% lower U_{crit} in normoxic cod that underwent surgery vs. those that did not (see Table 2.2 and Figure 2.1) also suggests that the post-surgery period (~ 18–24 hours) was not sufficient to allow for complete recovery.

The reduction in normoxic swimming performance (from 1.74 to 1.5 BL s^{-1}) associated with surgery/anaesthesia was not unexpected, as Butler et al. (1989) report that U_{crit} in cod is diminished by 13.5% following sham surgery. Nonetheless, it is difficult to ascribe a definitive cause for the diminished U_{crit} under normoxic conditions as maximum MO_2 and metabolic scope were similar between groups (~ 220 and 150 mg O_2 hr^{-1} kg^{-1}; ~ 198 and 135 mg O_2 hr^{-1} $kg^{-0.8}$) (Table 2.2) and comparable to literature values for this species at 7-11°C (Claireaux et al., 2000, 177 and 123 mg O_2 hr^{-1} $kg^{-0.8}$; ~ 200 and 155 mg O_2 hr^{-1} $kg^{-0.8}$, Lapointe et al. 2007 and Sylvestre et al., 2007). Further, maximum heart rate, S_V and Q (46 beats min^{-1}, 0.99 ml kg^{-1} and 45 ml min^{-1} kg^{-1}) in the surgery group are very comparable to recent *in situ* values for Atlantic cod hearts at 10°C (~ 42 - 54 beats min^{-1}; 0.92- 1.2 ml kg^{-1} and 45 – 50 ml min^{-1} kg^{-1}; Lurman et al. unpubl., Petersen et al., unpubl.; Chapter 3), and the scope for Q (21.5 ml min^{-1} kg^{-1}) was almost

identical to that obtained for cod fitted with Doppler flow probes that were subjected to a U_{crit} test following at least 48 hours of recovery (23 ml min^{-1} kg^{-1}, Webber et al., 1998). There are, however, two potential explanations. First, Lapointe et al. (2007) showed that cod in poor condition (condition factor 0.68) start burst-coast swimming (ie. switch to a mix of aerobic and anaerobic powered swimming) at lower speeds, and become exhausted at a reduced U_{crit}. However, we feel that an earlier initiation of burst-coast swimming is unlikely in this study as there were no significant ($P < 0.05$) differences in MO_2 between the two groups of fish at swimming speeds above 0.375 BL s^{-1}; this finding suggesting that the cost of transport was similar between the two groups. Second, Excess Post-Exercise Oxygen Consumption (EPOC: an index of the non-aerobic costs of exercise; Lee et al. 2003) was significantly lower in the surgery group. This latter result suggesting that U_{crit} was reduced not because burst-coast swimming was initiated at a lower velocity, but because the number or intensity of burst-coast events prior to exhaustion was diminished. Such a reduction in swimming effort associated with recovery from anaesthesia/surgery would be consistent with the conclusions of McFarlane and McDonald (2002) and Peake and Farrell (2006). These authors indicate that the cessation of activity and decreased swimming performance in U_{crit} tests is most likely a behavioural response (i.e. they are disinclined to swim at maximum levels).

2.5.3. Effects of Acute Hypoxia and Hypoxic-Acclimation: Measurements at Rest

Acclimation to chronic hypoxia ($P_wO_2 \sim 8$ kPa) resulted in elevated routine (i.e. measured at 0.25 BL s^{-1}) and standard metabolic rates (Table 2.3, Figure 2.2) in normoxia as compared to normoxic-acclimated cod. This result contrasts with the findings of

Bushnell et al. (1984) who reported that hypoxic-acclimation did not influence, or significantly reduce, normoxic MO_2 values in resting rainbow trout. The disparity in results between this study and Bushnell et al. (1984) could be related to methodological differences between the two studies. Bushnell et al. (1984) only acclimated their trout to hypoxia for 3 weeks, the level of hypoxia during acclimation ($P_wO_2 \sim 5.6$ kPa) was more severe than utilized in the present study, and the hypoxia-acclimated trout were only allowed a brief period at normoxic levels of oxygen prior to measurements of MO_2. However, it may also be due to inter-specific differences in how teleost fish are affected by chronic hypoxia. For example, while Lomholdt and Johansen (1979) report that carp (*Cyprinus carpio*) acclimated to a P_wO_2 of 4 kPa for 4 weeks had a reduced MO_2 when they were returned to high oxygen conditions, Pichavant et al. (2000) showed that hypoxic-acclimation (45 days at a $P_wO_2 \sim 10$ kPa) did not alter the routine MO_2 of turbot (*Scophthalmus maximus*) at any water PO_2 level.

Interestingly, the elevated routine and standard metabolic rates in hypoxic-acclimated fish were achieved with lower values of S_V and Q as compared with normoxic-acclimated individuals (0.49 vs. 0.73 ml kg^{-1} and 17.0 vs. 21.3 ml min^{-1} kg^{-1}, respectively). There are a number of possible explanations as to why Q and S_V were lower in the hypoxic-acclimated cod as compared to normoxic-acclimated fish, despite the elevated MO_2. These include a hypoxia-induced decrease in heart size, a loss of pumping capacity related to hypoxia-induced myocardial damage, and/or increases in blood oxygen transport and/or tissue O_2 extraction efficiency. Although, the first explanation can be excluded as subsequent studies (Petersen and Gamperl, unpubl.; Chapter 3) have shown that relative ventricular mass (RVM) is not different between

hypoxic- and normoxic-acclimated cod, later discussion will reveal that all of the three other possibilities may have played an important role.

Standard and routine metabolic rates decreased slightly (by 16 and 20%) when the non-surgery group was acutely exposed to a P_wO_2 8-9 kPa (Table 2.2). This magnitude of decrease in routine MO_2 is consistent with recent data on juvenile cod at 10°C under similar experimental conditions (Gamperl et al., 2009), but in contrast to previous studies on both Atlantic (at 10°C) and Greenland cod (*Gadus ogac*) (at 4.5°C) where no significant decrease in routine MO_2 was noted until the fish's critical oxygen tension (Steffensen et al., 1994; Schurmann and Steffensen, 1997). A significant decrease in routine MO_2 was also recorded in the hypoxic-acclimated cod when water oxygen content was reduced to 8-9 kPa (Table 2.3). In contrast to the non-surgery and hypoxia-acclimated groups, acute hypoxia did not significantly affect routine MO_2 in the normoxic-acclimated group. However, it is unlikely that this disparity represents a difference in the way that the groups responded to hypoxia. This is because SMR and routine MO_2 values in the normoxic-acclimated group were lower than those for the non-surgery group and hypoxia-acclimated groups under normoxia. Furthermore, routine MO_2 decreased by a similar magnitude when water oxygen levels were reduced prior to the hypoxic swim, albeit non-significantly (Figure 2.3D). At a P_wO_2 of 8-9 kPa hypoxic-acclimated cod had slightly (by ~ 14%), but not significantly, higher values for SMR and routine MO_2. The lack of a change, or slight increase, in SMR and routine MO_2 for hypoxic-acclimated cod when measured under hypoxia (P_wO_2 ~ 8 kPa, 40% O_2 saturation) is in agreement with Bushnell et al. (1984) for rainbow trout. However, it is in contrast to studies on flounder (*Platichthys flesus*) (Kerstens et al., 1979) and carp

(Lomholt and Johansen, 1979), where fish acclimated to P_wO_2 ~ 4 kPa had oxygen consumption values between 30 and 100% higher than normoxic-acclimated individuals at this P_wO_2.

When normoxic-acclimated cod were exposed to acute hypoxia, f_H and S_V did not change significantly, although Q did increase slightly after 1 hour at 8-9 kPa, as compared with the value recorded at the start of day 2 (Table 2.3, Figure 2.3). The lack of hypoxic bradycardia, and concomitant increase in S_V, in this study is in clear contrast to previous studies on cod (Fritsche and Nilsson, 1989), and numerous other teleost species (e.g. rainbow trout, *Oncorhynchus mykiss* Wood and Shelton, 1980; lingcod, *Ophiodon elongates*, Farrell, 1982; smallmouth bass, *Micropterus dolomieu*, Furimsky et al., 2003; short-horned sculpin, *Myoxocephalis scorpius*, MacCormack and Driedzic, 2004). This was most likely due to the depth of hypoxia, as: 1) the level of hypoxia (P_wO_2) used by Fritsche and Nilsson (1989) was ~ 5 kPa as compared to 8-9 kPa in the present study; 2) the S_{crit} for Atlantic cod at 10°C is at or below the lowest level of hypoxia used in this study (Schurmann, and Steffensen, 1997; Gamperl et al., 2009); and 3) Petersen and Gamperl (unpubl.; Chapter 5) have shown that f_H falls (and S_V increases) rapidly in normoxic- and hypoxic-acclimated cod when P_wO_2 falls below ~ 5 kPa; f_H and S_V in normoxic-acclimated cod ~ 20 beats min^{-1} and 1.3 ml kg^{-1}, respectively at 2.7 kPa. However, we cannot exclude the possibility that the rapid induction of hypoxia in Fritsche and Nilsson (1989) (i.e. over ~ 60 sec), versus exposure to graded hypoxia over a period of 3 hr (present study), contributed to the contrasting effects of hypoxia on f_H between the two studies.

2.5.4. Effects of Acute Hypoxia and Hypoxic-Acclimation: Exercise

When the two normoxic-acclimated groups (i.e. surgery and non-surgery) were exposed to a P_wO_2 of 8-9 kPa, U_{crit} was reduced by 33 and 29%, respectively. This decrease in U_{crit} is very similar to that reported by Dutil et al. (2007) for 7°C cod swum at 8.5 kPa (1.72 vs 1.26 BL s^{-1}), and comparable to those for rainbow trout (approx. 25% at 5.6 kPa; Bushnell et al., 1984) and coho salmon (*Oncorhynchus kisutch*, 27% at ~ 8.5 kPa; Dahlberg et al., 1968) swum at 15 and 20°C, respectively. However, it is much greater than that recorded for smallmouth bass at 25°C (10% at ~ 8.5 kPa; Dahlberg et al., 1968) and mullaway (*Argyrosomus japonicus*) at 21-23°C (17% at both ~ 10 and 5 kPa; Fitzgibbon et al., 2007). When combined, these data suggest that there are considerable intra-specific differences in the sensitivity of swimming performance to reduced oxygen levels, and that this variation is related to the likelihood that a species will encounter hypoxic conditions during its life history. For example, most salmonids inhabit well-aerated fluvial environments and cod normally avoid water oxygen levels less than 9 kPa (40-45% saturation) (Claireaux et al., 2005). In contrast, largemouth bass (*Micropterus salmoides*) prefer shallow, warm, weedy areas (Heidinger, 1975) and the mullaway spends its early life history in estuaries (Fitzgibbon et al., 2007), both environments where water quality and oxygenation can vary considerably.

The reduced swimming performance of trout during hypoxia was attributed by Jones (1971) to an increase in the metabolic cost of both cardiac and branchial pumps, which he suggested placed a constraint on maximum performance. Although this was later disputed by Farrell and Steffensen (1987) who showed that fish shift to ram ventilation at high swimming speeds, it is clear that reduced metabolic scope (by 55 and

80% in non-surgery and surgery groups, respectively; Table 2.2) restricted the swimming performance of the normoxic-acclimated cod during acute hypoxic exposure; and that this was, in large part, related to a diminished maximum cardiac performance. The dependence of fish swimming performance on metabolic scope is well established (e.g. see Fry, 1971; Arnott et al., 2006; Chatelier et al., 2006), and the magnitude of the decreases in metabolic scope when the cod were swum under hypoxic conditions is consistent with other studies that have examined the relationship between metabolic scope and reduced water oxygen levels in this species. For example, at a P_wO_2 of 8-10 kPa cod forced to swim in respirometers to U_{crit} show reductions in metabolic scope ranging from 65-75% (Claireaux et al., 1995; Dutil et al., 2007), while scope calculated on free-living fishes using limiting oxygen concentration curves (Claireaux and Lagardère, 1999) falls by approx. 53% (Claireaux et al., 2000). Further, myocardial performance is a primary factor limiting active metabolic rate and U_{crit} in active teleosts (Farrell, 2002; Claireaux et al., 2005; Clark et al., 2005), and maximum Q was reduced by 47% when normoxic-acclimated cod were swum under hypoxic conditions. While a diminished maximum Q under hypoxic as compared to normoxic conditions (from 45.5 to 34.6 ml min^{-1} kg^{-1}) was not unexpected (e.g. see Hanson et al., 2006), we were surprised to find that the reduction in Q was solely related to a lower maximum f_H (and scope for f_H); maximum S_V reaching the same value as measured under normoxia (0.99 ml kg^{-1}). This is because: 1) venous PO_2 would be expected to be well below 2 kPa assuming that values in swimming cod and rainbow trout (Steffensen and Farrell, 1998; Farrell and Clutterham, 2003) are similar; 2) the cod heart does not have a coronary blood supply and is thus totally dependent upon venous oxygen to support cardiac function; and 3) venous

blood becomes hyperkalemic and acidotic, in addition to severely hypoxemic, during strenuous exercise (Graham et al., 1982; Holeton et al., 1983; Thomas et al., 1987; Nielsen and Lykkeboe, 1992; Holk and Lykkeboe, 1998), and the evidence to date (Axelsson et al., 1988; Lurman et al., unpubl) suggests that the cod myocardium is insensitive to adrenergic stimulation at catecholamine concentrations well above plasma levels measured during exhaustive swimming (Axelsson and Nilsson, 1986; Butler et al., 1989). The latter is a particularly relevant point given that all three changes in blood chemistry have a significant negative effect on myocardial contractility, and adrenergic stimulation appears to be critical for maintaining maximum cardiac performance during prolonged swimming (Hanson et al. 2006; Hanson and Farrell, 2007).

The obvious question is how did the normoxic-acclimated cod in our experiments maintain maximum S_V in the face of deteriorating venous blood chemistry? One possibility is that nervous adrenergic stimulation of the cod heart was supporting cardiac function, a hypothesis consistent with Axelsson (1988) who concluded that adrenergic tonus on the cod heart at rest and during exercise is of nervous origin. Further, the lower heart rate in normoxic-acclimated cod swum under acute hypoxia, would have afforded a number of direct benefits with respect to myocardial oxygen delivery and utilization. These include: an increase in the diastolic residence time of blood in the lumen of the heart, and thus increased time for O_2 diffusion; an increase in myocardial contractility through the negative force-frequency effect; a decrease in myocardial oxygen demand through a reduction in the rate of pressure (tension) development (dP/dt); and finally, the increased S_V concomitant with the decreased f_H would have resulted in a stretching of the cardiac chambers, and a reduction of the diffusion distance for oxygen (see Farrell, 2007).

Finally, Sandblom and Axelsson (2005, 2006) have shown that venous capacitance is actively modulated in fish in response to hypoxia, and it is likely that increased cardiac preload associated with increased venous tone and the lower heart rate allowed the normoxic-acclimated cod to maintain maximum S_V when swum at P_wO_2 8-9 kPa.

What is not so clear, however, is what factor(s) mediated the reduction in maximum f_H (and scope for f_H) in the normoxic-acclimated cod swum under hypoxic conditions. This is because, in contrast to the rainbow trout (e.g. see Gamperl et al., 2004; Faust et al., 2004), acute exposure of the *in situ* cod heart to severe hypoxia does not affect f_H during resting or maximum levels of cardiac performance (Petersen and Gamperl, unpubl; Chapter 3), and maximum f_H and scope for f_H were not different in hypoxia-acclimated cod when swum under hypoxic and normoxic conditions (Figure 2.2; Table 2.3). Further, it is unlikely that the lowered f_H was the result of the stimulation of branchial O_2 receptors as this level of hypoxia (P_wO_2 8-9 kPa) did not elicit bradycardia under resting conditions. This raises the distinct possibility that venous O_2 receptors located at or before the heart, as proposed by Barrett and Taylor (1984), were stimulated by severe hypoxemia associated with exercise under hypoxic conditions and mediated a reduction in heart rate through the efferent limb of the cardiac vagus.

Cod chronically acclimated to moderate hypoxia (P_wO_2 8-9 kPa) had values of maximum MO_2, metabolic scope and U_{crit} under normoxia and hypoxia that were not significantly different from those measured in normoxia-acclimated fish (Table 2.3). These results are in agreement with the results of Bushnell et al. (1984) and Kutty (1968), who showed that neither the swimming speed – oxygen consumption relationship or U_{crit} values were altered when rainbow trout and goldfish, respectively, were acclimated to

hypoxic conditions. Nonetheless, they are surprising given that maximum Q was lower in hypoxic-acclimated fish at all swimming speeds in both U_{crit} tests, and that maximum Q during the normoxic swim test was 23% lower in hypoxic-acclimated cod as compared to normoxic-acclimated individuals due to diminished values for S_V (Figure 2.2, Table 2.3). This apparent discrepancy is resolved, however, when the relationship between Q and oxygen consumption is examined for the two groups (Figure 2.4). During both the normoxic and hypoxic swims, hypoxic-acclimated cod consumed more oxygen for a given cardiac output. For example, at a Q of 30 ml min^{-1} kg^{-1}, hypoxic-acclimated cod consumed approx. 185 ml O_2 h^{-1} kg^{-1} when swimming under normoxic conditions as compared with approx. 130 ml O_2 h^{-1} kg^{-1} in normoxic-acclimated fish. This upward shift in the relationship between Q and oxygen consumption following long-term acclimation to moderate hypoxia was likely due to increases in both blood oxygen transport capacity and tissue O_2 extraction efficiency: 1) several authors have reported increases in haematocrit, blood haemoglobin levels and/or haemoglobin oxygen affinity in response to hypoxic acclimation (e.g. Bushnell et al., 1984; Driedzic et al., 1985; Timmerman and Chapman, 2004); and 2) subsequent experiments in our lab have shown that tissue O_2 extraction efficiency is significantly enhanced (by 15%) in hypoxic-acclimated cod under normoxic conditions, while blood haemoglobin levels are slightly or significantly (depending on water PO_2) higher in hypoxic-acclimated cod at rest (Petersen and Gamperl unpubl.; Chapter 5). Further, although cod heart function does not appear to be modulated by circulating catecholamines, these hormones activate sodium/proton exchange in cod red blood cells (and presumably improve blood oxygen carrying

capacity; Berenbrink and Bridges, 1994), and hypoxic-acclimated cod have significantly higher stress-induced catecholamine levels (Petersen and Gamperl, unpubl.; Chapter 5).

One of the major findings of this study was that hypoxia-acclimated cod had significantly lower values for resting and maximum S_V and Q in both swim tests, and a significantly lower scope for S_V when swum under hypoxic conditions, as compared with the normoxic-acclimated group (Figure 2.2, Table 2.3). The most obvious explanation for this diminished cardiac function is that long-term acclimation to hypoxia had a direct negative influence on myocardial performance. Indeed, this appears to be the case, given recent *in situ* studies (Petersen and Gamperl, unpubl.; Chapter 3) which show that hypoxic-acclimation reduces maximum Q and S_V by 19 and 16% under oxygenated conditions, respectively. However, it is presently unclear whether this loss of pumping capacity was related to remodelling of the myocardium, the dysfunction of viable myocardium, myocardial necrosis/damage or a combination of all factors. This is because there are very few studies on the effects of chronic hypoxia on the fish heart, and the results of acute and chronic studies on cardiac physiology are often contradictory. For example, while 3 weeks of acclimation to a P_wO_2 of 5 kPa (25% air saturation) induced myocardial degeneration and subsequent fibrosis in the flounder (*Platichthys flesus*) heart (Lennard and Huddart, 1992), both Gamperl et al. (2001) and Overgaard et al. (2004) were unable to find evidence of irreversible myocardial damage in rainbow trout hearts following acute (15 – 20 min.) exposure to severe hypoxia (~ 1 kPa). Further, although Driedzic et al. (1985) did not report absolute values for myocardial tension development, these authors showed that 4-6 weeks of hypoxic acclimation (at 6 kPa, 30% air saturation) enhanced the contractility of *Zoarces vivparous* normoxic myocardial strips under

conditions of elevated calcium. In contrast, the results of Gamperl et al. (2001) and Overgaard et al. (2004) suggest that the trout heart is 'stunned' following brief periods of work under anoxic/severe hypoxic conditions, and Bolli and Marban (1999) suggest that the loss of contractility associated with 'stunning' stems from a reduction in Ca^{2+} responsiveness caused by oxygen radical related damage and/or Ca^{2+} overload. Clearly, more research needs to be conducted before the mechanism(s) mediating the diminished pumping capacity of hearts from hypoxic-acclimated fishes can be understood.

Despite the diminished S_V, hypoxic-acclimated cod were able to increase f_H during the hypoxic swim to levels measured during normoxia (Figure 2.2, Table 2.3). This resulted in them having a significantly greater scope for f_H (12.6 vs. 5.8 beats min^{-1}), and allowed them to achieve the same maximum Q, as compared to normoxic-acclimated fish when swum at 8-9 kPa. The ability to elevate f_H during the hypoxic U_{crit} test (in direct contrast to the normoxic-acclimated group) was presumably retained in hypoxia-acclimated cod because their hearts had a reduced pumping capacity as compared with normoxic-acclimated fish, and this was the only way that they could elevate Q to meet the demands of maximal exercise. The mechanism(s) resulting in the differential regulation of f_H in the two groups when swum under hypoxic conditions cannot be ascertained from the present study or the literature. However, this result, in combination with recent data showing that rainbow trout at 24°C can maintain Q even when f_H is cut in half using the pharmacological agent zetabradine (Gamperl et al., 2008), highlights the tremendous plasticity in how fish cardiorespiratory physiology responds to environmental challenges and that our understanding of control mechanisms that mediate myocardial function and adaptation in this taxa is far from complete.

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2.7. References

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Chapter 3

In situ Cardiac Function in Atlantic Cod (*Gadus morhua*): Effects of Acute and Chronic Hypoxia.

3.1. Abstract

Recent *in vivo* experiments on Atlantic cod (*Gadus morhua*) acclimated to chronic hypoxia (6-12 weeks at 10°C; P_wO_2 ~ 8- 9 kPa) revealed a considerable decrease in the heart's pumping capacity. To examine whether this diminished cardiac performance was due to the direct negative effects of chronic moderate hypoxia on the cod's myocardium (as opposed to alterations in neural and/or hormonal control), we measured the resting and maximum *in situ* function of hearts from normoxic- and hypoxic-acclimated cod: 1) when initially perfused with oxygenated saline; 2) at the end of a 15 minute exposure to severe hypoxia (PO_2 ~ 0.6 kPa); and 3) 30 minutes after the hearts had been reperfused with oxygenated saline. Although hypoxic acclimation did not influence resting (basal) *in situ* cardiac performance during oxygenated or hypoxic conditions, it caused a decrease in maximum cardiac output (Q_{max}) under oxygenated conditions (from 49.5 to 40.3 ml min^{-1} kg^{-1}; by 19%), this difference due to diminished values for maximum S_V and scope for S_V. In contrast, although severe hypoxia reduced Q_{max} in both groups to approx. 20 ml min^{-1} kg^{-1}, the hearts of hypoxic-acclimated fish were better able to sustain this level of Q under hypoxia, and the recovery of Q_{max} (as compared to initial values under oxygenated conditions) was significantly improved (94 vs. 83%) as compared with normoxic-acclimated individuals. These data show that hypoxic acclimation has a direct effect on cod myocardial function/physiology, and when considered in the context of recent research, suggest that the cod heart shows some adaptations to prolonged hypoxia.

3.2. Introduction

Hypoxia is encountered by many fish species, and exposure to low oxygen environments results in complex behavioural and physiological responses (Pihl et al., 1991; Val et al., 1995; Van Ginneken et al., 1995; Dalla Via et al., 1998). Of these, changes in cardiovascular function have been of particular interest, and a large amount of information currently exists on the *in vivo* cardiovascular responses of teleosts to short-term (acute) hypoxia (Wood and Shelton, 1980; Farrell, 1982; Bushnell et al., 1984; Glass et al., 1990, 1991; Perry et al. 1999; Sandblom and Axelsson, 2005). In contrast, only one study has investigated the effect of chronic (weeks of) hypoxia on fish *in vivo* cardiovascular function (Petersen and Gamperl; unpubl.; Chapter 2). This study showed that cod acclimated to hypoxia (P_wO_2 8-9 kPa, 40% air saturation) had significantly lower values for resting and maximum stroke volume and cardiac output, and a significantly lower scope for stroke volume during a hypoxic swim as compared with normoxic-acclimated fish. While this work has provided novel insights into how fish cardiorespiratory physiology is impacted by prolonged exposure to hypoxia, the reason(s) for the diminished cardiac function in hypoxia-acclimated cod is not clear. For example, stroke volume in fishes is controlled by aneural and neural factors such as cardiac filling/venous pressure, blood oxygen levels and biochemistry, myocardial contractility, circulating hormones, and by alterations in cholinergic and adrenergic nervous activity (Kiceniuk and Jones, 1977; Farrell, 1984; Axelsson, 1988; Axelsson and Nilsson, 1986; Satchell, 1991; Farrell, 1991; Zhang et al., 1998; Sandblom and Axelsson, 2005, 2006; Hanson et al., 2006; Hanson and Farrell, 2007). Further, while 3 weeks of acclimation to

a P_wO_2 of 5 kPa (25% air saturation) induced myocardial degeneration and subsequent fibrosis in the flounder (*Platichthys flesus*) heart (Lennard and Huddart, 1992), Driedzic et al. (1985) showed that myocardial strips from eelpout (*Zoarces vivparous*) acclimated to a P_wO_2 of 6 kPa (~ 30% air saturation) for 4-6 weeks were better able to sustain peak tension development during anoxia in the presence of elevated Ca^{2+} levels.

In situ heart preparations, first developed for fish by Farrell et al. (1982), are devoid of any nervous and hormonal input, are extremely robust and tractable, and perform at maximum levels typical of those measured *in vivo* (Farrell et al., 1985; Farrell et al. 1989; Hanson et al., 2006; Hanson and Farrell, 2007). Furthermore, they have proven to be a very valuable tool for understanding teleost heart function during periods of oxygen deprivation (Farrell et al., 1989; Arthur et al., 1992; Hanson and Farrell, 2007), the importance of circulating catecholamines in supporting cardiac function under conditions experienced during strenuous exercise (e.g. hypoxemia, hyperkalemia and acidosis: Hanson et al., 2006; Hanson and Farrell, 2007), and for elucidating several aspects of preconditioning in the teleost heart (Gamperl et al., 2001, 2004; Faust et al., 2004; Overgaard et al., 2004b). Thus, the present study used *in situ* heart preparations to investigate the effects of hypoxic acclimation on the normoxic and hypoxic performance of the Atlantic cod heart, and ultimately to determine whether the reduced *in vivo* cardiac performance observed in hypoxic-acclimated cod (Petersen and Gamperl, unpubl. Chapter 2) was a direct result of a decrease in the heart's pumping capacity.

3.3. Materials and Methods

These studies were conducted in accordance with the guidelines of the Canadian Council on Animal Care, and approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (Protocol # 05-03-KG).

3.3.2. Experimental Animals

Experiments were performed on adult (0.53 ± 0.04 kg; range 0.30 – 0.86 kg) Atlantic cod (*Gadus morhua*) at the Ocean Sciences Centre (OSC; Memorial University, St. John's, Newfoundland, Canada). Cod were obtained from stocks hatched at the Aquaculture Research and Development Facility (ARDF) and held in sea-cages at Hermitage Bay (Newfoundland, Canada) for approximately 18 months before being transported back to the OSC. At the OSC, the fish were held in a 12,000 litre tank supplied with aerated seawater at 10°C for at least 2 months prior to being moved to the acclimation tanks. The fish were fed a commercial cod diet three times a week, and maintained on ambient photoperiod.

3.3.3. Experimental Conditions and Surgery

Prior to the experiments, 40 fish from the holding tank were acclimated at a P_wO_2 of 19.4 ± 0.1 kPa or 8.6 ± 0.1 kPa in ~ 1300 litre tanks (20 fish/tank) for 6-12 weeks at 10 ± 1 °C as described in Petersen and Gamperl (unpubl.; Chapter 2). The normoxic fish were fed three times a week with commercial pellets at a ration equal to that consumed by the hypoxic group (see chap 2 for further detail).

The fish were netted and anaesthetized in seawater containing tricaine methane sulphonate (MS-222, 0.1 g L^{-1}) until ventilatory movements ceased. The fish were then weighed and measured, before being transferred to an operating table where chilled (4°C) oxygenated seawater, containing a lower dose of MS-222 (0.05 g L^{-1}), was continuously irrigated over their gills. On the operating table the fish was placed on a wetted sponge in a supine position and injected with 1.0 ml of heparin (50 IU ml^{-1} of saline solution) (Sigma Chemical Co., St Louis, MO, USA) *via* the caudal vein, and an *in situ* heart preparation was obtained as described by Farrell et al. (Farrell et al., 1986, 1989) with some minor modifications. Briefly, the peritoneal cavity was exposed through a midline incision and by cutting through the abdominal wall in a ventral-dorsal direction just posterior to the pectoral fins. Blood flow to the stomach, intestines and other abdominal organs was stopped by tying off the gastrointestinal tract, inferior to the liver, with umbilical tape. Then, the abdominal and digestive organs were carefully removed to permit proper placement of the input cannula whilst keeping the liver intact. A hepatic vein was selected for cannulation, and after the other one was tied off with 3-0 silk suture, a small cut was made in the hepatic vein and a steel cannula (0.9 mm outer diameter, O.D.) was inserted and tied in place. At this point, perfusion of the heart with ice-cold (4°C) oxygenated saline was begun, and the 1st and 2nd gill arches were cut on each side of the fish to prevent excessive pressure development by the heart; the level of saline in the perfusion bottle set at the same height as the heart (i.e. 0 kPa input pressure) in order to obtain basal cardiac output and prevent cardiac stretch.

The lower jaw and operculum were then removed, the 1st and 2nd gill arches cut away, and the 3rd and 4th gill arches cut in half and clamped with cable ties (4") to prevent

leakage. Finally, the isthmus between the 2nd and the 3rd gill arches was cut to expose the ventral aorta in cross-section, the ventral aorta was dissected free from the surrounding tissue, and a steel output cannula (0.8 mm O.D.) was inserted into the ventral aorta and tied in place with 3-0 silk suture.

After the output cannula was secured in place, the ducts of Cuvier were tied off by passing a large needle with attached silk suture (1-0) from the corner of the opercular cavity into the muscle of the abdominal wall, and then into the oesophagus and back into the buccal cavity. When this suture was subsequently pulled tight, it occluded the ducts of Cuvier and other veins entering the heart, and crushed the cardiac branches of the vagus nerve; crushing of the nerves confirmed by a noticeable pectoral fin twitch and transitory cardiac arrest. This procedure ensured that any fluid entering the heart was from the input cannula, and that nervous stimulation of the heart was prevented during the experiment. Once surgery was completed, the fish was bisected just posterior to the pectoral fins, and placed in a water-jacketed saline-filled bath maintained at the fish's acclimation temperature.

3.3.4. Stabilization

After placing the *in situ* preparation in the experimental bath, the input cannula was attached to an adjustable constant-pressure head that was used to manipulate atrial filling pressure (P_{in}), and the output cannula was connected to tubing, the height of which could be adjusted to control end-diastolic pressure (P_{out}). The heart was then perfused with oxygenated physiological saline (see recipe below) from temperature controlled (10°C) water-jacketed bottles. Output pressure (P_{out}) was maintained at 2 kPa during the

first 10 min. to let the heart recover from surgery, and to prevent excessive cardiac work while input pressure (P_{in}) was being set to a physiologically relevant resting cardiac output (Q, 16 ml min^{-1} kg^{-1}) (Axelsson and Nilsson, 1986; Fritsche and Nilsson, 1989; Webber et al., 1998). After this initial period, P_{out} was increased to a physiological output pressure of 5 kPa (Axelsson and Nilsson, 1986) and the heart was allowed to stabilize for 15 min. at this resting (basal) cardiac output.

3.3.5. Experimental Protocol

A schematic diagram of the entire protocol can be seen in Figure 3.1. After stabilization was complete, resting cardiac parameters (input pressure, P_{in}; heart rate, f_H; cardiac output, Q; stroke volume, S_V; and power output, PO) were recorded, and then maximum cardiac output (Q_{max1}) was determined by increasing P_{in} from the height required to achieve resting cardiac output (~ 0 to 0.05 kPa) to 0.4 kPa, and then in a stepwise fashion to 0.5, to 0.55 and finally to 0.6 kPa. During the Q_{max1} test, P_{out} was maintained at 5 kPa, and each increase in P_{in} was maintained for approximately 30 seconds to allow enough time for cardiac functional parameters (Q_{max}, f_{Hmax}, S_{Vmax} and PO) to stabilize and be recorded, but short enough to avoid excessive cardiac stretch/myocardial damage. After determining Q_{max1}, input pressure was again reduced to levels required to obtain resting cardiac output and the heart was allowed to recover for 10 min.

After 10 min. of recovery under oxygenated conditions, the hearts were exposed to 15 min. of severe hypoxia (perfusate PO_2 of ~ 0.6 kPa), during which time P_{in} was not adjusted. This allowed for a determination of whether the heart's ability to maintain basal

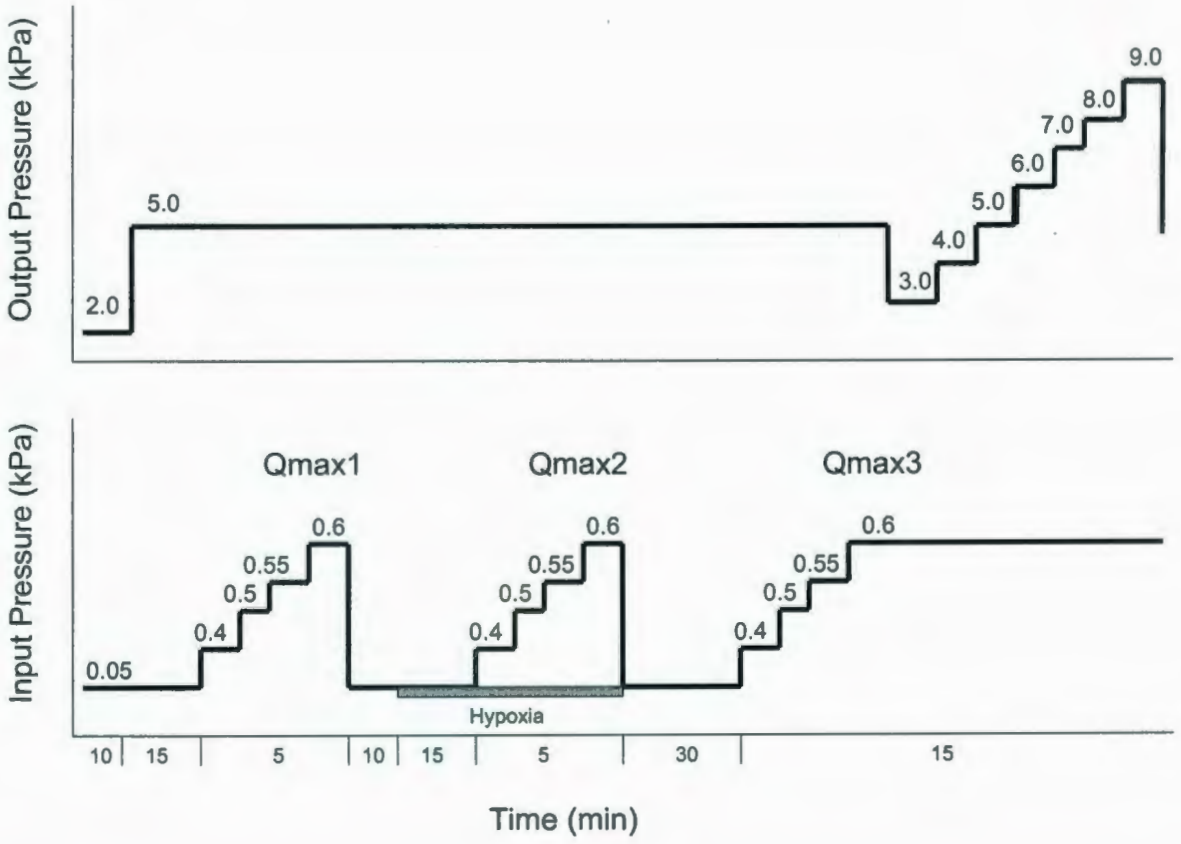

Figure 3.1. Experimental protocol used to assess the resting and maximal cardiac performance on *in situ* hearts from normoxic- and hypoxic-acclimated Atlantic cod. Lower and upper panel shows input and output pressures, respectively. P_{out} was normally set to a physiologically realistic value of 5 kPa: however a sub-physiological level of P_{out} (2-3 kPa) was used for the first 10 min. of the protocol to let the heart recover from surgery. Then P_{out} was raised to 5 kPa and P_{in} continuously adjusted to give a cardiac output of 16 – 17 ml min^{-1} kg^{-1}. Resting conditions were then recorded for 15 min. while the heart was perfused with oxygenated saline. The next set of steps indicates the first maximum cardiac output test (Q_{max}), where P_{in} was raised sequentially from 0.05 kPa to 0.6 kPa. The heart was then left to recover for 10 min. and was then exposed to severe hypoxia (PO_2 0.6 kPa, grey bar) for 15 min. without adjusting P_{in}. This was followed by a second Q_{max} test, identical to the first one, but this time in hypoxic saline. Subsequently the heart was allowed to recover at 0.05 P_{in} in oxygenated saline for 30 min. before a final normoxic Q_{max} test was performed. Finally, while P_{in} remained at 0.60 kPa, output pressure was decreased from 5 kPa to 3 kPa and then raised in 1 kPa steps until the heart could no longer pump (~9 kPa).

levels of performance during severe hypoxia differed between normoxic- and hypoxic-acclimated cod. This 15 min. of hypoxia was followed by a second Q_{max} test (Q_{max2}), after which the hearts were perfused with oxygenated saline and allowed to recover at resting cardiac output (16 ml min^{-1} kg^{-1}) for 30 min. Most hearts survived this hypoxic exposure, but some (2 hearts) stopped pumping either at the end of the 15 min. of basal cardiac function or during the Q_{max2} test. After 30 min. of recovery in oxygenated saline a third Q_{max} test (Q_{max3}) was performed, and this was followed by a maximum power output (PO_{max}) test. This final test was performed while the input pressure of the heart remained at 0.6 kPa (i.e. that used to obtain Q_{max}), and involved decreasing P_{out} from 5 kPa to 3 kPa, and then increasing P_{out} in 1 kPa steps until the heart could no longer pump (or until an output pressure of 9 kPa was reached). The time spent at each level of output pressure was just long enough to allow cardiac performance to stabilize, approximately 20-30 seconds.

After each experiment, the heart was tested to ensure that no leaks were present. This was done by clamping the input perfusate line with a pair of haemostats and ensuring cardiac output fell to zero, then raising the output tube to ~ 10 kPa and ensuring that no significant backflow occurred. Data from hearts that 'leaked' were excluded from analysis. The hearts were then dissected from the fish and the cardiac chambers were separated, blotted dry, and weighed.

3.3.6. Experimental Solutions

Hearts were perfused with physiological marine teleost saline during surgery and during the experimental period. This saline (pH 7.76 at 12 °C) contained (in mmol L^{-1}):

181.3 NaCl; 1.99 $MgSO_4*7H_2O$; 5.0 KCl; 2.30 $CaCl_2*2H_2O$; 1.01 $NaH_2PO_4*H_2O$; 7.33 Sodium TES base ($C_6H_{14}NO_6SNa$); 2.58 TES acid ($C_6H_{15}NO_6S$); 5.55 dextrose. The TES buffer system was used to simulate the buffering capacity of cod plasma and epinephrine (10 nmol l^{-1}) was added to the perfusate to ensure the long-term viability of the *in situ* heart (Graham and Farrell, 1989). These chemicals were obtained from Fisher Scientific (Fair Lawn, NJ, USA), with the exception of the TES salt and adrenaline bitartrate salt, which were purchased from Sigma Chemical Co. (St Louis, MO, USA). The saline was continuously gassed with oxygen during both surgery and when the heart was not being exposed to severe hypoxia during the experiment. To achieve severe hypoxia, the saline in the perfusion bottles was gassed with pure N_2 for at least 60 min. before the hypoxic trial, and saline in the experimental bath was gassed with pure N_2 beginning approximately 5 min. before the onset of the hypoxic experiment. The use of Masterflex® tubing, with low oxygen permeability (Tygon Food, ser. 6-419, Cole Parmer), to deliver saline to the *in situ* hearts further ensured that oxygen from external sources was minimized.

3.3.7. Data Collection and Analysis

Input and output pressures were measured using Gould (P23 ID, Oxnard, CA, USA) and Grass (PT300, Warwick, RI, USA) pressure transducers, respectively, and cardiac output was measured with a 2N in-line flow probe in conjunction with a T206 flow meter (Transonic Systems Inc., Ithaca, NY, USA). Input and output pressures were corrected to account for cannula resistance between the point of measurement and the heart (using predetermined calculations from Faust et al., 2004), and the pressure

transducers were calibrated daily against a static column of water; with zero pressure equal to the level of saline in the bath. Pressure and flow signals were collected at a sample rate of 20 Hz, and filtered and amplified, using a Model MP100A-CE data acquisition system (BIOPAC Systems Inc., Santa Barbara, CA, USA), and the acquired signals were stored and analyzed using AcqKnowledge Software (BIOPAC Systems Inc.) installed on a 300 MHz Macintosh G3 computer.

Cardiac function was continuously recorded throughout the experiment by measuring input pressure (P_{in}) and output pressure (P_{out}), cardiac output (Q, ml min^{-1} kg^{-1}), heart rate (f_H, beats min^{-1}), and stroke volume (S_V, ml kg^{-1}). Although data were collected continuously, cardiac function was only analyzed at specific intervals during each experiment. Resting cardiac parameters (Q, f_H, and S_V) were measured just prior to each of the Q_{max} tests. Maximum cardiac function was quantified by measuring Q_{max}, f_{Hmax}, S_{Vmax} and power output (PO_{max}). The first three parameters were measured at an input pressure of 0.6 kPa during the maximum cardiac output tests. Maximum power output for each fish was calculated by fitting a second or third order regression to the power output vs. P_{out} relationship. These data were then used to calculate the mean PO_{max} for each group. Heart rate (f_H, beats min^{-1}) was calculated by counting 20 systolic peaks, dividing by the measurement period (sec), and multiplying by 60. Cardiac output (ml min^{-1} kg^{-1}) was calculated by dividing absolute flow (ml min^{-1}) by the body mass of the fish (kg). Stroke volume (S_V, ml kg^{-1}) and power output (mW g $ventricle^{-1}$) were calculated as follows:

$$S_V = Q/f_H$$

$$PO = Q \times (P_{out}-P_{in}) \times a) / M_v.$$

Where P_{out} and P_{in} are output and input pressures (in cm H_2O) respectively, Q is cardiac output (in ml min^{-1}), M_v is ventricle mass (g) and a=0.098 (mW min ml^{-1} cm H_2O^{-1}) is a conversion to milliwatts.

3.3.8. Statistical Analyses

Statistical analyses were carried out using SPSS (v. 13.0; SPSS, Chicago, IL, USA). Paired t-tests and one-way ANOVAs were used to test for statistical differences within and between groups, respectively, for: 1) body and cardiac morphometrics (Table 3.1); 2) resting cardiac parameters (Table 3.2); 3) routine, maximum and scope of cardiac parameters (Table 3.3); and 4) all maximum cardiac parameters (Table 3.4). GLM repeated measures analyses were used to determine the effect of time and acclimation condition on cardiac parameters (f_H, S_V, Q and PO) when hearts were exposed to either oxygenated or hypoxic saline (see Figure 3.2). This analysis was also performed to determine the effect of: 1) P_{in} and acclimation condition on Q_{max1}, Q_{max2}, Q_{max3}; and 2) P_{out} and acclimation condition on PO_{max}. These analyses were followed by Dunnett's *post-hoc* tests to determine when there was a significant change in a cardiac parameter from the resting value within each group. One-way ANOVAs were used to determine significant differences at each time point (Figure 3.2) or each P_{in}/P_{out} (Figures 3.3 and 3.5) between the normoxic and hypoxic groups. One-way ANOVAs were also performed to determine significant differences between Q_{max1} and Q_{max3} within each group (Figure 3.4). Unless otherwise stated, a result was considered significant when $P < 0.05$. All data presented in the text, figures and tables are means ± standard error of the mean (SEM).

3.4. Results

3.4.1. Cardiac Morphometrics and Resting (Basal) Performance

Hypoxic acclimation did not affect the cod's body mass, condition factor, ventricular mass or RVM (Table 3.1), or basal *in situ* cardiac performance under oxygenated conditions (Table 3.2). In oxygenated saline, resting f_H was 61.9 ± 2.3 beats min^{-1} and 60.4 ± 3.0 beats min^{-1} in the normoxic- and hypoxic-acclimated groups, respectively. Further, at this f_H, hearts from both groups required a slightly positive P_{in} (means 0.05 vs. 0.08) and a S_V of approx. 0.28 ml kg^{-1} to achieve a cardiac output of 16 – 17 ml min^{-1} kg^{-1}.

During the 15 min. of acute severe hypoxia (saline PO_2 ~ 0.6 kPa) (during which time input pressure was not adjusted) changes in cardiac function were almost identical in the two groups. Q decreased gradually in both groups, this decrease in Q becoming significant after approx. 8 min. of hypoxic exposure, and Q after 15 min. of severe hypoxia falling from ~ 16 to ~ 10 ml min kg^{-1} (i.e. by approx. 35%) (Figure 3.2; Table 3.3). This diminished Q was mirrored by changes in PO, and was the sole result of hypoxia-induced reductions in S_V. For example, in both groups, only slight (~2-3 beat min^{-1}) decreases in f_H were observed while S_V fell by 27 and 38% in normoxic- and hypoxic-acclimated fish, respectively.

Following 30 min. of recovery in oxygenated saline, values for P_{in}, Q, f_H, and S_V were nearly identical between groups, and to those measured at the start of the experiment (i.e. prior to Q_{max1}) (Table 3.2). The only exception was the value for P_{in} which was 0.1 kPa higher in hearts from hypoxia-acclimated cod ($P < 0.10$). However, this result was

not surprising given that f_H was approx. 4 beats min^{-1} (but not significantly) lower in the hearts from hypoxia-acclimated cod.

3.4.2. Maximum Cardiac Output and Power Output Tests

Although prolonged hypoxic exposure (~8-9 kPa; 40% air saturation) did not influence resting (basal) *in situ* cardiac performance during oxygenated or hypoxic conditions, there were several important differences in maximum cardiac performance between the two groups. First, hypoxic acclimation caused a decrease in maximum cardiac output (from 49.5 to 40.3 ml min^{-1} kg^{-1}; by 19%, Table 3.3) and the scope for Q (from 32.8 to 23.8 ml min^{-1} kg^{-1}; by 28%, Table 3.3) during the initial oxygenated Q_{max} test (Q_{max1}). This difference was again due to diminished values for maximum S_V and scope for S_V in hearts from hypoxia-acclimated cod; the decrease in f_H due to myocardial stretch (~7 beats min^{-1}) was similar between the two groups (Figure 3.3A; Table 3.3). Second, while exposure to 15 min. of severe hypoxia reduced maximum Q and S_V to similar levels in both groups (Q to 18 and 23 ml min^{-1} kg^{-1} and S_V to 0.34 and 0.44 ml $beat^{-1}$): 1) the decrease in maximum Q between Q_{max1} and Q_{max2} was much greater in normoxic vs. hypoxic-acclimated cod (~ 31.5 vs. 17.2 ml min^{-1} kg^{-1}; Table 3.4); and 2) Q remained constant in the hypoxic-acclimated cod hearts as P_{in} was increased from 0.4 to 0.6 kPa, yet fell significantly from ~ 26.5 to 18 ml min^{-1} kg^{-1} in the normoxic-acclimated group (Figure. 3.3B). Finally, although maximum Q and S_V values following 30 min. of recovery from hypoxia were similar to initial values (i.e. during Q_{max1}) in the hypoxic-acclimated group, both these parameters were lower (Q significantly) in normoxic-acclimated fish during Q_{max3}. This resulted in % recovery values of only

approx. 83% for maximum Q and S_V in normoxic-acclimated cod, whereas values for hypoxic-acclimated cod were 93.5 and 90.1%, respectively. Collectively, these results show that while hearts from hypoxic-acclimated cod have a reduced maximum pumping capacity as compared to those from normoxic-acclimated individuals, they can maintain maximum cardiac function better when faced with acute hypoxia, and their recovery is enhanced following an acute hypoxic insult.

Power output increased slightly in both groups as P_{out} was raised from 3 to 5 kPa. However, it decreased rapidly thereafter, with PO at 8 kPa falling to < 0.05 mW g^{-1} ventricle (Figure 3.5). Although PO was somewhat lower in hearts from hypoxic-acclimated cod at output pressures from 3-5 kPa, there was no significant difference in maximum power output between the two groups (normoxic-acclimated 4.7 ± 0.5 mW ventricle^{-1}; hypoxic-acclimated 4.0 ± 0.8 mW ventricle^{-1}).

Table 3.1. *Body and cardiac morphometrics for Atlantic cod acclimated to either normoxia [water oxygen partial pressure (P_wO_2) 21 kPa] or hypoxia (P_wO_2 8-9 kPa) for 6-12 weeks.*

	Body and Cardiac Morphometrics				
	Animal Mass (kg)	**Length (cm)**	**Heart Mass (g)**	**Relative Ventricular Mass (RVM)**	**Condition Factor (K)**
Normoxic	0.49 (0.05)	39.3 (1.2)	0.35 (0.03)	0.071 (0.003)	0.80 (0.03)
Chronic Hypoxic	0.56 (0.05)	40.1 (1.3)	0.40 (0.05)	0.072 (0.003)	0.85 (0.03)

Values shown are means ± SEM (N=8 for each group). Mass was determined prior to surgery.

Table 3.2. *Resting (basal) cardiac parameters for* in situ *hearts from normoxic- and hypoxic-acclimated cod under a variety of test conditions. Input pressure (P_{in}) was that required by each heart to maintain a cardiac output of 16 – 17 ml min^{-1} kg^{-1} under oxygenated conditions, or at the onset of the hypoxic exposure.*

	Oxygenated Saline			*Hypoxia*			*Recovery (Oxygenated Saline)*		
	P_{in} (kPa)	**Stroke Volume (ml kg^{-1})**	**Heart Rate (beats min^{-1})**	**P_{in} (kPa)**	**Stroke Volume (ml kg^{-1})**	**Heart Rate (beats min^{-1})**	**P_{in} (kPa)**	**Stroke Volume (ml kg^{-1})**	**Heart Rate (beats min^{-1})**
Normoxic	0.05 (0.03)	0.27[a] (0.01)	61.9 (2.3)	0.02 (0.03)	0.19 (0.02)	59.9 (2.3)	0.03[+] (0.04)	0.26 (0.02)	64.0 (3.7)
Chronic hypoxic	0.08 (0.02)	0.28[a] (0.01)	60.4 (3.0)	0.08 (0.03)	0.18 (0.03)	57.6 (2.4)	0.13 (0.06)	0.28 (0.02)	59.7 (3.5)

Values shown are means ± SEM (N=8 for each group)

[a]Indicates a significant difference ($P<0.05$) between parameters measures under oxygenated and hypoxic conditions within each acclimation condition

[+]Indicates a significant ($P<0.10$) difference between groups (normoxic vs. hypoxic acclimation) within a particular test.

Table 3.3. *Routine, maximum and scope for cardiac parameters (f_H: heart rate; S_V: stroke volume; Q: cardiac output) measured using* in situ *cod hearts from normoxic- and hypoxic-acclimated (water PO_2 ~ 8-9 kPa) individuals. Maximum cardiac output tests (see Figure 3.1) were initially performed while hearts pumped oxygenated saline, and then after 15 min. of exposure to hypoxic saline (PO_2 ~0.6 kPa).*

	Normoxic Acclimated						***Hypoxic Acclimated***					
	Oxygenated Saline			**Hypoxic saline**			**Oxygenated Saline**			**Hypoxic Saline**		
	f_H (beats min^{-1})	S_V (ml kg^{-1})	Q (ml min^{-1} kg^{-1})	f_H (beats min^{-1})	S_V (ml kg^{-1})	Q (ml min^{-1} kg^{-1})	f_H (beats min^{-1})	S_V (ml kg^{-1})	Q (ml min^{-1} kg^{-1})	f_H (beats min^{-1})	S_V (ml kg^{-1})	Q (ml min^{-1} kg^{-1})
Routine	61.9 (2.3)	0.27[a] (0.01)	16.7[a] (0.1)	59.9 (2.3)	0.19 (0.02)	10.9 (1.1)	60.4 (3.0)	0.28[a] (0.01)	16.5[a] (0.3)	57.8 (2.7)	0.18 (0.03)	10.3 (1.4)
Max	54.1 (1.9)	0.92[a*] (0.04)	49.6[a*] (2.3)	55.2 (2.3)	0.34 (0.07)	18.0 (3.2)	53.0 (2.4)	0.77[a] (0.08)	40.3[a] (4.0)	53.0 (2.3)	0.44 (0.03)	23.1 (1.7)
Scope	7.8 (0.9)	0.65[a*] (0.04)	32.8[a*] (2.4)	4.8 (1.4)	0.20 (0.04)	9.8 (1.5)	7.4 (1.5)	0.49[a] (0.08)	23.8[a] (3.9)	3.8 (1.2)	0.20 (0.04)	10.1 (2.1)

Values shown are means ± SEM (N=8 for each group).

[a]Indicates a significant difference ($P<0.05$) between hearts perfused with oxygenated and hypoxic saline within each acclimation condition.

*Indicates a significant ($P<0.10$) difference between groups (normoxic vs. hypoxic acclimation) within a particular test condition.

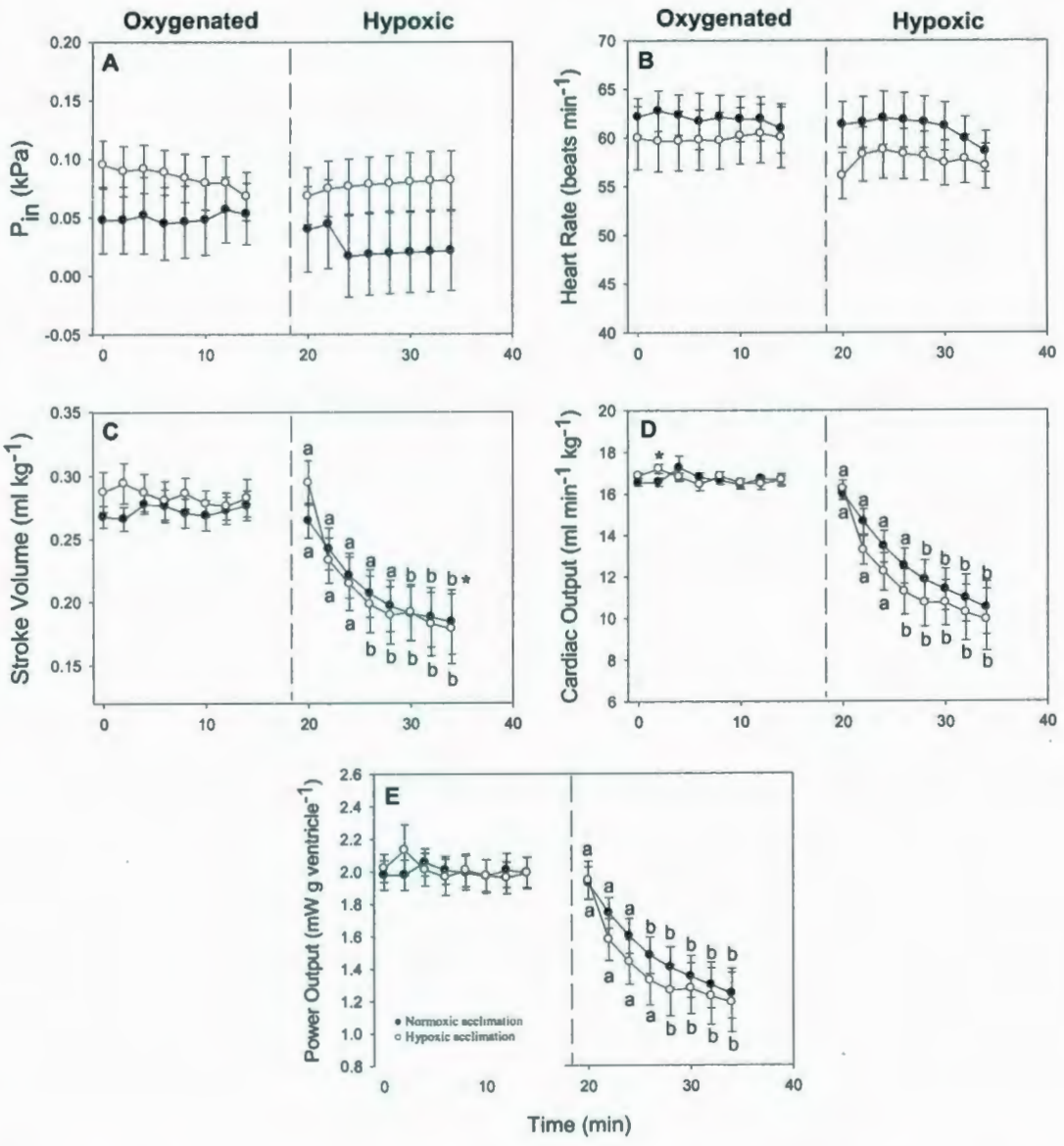

Figure 3.2. Effect of acute hypoxia on the (A) input pressure, P_{in}; (B) heart rate; (C) stroke volume; (D) cardiac output and (E) power output of *in situ* hearts from normoxic- (•, P_wO_2 of 21 kPa) and hypoxic-acclimated (o, P_wO_2 of 8-9 kPa) cod. The isolated hearts were first exposed to oxygenated saline where cardiac output was maintained at 16 -17 ml min^{-1} kg^{-1} by adjusting P_{in} (left hand side of each figure). Then, the hearts were exposed to 15 min. of severe hypoxia (saline PO_2 of 0.6 kPa) where P_{in} was held constant (right panel of each figure). Values are means ± SEM (N=8 for each group). *Value significantly ($P<0.05$) different between normoxic and hypoxic acclimated groups. Dissimilar letters indicate values that were significantly different from those recorded at time 0 during the acute hypoxic exposure.

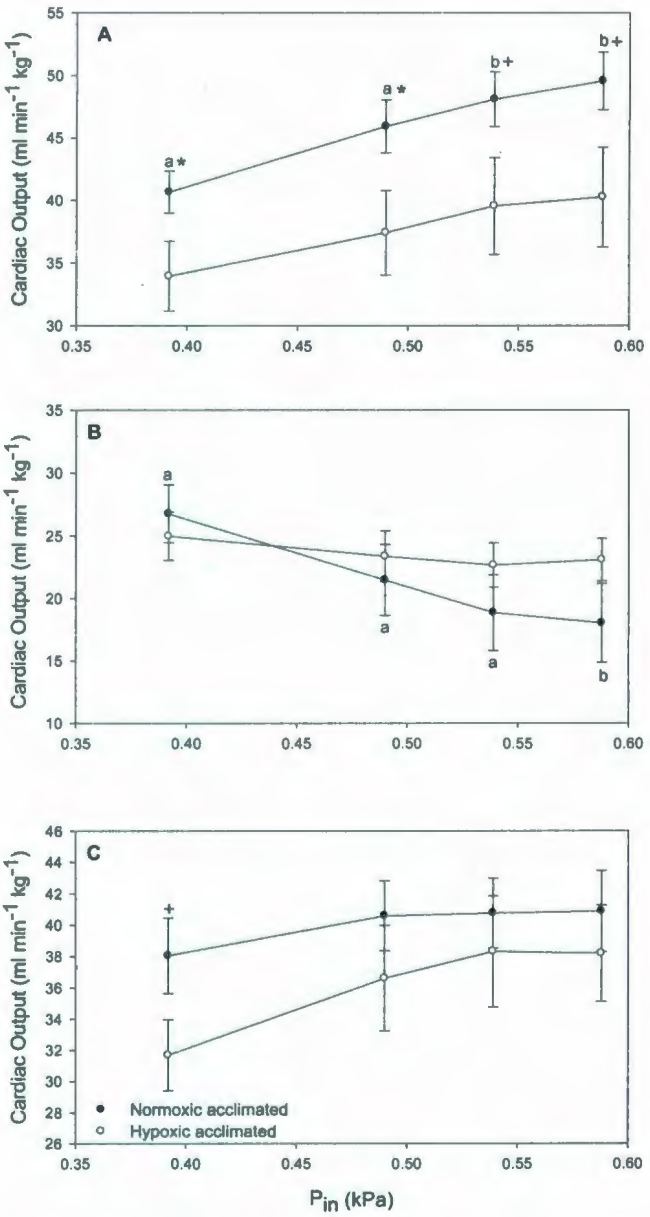

Figure 3.3. The relationship between input pressure (P_{in}) and cardiac output for cod *in situ* hearts during 3 maximum cardiac output (Q_{max}) tests. (A) Initial maximum cardiac output test (Q_{max1}) in oxygenated saline; (B) Q_{max2} test performed after hearts were exposed to severe hypoxia (saline $PO_2 \sim 0.6$ kPa) for 15 min.; (C) the final Q_{max} test (Q_{max3}), performed 30 min. after the hearts were allowed to recover from the severe hypoxic exposure (• normoxic-acclimated cod; o hypoxic-acclimated cod). Values are means ± SEM (N=8 for each group). *Value significantly different ($P<0.05$) between normoxic- and hypoxic-acclimated groups. $^{+}$Value significantly different ($P<0.10$) between normoxic- and hypoxic-acclimated groups at 0.55 kPa ($P=0.075$) and 0.60 kPa ($P=0.064$) in (A) and $P=0.080$ at 0.4 kPa in (C). Dissimilar letters indicate values within the normoxic-acclimated group that were significantly different from those recorded at 0.40 kPa.

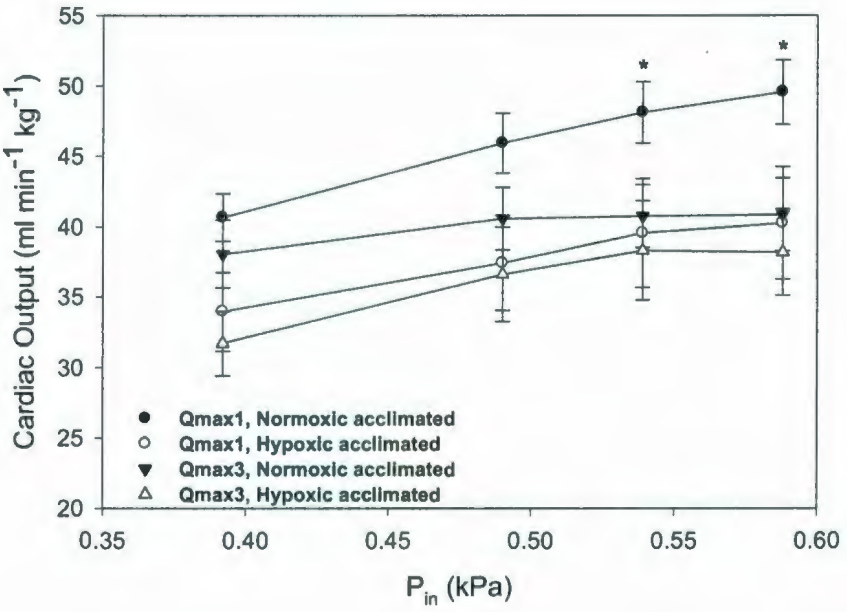

Figure 3.4. The relationship between input pressure (P_{in}) and cardiac output for cod *in situ* hearts during the Q_{max} tests performed in oxygenated saline. Q_{max1} is the initial Q_{max} test and Q_{max3} is the third Q_{max} test, performed 30 min. after the hearts were allowed to recover from severe hypoxia (see legend for Figure 3.1). Values are means ± SEM. (N=8 for each group).*Value significantly different ($P<0.05$) between Q_{max1} and Q_{max3} for the normoxic group.

Table 3.4. *Maximum cardiac parameters for* in situ *hearts from cod acclimated to normoxia or hypoxia (water PO_2 ~ 8-9 kPa) for 6-12 weeks. Each heart was given 3 maximum cardiac output (Q_{max}) tests. An initial Q_{max} test in oxygenated saline (Q_{max1}); Q_{max2}, a test performed after hearts were exposed to severe hypoxia (saline PO_2 ~ 0.6 kPa) for 15 min.; and a final Q_{max} test (Q_{max3}), performed 30 min. after the hearts were allowed to recover from the severe hypoxic exposure.*

	Q_{max}				S_{Vmax}				f_{Hmax}			
	max1	**max2**	**max3**	**% rec**	**max1**	**max2**	**max3**	**% rec**	**max1**	**max2**	**max3**	**% rec**
Normoxic	49.6* (2.3)	18.0[a] (3.2)	40.9[a] (2.6)	83.2* (5.7)	0.9* (0.04)	0.3[a] (0.07)	0.8 (0.06)	82.3 (5.81)	54.1 (1.9)	55.2 (2.3)	55.0 (2.2)	101.7 (2.2)
Chronic Hypoxic	40.3 (4.0)	23.1[a] (1.7)	38.2 (3.1)	93.6 (4.1)	0.8 (0.08)	0.4[a] (0.03)	0.73 (0.06)	90.1 (4.55)	53.0 (2.4)	53.0 (2.3)	53.0 (3.0)	102.7 (2.7)

Values shown are means ± SEM (N=8 for each group). % rec = % recovery: ((max3 ÷ max1)/max1) x 100 within each acclimation condition.
[a]Significant difference ($P<0.05$) between max1, and max2 or max3, within each acclimation condition.
*Significant difference ($P<0.10$) between groups (normoxic- vs. hypoxic-acclimation) within a particular test condition.

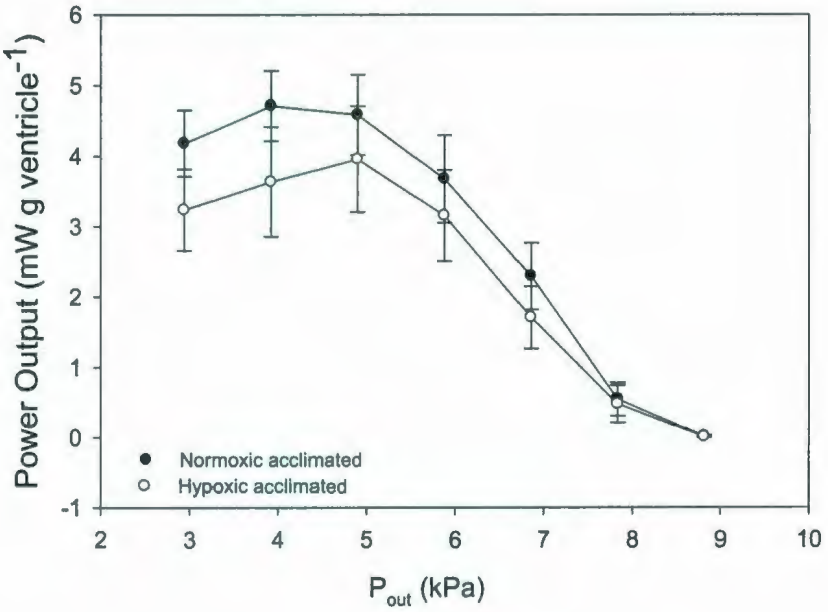

Figure 3.5. The effect of increased output pressure (P_{out}) on the myocardial power output of *in situ* Atlantic cod hearts from normoxic- or hypoxic-acclimated individuals. These measurements were taken while input pressure was left at the level (0.6 kPa) used to obtain maximum cardiac output. Values are means ± SEM (N=8 for each group). There was no statistical difference in maximum power output (see Table 3.3) between the two groups even though maximum power output was routinely lower in the hypoxic acclimated group.

3.5. Discussion

This *in situ* study confirms earlier *in vivo* measurements showing that stroke volume and cardiac output during well-oxygenated conditions are significantly reduced in hypoxic-acclimated (> 6 weeks at P_wO_2 8-9 kPa) cod as compared with normoxic-acclimated conspecifics. Thus, this research strongly suggests that *in vivo* cardiac function in hypoxia-acclimated cod was not lower because of alterations in nervous/humoral control or venous vascular tone, but because of the direct effects of prolonged hypoxia on the myocardium. Interestingly, the present study also showed that hearts from hypoxia-acclimated cod could better maintain maximium performance when faced with severe hypoxia (PO_2 ~ 0.6 kPa), and recovered better than hearts from normoxic-acclimated fish following an acute severe hypoxic insult. These latter results suggest that although their normoxic performance may be reduced, they may be better able to perform repeatedly under conditions of limited O_2 supply.

3.5.1. Resting (Basal) Cardiac Performance

I adjusted cardiac output to 16 - 17 ml min^{-1} kg^{-1} according to *in vivo* data for cod which indicates that resting Q for this species at 10°C is between 17 (Axelsson and Nilsson, 1986) and 19 ml min^{-1} kg^{-1} (Axelsson, 1988; Fritsche and Nilsson, 1989). This Q, however, was lower than resting *in vivo* values for normoxic-acclimated fish obtained from the same cod stock (23.1 ±1.8 ml min^{-1} kg^{-1}, Petersen and Gamperl, unpubl.; Chapter 2). I used 16 ml min^{-1} kg^{-1} because Petersen and Gamperl (unpubl.; Chapter 2) suggested that their cod had not recovered fully from surgery/anaesthesia, a conclusion

supported by the significantly lower critical swimming speed for these fish vs. non-surgery controls, and because this value has been previously used in our lab for studies utilizing *in situ* cod hearts (e.g. Mendonca et al., 2007; Gamperl and Genge, unpubl.). The resting (basal) S_V recorded for *in situ* hearts from normoxic-acclimated cod (0.27 ± 0.01 ml kg^{-1}) corresponds well with previous data for most normoxic *in situ* cod heart preparations (approx. 0.3 ml kg^{-1}) (Mendonca et al. 2007; Gamperl and Genge, unpubl), but is lower than the *in vivo* S_V recorded in normoxic-acclimated Newfoundland cod (0.60-0.73 ml kg^{-1}) (Gollock et al., 2006; Petersen and Gamperl, unpubl.; Chapter 2) and North Sea cod (0.39 to 0.51 ml kg^{-1}) (Axelsson and Nilsson, 1986; Axelsson, 1988; Fritsche and Nilsson, 1989). The lower S_V *in situ,* as compared to *in vivo,* results because the cholinergic nervous tone on the cod heart (Axelsson and Nilsson, 1986) is eliminated during surgical procedures used to obtain the *in situ* heart preparation, and a lower S_V is thus required to achieve resting Q at the heart's intrinsic rate. For example resting f_H *in vivo* is approx. 25 – 40 beats min^{-1} at 10°C (Jones et al., 1974; Pettersson and Nilsson, 1980; Wahlqvist and Nilsson, 1980; Smith et al., 1985; Axelsson and Nilsson, 1986; Axelsson, 1988; Butler et al., 1989; Fritsche and Nilsson, 1989; Webber et al., 1998; Gollock et al., 2006) whereas *in situ* heart rates are generally in the range of 50 – 60 beats min^{-1} (Mendonca et al., 2007; Gamperl and Genge, unpubl., present study).

An interesting observation was that even though *in situ* heart rate was not affected by severe hypoxia in either normoxic- or hypoxic-acclimated hearts, heart rates in the latter group were comparable, or slightly lower than for the normoxic group (e.g. see Figure 3.2). This is contrary to findings *in vivo* where heart rate in cod acclimated to

chronic hypoxia was always significantly higher than in normoxic-acclimated animals (Petersen and Gamperl, unpubl.; Chapters 2, 4 and 5). As resting catecholamine levels do not differ between normoxic- and hypoxic-acclimated cod (Petersen and Gamperl, unpubl.; Chapter 5), these data indicate that alterations in adrenergic (increase) or cholinergic (decrease) nervous tone mediated the higher f_H in hypoxic-acclimated fish *in vivo*.

During the 15 min. of severe hypoxia (saline PO_2 ~0.6 kPa) cardiac output and power output decreased gradually in both groups, with Q at the end of the hypoxic period only ~ 65% of initial values. Further, this decrease was almost entirely the result of a hypoxia-induced reduction in stroke volume as only slight decreases in heart rate were observed while S_V fell by 27 and 38 % in normoxic- and hypoxic-acclimated fish, respectively. This magnitude and rate of decrease in Q and S_V (myocardial performance) are very similar to that measured for rainbow trout (*Oncorhynchus mykiss*) and cod myocardial strips (Hartmund and Gesser, 1996) and for the *in situ* trout heart (Gamperl et al., 2001; Faust et al., 2004; Gamperl et al., 2004; Overgaard et al., 2004a). Further, the lack of a hypoxia-induced fall in *in situ* f_H at 10°C is in agreement with data for the eel (*Anguilla dieffenbachii*; Davie et al., 1992), dogfish (*Squalus acanthias*; Davie and Farrell, 1991) and rainbow trout (Overgaard et al. 2004a), although Faust et al. (2004) report that heart rate in rainbow trout falls by 30% after 15 min. of severe hypoxia. While more research is clearly needed to clarify how the pacemaker cells of teleost fish respond to severe hypoxia/anoxia, and thus how these conditions affect intrinsic heart rate, there is a distinct difference in how cod hearts respond to hypoxia *in situ* (present study) vs. *in*

vivo (Petersen and Gamperl, unpubl. Chapter 5). In the *in vivo* study, heart rate was reduced when cod were exposed to hypoxia, with f_H falling from 32 to 18 beats min^{-1} at the point at which cod lost equilibrium (water $PO_2 \sim 2.7$ kPa). Again, these data support previous *in vivo* studies which demonstrated the importance of alterations in adrenergic and cholinergic nervous function in the regulation of teleost f_H during hypoxia (Wood and Shelton, 1980; Fritsche and Nilsson, 1989; Fritsche, 1990).

Following 30 min. of recovery in oxygenated saline values for P_{in}, Q, f_H and S_V were almost identical between groups, and also to those measured at the start of the experiment. The only exception being P_{in}, which was 0.1 kPa higher in hypoxic-acclimated as compared to normoxic-acclimated hearts; an increase likely due to heart rate in this group being 4 beats min^{-1} lower than in the former. It is quite surprising that the cod heart did not require a substantially higher P_{in} to maintain a resting Q of 16 – 17 ml min^{-1} kg^{-1} after recovering from severe hypoxia as this has been routinely reported for the rainbow trout heart (Gamperl et al., 2001; Faust et al., 2004; Gamperl et al., 2004; Overgaard et al., 2004a). This difference may be due to the presence of only spongy myocardia in cod, as compared with both compact and spongy myocardium in the trout. However, a definitive answer as to why the cod heart retains its sensitivity to filling pressure following exposure to acute severe hypoxia and reperfusion awaits further study.

3.5.2. Maximum Cardiac Function: Normoxia

When input pressure was increased in normoxic-acclimated hearts, maximum Q in oxygenated saline was 49.6 ± 2.3 ml min^{-1} kg^{-1}, and maximum S_V and f_H at maximum Q

were 0.92 ± 0.04 ml kg^{-1} and 54.1 ± 1.9 beats min^{-1}. These values for Q and S_V are very similar to *in vivo* values reported for 10°C acclimated cod that were exercised to exhaustion (Petersen and Gamperl, unpubl.; Chapter 2: ~ 45 ml min^{-1} kg^{-1} and 0.99 ml kg^{-1}, respectively) and comparable to those recorded for cod exposed to a temperature increase up to their critical thermal maximum when the elevation in f_H is taken into account (Gollock et al., 2006; ~ 52.6 ml min^{-1} kg^{-1} and 0.78 ml kg^{-1}). Further, the reported values are in-line with other studies using *in situ* cod hearts although there is some variation between studies (Q, 48 – 58 ml min^{-1} kg^{-1}; S_V, 0.95 – 1.2 ml kg^{-1}: Mendonca et al., 2007; Lurman et al., unpubl; Gamperl and Genge, unpubl.).

A very significant finding was that maximum Q and S_V were 19 and 28% lower, respectively, under oxygenated conditions in hearts from hypoxic-acclimated cod, and that these reductions mirrored those seen *in vivo* (~ 25%) when normoxic- and hypoxic-acclimated cod were given a U_{crit} test under normoxic conditions (Petersen and Gamperl, unpubl.; Chapter 2). These data strongly suggest that the reduced *in vivo* capacity of hearts from hypoxic-acclimated cod to pump during exhaustive exercise was not the result of alterations in the ability of nervous or hormonal mechanisms to stimulate cardiac function, or of the fish to modulated venous (filling) pressure (e.g. see Sandblom and Axelsson, 2005, 2006), but a direct effect of chronic hypoxia on the cod's myocardium.

There has been very little research on the effects on chronic hypoxia on fish cardiac physiology/morphology. However, there are at least three potential explanations for the poor pumping capacity of *in situ* hearts from hypoxic-acclimated fish under oxygenated conditions. First, it is possible that the cod myocardium was damaged by

constant exposure to low oxygen conditions. Such a conclusion would be consistent with the findings of Lennard and Huddart (1992) who showed that cardiomyocytes in flounder (*Platichthys flesus*) subjected to 3 weeks of hypoxia (water $PO_2 \sim 5$ kPa) showed striking changes in mitochondrial morphology (decreased size, budding and necrosis) and evidence of myofibril degeneration. However, the level of hypoxia utilized in this study (8-9 kPa) was not nearly as severe as that used by Lennard and Huddart (1992), and several studies have shown that, at least in the trout heart, acute (< 30 min.) exposure to severe anoxia (perfusate $PO_2 \leq 1$ kPa) does not result in myocardial necrosis or a disruption in myocardial energetic and enzymatic status (Faust et al., 2004; Overgaard et al., 2004a,b). These data, thus question whether myocardial damage/necrosis was experienced by our hypoxic-acclimated cod. Second, it is possible that there was no myocardial damage in cod acclimated to 8-9 kPa (40% air saturation) for 6-12 weeks, and that hypoxia-induced myocardial remodelling reduced the maximum S_V of the heart. Although, the similar RVM in hypoxic- and normoxic-acclimated cod (0.71 and 0.72%, respectively) provides some evidence against extensive cardiac remodelling in the chronically hypoxic cod, Marques et al. (2008) showed that acclimation of zebrafish (*Danio rerio*) and the cichlid (*Haplochromis piceatus*) to a P_wO_2 2 kPa (10% air saturation) for 21 days increased cardiac myocyte density and that this resulted in a smaller ventricular outflow tract and reductions in the size of the central ventricular cavity and lacunae. Such a decrease in the capacity of the ventricle to fill with blood would certainly explain why maximum *in situ* and *in vivo* S_V were reduced by 28 and 25%, respectively, in the hypoxia-acclimated cod. Finally, it is possible that the hearts of hypoxic-acclimated cod were 'stunned' (i.e. experiencing mechanical dysfunction that

persists after reoxygenation/reperfusion despite the absence of irreversible damage; see Bolli and Marban, 1999) when initially tested under oxygenated conditions, and thus that the decreased pumping capacity of hearts from hypoxia-acclimated cod represented a reduced functional capacity that was not related to major morphological/structural alterations of the myocardium. This conclusion would be consistent with the findings of a number of authors who showed that the trout heart is stunned when acutely exposed to severe hypoxia (Faust et al., 2004; Overgaard et al., 2004a,b). However, I feel it is unlikely that the decrease in performance during the initial test under oxygenated conditions was the result of stunning. This is because the hearts of hypoxia-acclimated cod showed no deficit in cardiac performance after exposure to severe hypoxia and reperfusion (see below). More studies are clearly needed to determine the exact cause of the observed reduction in pumping capacity in hypoxic-acclimated cod. However, currently the most plausible explanation would be that a remodelling has taken place in the hypoxic-acclimated cod heart and that this has resulted in a reduced maximum S_V.

3.5.3. Maximum Cardiac Function: Hypoxia and Reperfusion

When hearts from normoxic-acclimated fish were exposed to severe hypoxia (PO_2 $\leq$ 1 kPa) at 10°C, maximum Q was reduced to approximately one-third of initial values under oxygenated conditions (Table 3.3). This decrease in Q_{max} was attributed to a decrease in S_V as f_H did not change, and its magnitude is consistent with earlier studies on the effect of hypoxia on the cardiac performance of isolated trout and dogfish hearts. For example, Q_{max} in 10°C *in situ* trout hearts decreased by 25 - 50 % when perfusate PO_2 was lowered to ~ 3.3 kPa (Farrell et al., 1989; Hanson et al., 2006) and Davie and Farrell

(1991) showed that Q_{max} in an isolated 15°C dogfish heart preparation was decreased by 59% at 1 kPa. However, it is much greater than the < 20% reduction in Q_{max} experienced by 15°C eel hearts exposed to a PO_2 of 1.6 kPa (Davie et al., 1992), although these hearts were only exposed to 8 min. of hypoxia before Q_{max} was assessed.

Exposure of hearts from normoxic-acclimated cod to hypoxia, including a Q_{max} test at the end of the 15 min. hypoxic period, resulted in a ~ 17% reduction in Q_{max} after 30 min. of recovery as compared with initial Q_{max} values. Further, although I did not measure PO_{max} at the beginning of the experiment, a comparison of my PO_{max} data with other studies on cod hearts where PO_{max} was only evaluated under oxygenated conditions (Figure 3.6) indicates that hypoxic exposure reduced PO_{max} by ~ 30% (4.7 kPa vs. ~ 7 mW g ventricle^{-1}, respectively) and shifted the P_{out} where PO_{max} occurred from ~ 6-7 to 4 kPa. These hypoxia-induced reductions in Q_{max} and PO_{max} are very similar to values reported for 10°C acclimated trout (Faust et al., 2004; Overgaard et al., 2004a,b) and cod (Gamperl and Genge, unpublished), and were likely associated with stunning of the myocardium and not myocardial damage or disruptions in energy metabolism.

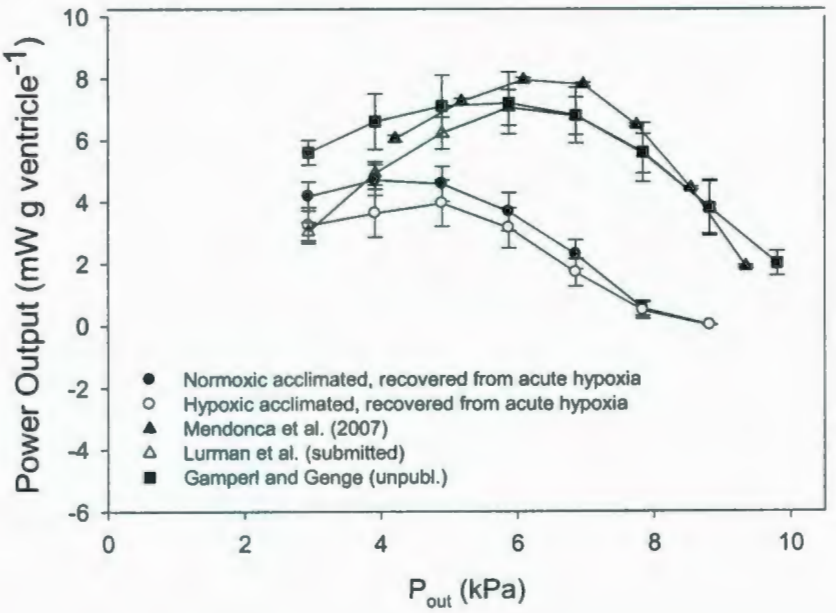

Figure 3.6. Comparison of the relationship between power output and output pressure (P_{out}) for hearts in the current study (ie. after recovery from 15 min. of severe hypoxia) with that obtained in several other studies on Atlantic cod *in situ* cardiac performance where the hearts did not experience a hypoxic insult prior to the maximum power output test. In these other studies, all fish were acclimated to normoxic conditions.

This conclusion is based on the fact that several studies on trout have failed to demonstrate myocardial necrosis after anoxic/severe hypoxia periods as long as 30 min. (Gamperl et al., 2001; Faust et al., 2004; Overgaard et al., 2004a,b), and Overgaard et al. (2004a) reported that the energetic state of trout hearts exposed to 20 min. of anoxia and 30 min. of reperfusion was similar to that measured in hearts constantly perfused with oxygenated saline.

When compared to hearts of normoxic-acclimated cod, those of hypoxia-acclimated cod were better able to maintain Q_{max} during severe hypoxia and showed significantly enhanced recovery following 30 min. of reperfusion with oxygenated saline (Figures 3.3 and 3.4; Table 3.4). These results suggest that acclimation to chronic hypoxia increases myocardial hypoxia tolerance, and are consistent with the substantial body of research that has been conducted on chronically hypoxic mammals [see Ostadal and Kolar (2007) for a review]. Further, they are in agreement with Driedzic et al. (1985) who showed that ventricular strips from hypoxia-acclimated (4-6 weeks; P_wO_2, 4-4.7 kPa) eelpout were better able to sustain peak tension development during anoxia in the presence of elevated levels of Ca^{2+} in the bathing media.

In fish, as in mammals, the loss of myocardial function during severe hypoxia/anoxia is primarily due to the inability of the heart to maintain the rate of ATP production through anaerobic metabolism (Farrell et al., 1985; Overgaard and Gesser, 2004), and changes in the intracellular environment of the myocyte that result (Godt and Nosek, 1989). For example, anaerobic metabolism leads to the production of lactate and a corresponding build up of hydrogen ions that diminishes hypoxic performance (Gesser and Poupa, 1974). In addition, cellular creatine phosphate levels decline during hypoxia

and this leads to an accumulation of intracellular phosphates (Arthur et al., 1992; Hartmund and Gesser, 1996), which further impairs contractility by reducing the calcium sensitivity of troponin C (Gesser and Jorgensen, 1982; Nosek et al., 1987). There are several strategies that can be utilized to balance cellular ATP demand and supply during hypoxia, including upregulating glycolytic energy production, and reducing energy demand to levels that can be supported by the reduced ATP availability. Measurements were not taken in this study to elucidate the mechanisms whereby hearts from hypoxic-acclimated cod were better able to sustain initial level of cardiac performance under severe hypoxia. However, data from the fish and mammalian literature points to several mechanisms that might have been important for balancing ATP supply and demand. First, it is possible that enhanced glucose uptake and glycolytic capacity were primary factors mediating the enhanced cardiac function of hearts from hypoxic-acclimated cod. Lennard and Huddart (1992) report that endogenous glycogen stores are increased in flounder myocytes after 3 weeks at a P_wO_2 of 5 kPa. Several studies have reported the reliance of hypoxic/anoxic teleost cardiac performance on extracellular glucose (Driedzic et al., 1985; Bailey et al., 2000; Gamperl et al., 2001; Clow et al., 2004), and Clow et al. (2004) showed that glucose uptake was increased 3-fold in the cod heart during hypoxia (~ P_wO_2 5 kPa). Finally, Martinez et al. (2006) report increases in myocardial hexokinase, pyruvate kinase and triose phosphate isomerase activity ranging from 18 – 28% in Gulf killifish (*Fundulus grandis*) exposed to a P_wO_2 of 3 kPa for 4 weeks, and Marques et al. (2008) showed increased expression of pyruvate kinase (by 2.7-fold) and aldolase b (by 4.3 fold) mRNA after zebrafish were exposed to 2 kPa P_wO_2 for 21 days. However, I feel this is unlikely as Hall et al. (2009) showed that increases in the expression of genes

related to glucose transport, uptake and metabolism are short-lived when cod are acclimated to hypoxia (P_wO_2 ~ 8.0 - 9.0 kPa) (i.e. hypoxia-acclimated cod do not appear to have an enhanced capacity for anaerobic metabolism). Second, a caveat with these experiments is that the heart cannot be made completely anoxic (i.e. PO_2 was ~ 0.6 kPa), and a small amount of aerobic metabolism can go a long way in supplying cellular ATP demands given the large amount of ATP generated per molecule of glucose (36 vs. 2 through anaerobic metabolism). Given that myoglobin plays an important role in oxygen metabolism in fish hearts at low extracellular PO_2s by facilitating oxygen diffusion from the extracellular space to the mitochondria (e.g. see Legate et al., 1998), one might expect to see an increase in myocardial myoglobin levels in hearts of chronically hypoxic individuals. However, this is unlikely as myoglobin levels in eelpout (Driedzic et al, 1985) and myoglobin mRNA levels cod (Hall et al., 2009) were unchanged following hypoxic acclimation. Third, Na^+/K^+ ATPase activity comprises 20-40% of cellular energy expenditure in excitable tissues (Rolfe and Brown, 1997), and Paajanen and Vornanen (2003) showed that hypoxic-acclimation reduces the Na^+/K^+ ATPase activity of crucian carp (*Carassius carassius*) cardiac homogenates by 33%. This overall ~10% saving in cellular energy expenditure may be an important component to the enhanced performance exhibited by hypoxia-acclimated cod. Finally, there are several other mechanisms that have been reported to confer hypoxia tolerance on the mammalian heart during periods of prolonged oxygen deprivation. Amongst these are ATP-sensitive potassium (K_{ATP}) channels [both sarcolemmal (sK_{ATP}) and mitochondrial (mK_{ATP})], nitric oxide (NO), HIF-1a, and various protein kinases (including PKC) (Kolar and Ostadal, 2004; Ostadal and Kolar, 2007). However, research on the importance of these mechanisms in conferring

cardioprotection in fishes is still in its infancy, and is often contradictory (see MacCormack and Driedzic, 2002; Chen et al., 2005; Rissanen et al., 2006; Marques et al., 2008).

With regards to the ability of fish hearts to recover maximum cardiac function following a period of oxygenated perfusion, Overgaard et al. (2004a): 1) showed that functional impairment of trout hearts following anoxic exposure occurs even though the energetic and biochemical status of the myocardium is not compromised (altered); and 2) suggested that increased levels of oxygen radicals were responsible for the stunning of trout hearts following recovery from severe hypoxia/anoxia. Based on this information, it could be hypothesized that the improved functional recovery shown by hearts of hypoxia-acclimated cod following 15 min. of severe hypoxia was due to an increased ability (relative to trout hearts) to protect the myocardium against the negative effects of reactive oxygen species (ROS). Indeed, this is a plausible explanation as Marques et al. (2008) showed that the expression of 6 genes important for protection against ROS were upregulated (by 2.1 to 6.5 fold) in zebrafish hearts following 21 days of acclimation to a P_wO_2 of 2 kPa.

3.5.4. Perspectives and Future Research

In summary, this study shows that acclimation to a moderate level of hypoxia (P_wO_2 ~ 8 kPa) has a direct negative impact on the maximum performance of the cod heart, but enhances its ability to maintain pumping capacity under conditions of oxygen shortage and to recover better following a period of oxygenated reperfusion. While these results may appear contradictory, and I presently have few mechanistic explanations for

how these differences in functional capacity might be mediated in fishes, I hypothesize based on the literature, that they reflect two different types of myocardial adaptation: 1) a remodelling of the heart (likely involving significant hyperplasia) that limits ventricular volume (stroke volume) but diminishes the workload of individual cardiomyocytes and prevents severely hypoxic/anoxic cores in the cardiomyocytes, and thus limits apoptosis and/or myocardial necrosis (Des Tombe et al., 2002; Laarse et al., 2005; Marques et al., 2008); and 2) a reprogramming of cellular metabolism that ameliorates the potentially negative effects of long-term hypoxia on the capacity for aerobic metabolism, and attenuates mitochondrial ROS production (Kelly, 2008; Popandreou et al., 2006; Kim et al., 2006). Whether these hypothese are correct, and applies to fishes in general, awaits a significant amount of research effort. However, I expect this effort will reveal novel insights into myocardial plasticity and adaptation in fishes (vertebrates), and the molecular and biochemical pathways that protect the heart from environmental insults that might normally lead to cardiac dysfunction, myocardial damage, and eventually mortality.

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Chapter 4

Importance of Adrenergic Stimulation for *in vivo* Cardiac Function in Normoxic and Hypoxic-Acclimated Atlantic Cod (*Gadus morhua*).

4.1. Abstract

Recent experiments show that the normoxic *in situ* Atlantic cod heart is minimally responsive to adrenergic stimulation, and that the hearts of hypoxic-acclimated (6-12 weeks at 10°C; P_wO_2 ~ 8- 9 kPa) cod have decreased maximal cardiac performance. However, at present, we have little knowledge of the *in vivo* response of the cod's cardiovascular system to adrenergic stimulation or whether the heart's responsiveness is affected by hypoxic-acclimation. Therefore, I determined the response of cod *in vivo* cardiac function to adrenergic stimulation by injecting increasing doses of epinephrine (0.2 - 4.0 μg kg^{-1}) into the afferent branchial artery of normoxic and hypoxic-acclimated individuals. Epinephrine injection did not result in positive chronotropy in either acclimation group, with the maximum increase in heart rate only 5 to 10% ($P > 0.05$). In contrast, stroke volume (S_V) and cardiac output (Q) responded to the highest doses of epinephrine, and the response was diminished in hypoxic-acclimated individuals. For example, these parameters increased by 25 and 35%, respectively, in normoxic-acclimated fish injected with the highest dose of epinephrine (4.0 μg kg^{-1}), whereas the increases in S_V and Q were only 12 and 15% in hypoxic-acclimated cod. Overall, these results: 1) provide direct *in vivo* evidence that the cod heart is not very responsive to increases in circulating catecholamines; and 2) show that hypoxic-acclimation reduces the already low sensitivity of this species' heart to adrenergic stimulation.

4.2. Introduction

The hormones epinephrine and norepinephrine are released from adrenergic nerve fibres that innervate the heart (Gannon and Burnstock, 1969; Holmgren, 1977) and vasculature (Randall and Stevens, 1967; Wahlqvist, 1980; Nilsson, 1984; Zhang et al., 1998), and are released into the circulation from chromaffin cells lining the posterior cardinal vein within the head kidney (Nandi, 1961; Reid et al., 1998). Generally, it is believed that the teleost heart needs a tonic, low level, of adrenergic stimulation to sustain resting cardiac performance (Graham and Farrell, 1989), and that circulating catecholamines are required for the teleost heart to reach its maximum pumping capacity (Farrell et al., 1996; Claireaux et al., 2005; Hanson et al., 2006). However, recent data suggest that adrenergic stimulation is not important for the normoxic performance of all fish hearts. For instance, no observable differences in resting or maximum heart rate, stroke volume, or cardiac output were reported for *in situ* normoxic cod hearts treated with 7 nM adrenaline as compared with those perfused with adrenaline-free saline (Gamperl and Genge, unpublished). The addition of 200 nM of epinephrine only resulted in minor enhancements in resting or maximum cardiac parameters in *in situ* cod hearts exposed to temperatures from 10 to 0°C (Lurman et al., unpubl.). Mendonca and Gamperl (2009) showed that the flounder (*Pleuonectes americanus*) heart is not dependent upon adrenergic stimulation at rest, and that cardiac function was not affected by catecholamine injections that raised circulating epinephrine and norepinephrine concentrations to those seen following a chase to exhaustion. Finally, Axelsson (1988) found that epinephrine only has positive inotropic effects on the cod atrium at

concentrations seen during severe stress or exhaustive exercise (> 100 nM: Axelsson and Nilsson, 1986).

Environmental hypoxia is one of several stressors that can cause the release of the hormones epinephrine and norepinephrine from the chromaffin tissue of fish (Butler et al., 1979; Fritsche and Nilsson, 1990; Perry et al., 1991; Kinkead et al., 1991). These catecholamines, in turn, initiate a series of responses aimed at alleviating the disruptive effects of the stressor on physiological and metabolic function (Perry and Wood, 1989; Thomas and Perry, 1992; Randall and Perry, 1992; Fabbri et al., 1998). For instance, catecholamines released during acute hypoxia and exhaustive exercise influence cardiorespiratory function by: 1) enhancing blood oxygen transport through activation of Na^+/H^+ exchangers in the erythrocyte cell membrane (Primmett et al., 1986; Jensen, 1991; Thomas et al., 1991; Perry and Gilmour, 1999), and by causing the release of red blood cells from the spleen (Kita and Itazawa, 1990; Pearson et al., 1992); 2) improving gill oxygen-diffusing capacity (Pettersson, 1983; Perry et al. 1985); and 3) alleviating the negative inotropic effects of hypoxia, acidosis and hyperkalemia on cardiovascular function (e.g. Farrell et al., 1983, 1986; Hanson et al., 2006; Hanson and Farrell, 2007). However, it is presently unclear how exposure to chronic (weeks of) hypoxia affects the ability of catecholamines to stimulate the fish heart. This is because no such studies have directly examined this question. Although studies on mammalian and avian hearts show that prolonged hypoxia (hours to weeks) results in a downregulation of cardiac β-adrenoreceptors (Voelkel et al., 1981; Bernstein et al., 1990, 1992; Rocha Singh et al., 1991; Marsh and Sweeney, 1989; Kacimi et al., 1992; Leon-Velarde et al., 2001), Gamperl et al. (1998) showed that salmonid myocardial β-adrenoreceptors are resistant to

downregulation during short-term (6 hours) hypoxia. Finally, it appears that considerable inter-specific differences exist in the characteristics of β-adrenoreceptor populations found in the spongy myocardium; this cardiac muscle comprising the entire myocardium of approx. 70% of fish species, and completely dependent upon the low oxygen content and partial pressure of venous blood for oxygen delivery (Farrell and Jones, 1992). For example, based on a 14% greater cell-surface β-adrenoreceptor density and similar binding affinity in the spongy myocardium vs. compact myocardium of chinook salmon (*Oncorhynchus tshawytscha*), Gamperl et al. (1998) suggested that the spongy myocardium of salmonid hearts was well adapted for sustained performance during moderate hypoxia. In contrast, Mendonca and Gamperl (2009) showed that although the heart of the winter flounder, a species that experiences very low oxygen concentrations when buried in soft sediments (Fletcher, 1975; Duthie, 1982; Pereira et al., 1999), has an exceptionally high number of β–adrenoreceptors, they are of very low affinity.

Given the expanding threat of hypoxia for marine organisms including cod (e.g. see Wu, 2002; Pollock et al., 2007; Chabot and Claireaux, 2008), and recent experiments showing that the normoxic *in situ* Atlantic cod heart is only minimally responsive to adrenergic stimulation and that the hearts of hypoxic-acclimated (6-12 weeks at 10°C; P_wO_2 ~ 8-9 kPa) cod have decreased cardiac performance (Lurman et al., unpubl.; Gamperl and Genge, unpubl.; Petersen and Gamperl, unpubl.; Chap 2 and 3), it is clear that I need a more complete understanding of how long-term hypoxic exposure affects the capacity of this species' heart to respond to biotic and/or abiotic stressors. To this end, I fitted normoxic- (P_wO_2= 21 kPa, > 6 weeks) and hypoxic- (P_wO_2= 8 kPa, > 6 weeks) acclimated cod with Transonic® flow probes and afferent branchial artery cannulae, and

measured cardiac variables following the sequential injection of increasing doses of epinephrine.

4.3. Materials and Methods

4.3.1. Experimental Animals

The Atlantic cod (*Gadus morhua*) used in this study (0.56 ± 0.02 kg; range= 0.37-0.83 kg) were transported from a sea-cage site at Hermitage Bay (Newfoundland, Canada) to the Ocean Sciences Centre (OSC, Memorial University of Newfoundland, St. John`s, Newfoundland). At the OSC, they were maintained in a 20,000 litre tank supplied with aerated seawater at 10 ± 1°C for at least 5 months prior to experimentation. During this period, the fish were fed a commercial cod diet 3 times a week (EWOS, Canada), and kept at a seasonally ambient photoperiod.

Prior to experimentation, 60 fish from the holding tank were acclimated at 10.8 ± 1.0 °C to either normoxia [water oxygen partial pressure (P_wO_2) 19.2±0.3 kPa] or hypoxia (P_wO_2 8.4±0.04 kPa) for at least 6 weeks in three 1 m^3 tanks. Each acclimation tank held 20 fish, oxygen levels in both tanks were controlled as previously described by Petersen and Gamperl (unpubl.; Chapter 2), and the feeding of normoxic fish was restricted to levels consumed by the hypoxic-exposed fish when fed to satiation (see chap 2 for further detail).

4.3.2. Surgical Procedures

Fish were netted and placed in aerated seawater containing tricaine methane sulfonate (MS-222, Finquel: 0.1 g L^{-1}) until ventilatory movements ceased. The fish were then weighed and measured before being transferred to a surgery table where oxygenated seawater containing MS-222 (0.05 g L^{-1}) continuously irrigated their gills. *In vivo* cardiac function was measured by placing a 2S or 2.5S Transonic flow probe® around the ventral aorta of the cod as previously described by Gollock et al. (2006). After the flow probe was carefully fitted around the vessel, it was connected to a flow meter (Transonic Systems Inc., model TS420) to check the probe's signal strength and the blood flow profile. Then, a polyethylene cannula (PE 50, Clay Adams) filled with heparinised (100 IU ml^{-1}) marine teleost saline (Driedzic et al., 1985) was occlusively inserted into the afferent branchial artery of the third gill arch (Sundin and Nilsson, 1992), and this cannula was secured in place by tying a 3-0 silk suture (American Cyanamid Company, Pearl River, NY) around the gill arch (see Plate 2.1 in Chap 2, pg. 38). Finally, the flow probe cable and afferent artery cannula were secured to the fish at 3 locations using with 3-0 silk sutures; one location close to the incision, a second under the pectoral fin, and a third just anterior to the dorsal fin.

Once surgery had been completed, fish were transferred to a Perspex black-box (60.5 cm long x 10.5 cm high x 10.5 cm deep) receiving aerated seawater (10°C) at a flow rate of ~ 1 L min^{-1}, and the cannula and flow probe lead were pulled through a small hole in the top of the box. Fish from both groups recovered quickly after being placed into the black-boxes, but were allowed to recover from surgery for at least 20 h prior to

experimentation. During the recovery period, the cannulae were flushed several times with heparinised saline to avoid clotting.

4.3.3. Experimental Protocol

After recovery, some of the cod from each acclimation group were sequentially injected with 0 (sham), 0.2, 0.5, 1.0, 2.0 and finally 4.0 $\mu g\ kg^{-1}$ of epinephrine using a 0.5 - 0.6 ml carrier volume of marine teleost saline, with 1.5 h between injections. This protocol was based on Gamperl et al. (1994b), and allowed cardiovascular parameters to return to "resting" (pre-injection) levels for approximately 1 hr prior to the next injection. All doses of epinephrine (bitartrate salt; Sigma Chemical CO., St. Louis, MO) were injected slowly (i.e. over approximately 20-30 sec.) through the afferent branchial artery cannula, and were followed by a 0.3 ml saline bolus to ensure that all epinephrine was delivered to the fish. Heart rate (f_H), and cardiac output (Q) were continuously recorded throughout the experiment. However, the recording of afferent branchial artery pressure (i.e. ventral aortic pressure) was briefly interrupted when the various doses of epinephrine were injected.

4.3.4. Measurement and Analysis of Cardiovascular Parameters

Cardiac output (Q) was recorded by connecting the probe lead to a flow meter (Transonic Systems, Model TS-420) that was interfaced with a MP100A-CE data acquisition system (BIOPAC systems Inc., Santa Barbara, CA) and a laptop running AcqKnowledge software (BIOPAC Systems Inc.). Data were recorded at a frequency of 20 Hz, and records of cardiac output were analyzed during the first 30 min. after each

epinephrine injection. Cardiac output (in ml $min^{-1}kg^{-1}$) was calculated in AcqKnowledge by dividing the raw data (ml min^{-1}) by the body mass of the fish (kg). Heart rate (f_H, beats min^{-1}) was calculated by counting 20 systolic peaks, dividing by the elapsed time (sec) and multiplying by 60 (sec/min). Finally, stroke volume (S_V, ml kg^{-1}) was calculated from Q/f_H.

Ventral aortic blood pressure was measured using a Grass (PT300, Warwick, RI, USA) pressure tranducer, which was calibrated daily against a static column of water. Unfortunately, however, pressure signals became highly variable in many fish following epinephrine injection, and thus only resting (pre-injection) data are reported for the two groups.

Sham injection of the marine teleost saline increased stroke volume and cardiac output slightly, but did not affect heart rate (Figure 4.1). Although not being significantly different overall (based on a one-way ANOVA and Dunnett`s post-hoc tests) from resting values (Figure. 4.1), I decided to account for these slight post-injection increases in Q and S_V. Thus, I converted all changes in cardiac parameters following the various doses of epinephrine into values of '% change' prior to performing statistical analysis. For example, % change in f_H was calculated as: [f_H (epi) - f_H (pre drug injection))/ f_H (pre drug injection)* 100] - [(f_H (saline) - f_H (pre-injection)/ f_H (pre-injection)) * 100].

4.3.5. Statistical Analyses

Independent t-tests were used to determine if hypoxic acclimation significantly affected cardiac and body morphometrics, and resting cardiovascular parameters. One-way ANOVA`s followed by Dunnett`s *post-hoc* tests were used to examine if post saline-

injection values of Q, f_H and S_V were different from resting values. GLM repeated measures analysis was used to test for the effects of hypoxic acclimation and time post-injection on cardiac parameters (Q, f_H and S_V) at each epinephrine dose, and this analysis was followed by one-way ANOVA`s at each time point to test for significant differences between the normoxic and hypoxic groups. This analysis was also used to evaluate whether hypoxic-acclimation and epinephrine dose had significant effects on the maximum increase in cardiac output and time to maximum cardiac output. Statistical analyses were carried out using SPSS (v.13.0; SPSS, Chicago, IL, USA). A result was considered significant when $P<0.05$. All data presented in the text, figures and tables are means ± standard errors (SEM).

4.4. Results

4.4.1. Cardiac Morphometric and Resting (basal) Cardiac Performance

Despite being fed equal amounts of food, fish acclimated to hypoxic water (P_wO_2 8-9 kPa) had significantly lower values for mass, ventricular mass and condition factor, but not relative ventricular mass (RVM) (Table 4.1). Resting (pre-injection) f_H was significantly higher in the hypoxic-acclimated cod as compared with normoxic individuals (46.7±1.6 vs 42.1±1.22 beats min^{-1}, Table 4.1). However, this difference in f_H was not reflected in the values for S_V, Q, or ventral aortic pressure (P_{va}). Values for Q and S_V were approximately 0.6 ml kg^{-1} and 26 ml min^{-1} kg^{-1}, respectively, in the two groups. Further although P_{va} was slightly higher in the hypoxic-acclimated fish (3.94 ±0.22 kPa)

this value was not significantly different from that measured in the normoxic group (3.50±0.45 kPa).

4.4.2. *In Vivo* Cardiac Response to Epinephrine Injection

There was no significant difference in the effect of 0.2, 0.5 and 1.0 μg kg^{-1} of epinephrine on heart rate between the two groups (GLM repeated measures) (Figure 4.2A-C). These doses of epinephrine, only resulted in increases in f_H of ~ 0-5% in hypoxic-acclimated cod, and ~5-10 % in normoxic-acclimated cod. Following the injection of 2.0 μg kg^{-1} of epinephrine (Figure 4.2D), f_H in the normoxic group increased by approx. 10% (i.e. by ~ 5 beats min^{-1}), and heart rate was elevated as compared to the hypoxic group at 2, 4 and 14 min. post-injection. The diminished responsiveness to adrenergic stimulation in the hypoxic-acclimated cod was also observed for f_H and stroke volume after the highest epinephrine dose (4.0 μg kg^{-1}, Figures 4.2E and 4.3E). This latter parameter only increased by approx. 10% in hypoxic-acclimated fish following doses of 0.2 – 2.0 μg kg^{-1} (Figures 4.3A-D), but increased by approx. ~20-25% in normoxic-acclimated fish after the injection of 0.5 and 4.0 μg kg^{-1} epinephrine (Figure 4.3B,E).

Post-injection changes in Q reflected those in f_H and S_V, with no differences observed between the groups at the 3 lowest epinephrine doses (Figures 4.4A-C), a transiently higher Q in normoxic-acclimated cod when injected with 2.0 μg kg^{-1} epinephrine (Figures 4.4D; increases ~ 20 vs. 5% in the two groups), and finally a larger and more sustained elevation in post-injection Q in the normoxic- vs. hypoxic-acclimated fish after the injection of 4.0 ug kg^{-1} epinephrine (Figure 4.4E). The increase in Q in normoxic-acclimated fish peaking at 34% above pre-injection values. The reduced ability

of epinephrine to increase Q in hypoxic-acclimated fish is also seen when the maximum increase in Q (in ml min^{-1} kg^{-1}) is plotted vs. injected dose (Figure 4.5A). Cardiac output increased by about 12 ml kg^{-1} min^{-1} at 4.0 μg kg^{-1} in normoxic-acclimated cod, and this value was significantly higher than measured following the injection of 0.2 μg kg^{-1}. In contrast, no significant dose effect was observed for the hypoxic-acclimated cod, and maximum Q was approx. 3 ml min^{-1} kg^{-1} lower (although not significantly; $P = 0.2$) than measured in the normoxic-acclimated group. Cardiac output peaked within 1.5 to 3.5 min. post-injection, and time to peak Q tended to increase with epinephrine dose and was generally longer in normoxic-acclimated fish (Figure 4.5B). However, only one significant difference was found. Time to peak Q was 3.5 $\pm$ 2.5 min. in normoxic-acclimated fish vs. 1.6 $\pm$ 0.5 min. in hypoxic-acclimated fish when given 1.0 μg kg^{-1} of epinephrine.

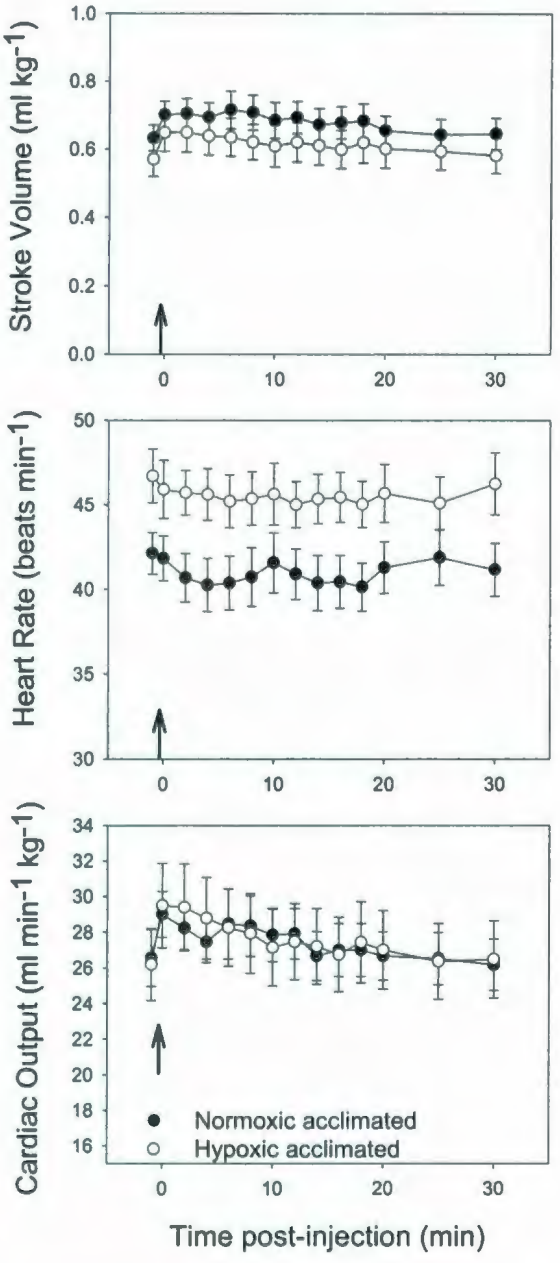

Figure 4.1. *In vivo* cardiac parameters (S_V, f_H and Q) in 6-10 week normoxic- (water oxygen partial pressure (P_wO_2) 21 kPa) and hypoxic- (P_wO_2 8-9 kPa) acclimated Atlantic cod. First data points are resting values before any manipulation. Arrow indicates time of bolus saline injection. One-way ANOVA with Dunnett's *post-hoc* test showed no significant difference ($P<0.05$) between resting and other time point values. Values are means ± SEM. N = 11-12.

Table 4.1. *Body and cardiac morphometrics, and* in vivo *resting cardiovascular parameters, in normoxic- (P_wO_2 ~ 19 kPa) and hypoxic- (P_wO_2 8-9 kPa) acclimated Atlantic cod.*

	Morphometrics					***Resting Cardiovascular Parameters***			
	Mass (kg)	**Length (cm)**	**Ventricular Mass (g)**	**Relative Ventricular Mass (RVM)**	**Condition Factor**	**Heart rate (beats min^{-1})**	**Stroke Volume (ml kg^{-1})**	**Cardiac Output (ml^{-1} min^{-1} kg^{-1})**	**Blood Pressure (kPa)**
Normoxic	0.55* (0.03)	40.6 (0.8)	0.51* (0.02)	0.096 (0.007)	0.82* (0.04)	42.1* (1.2)	0.63 (0.04)	26.6 (1.6)	3.5 (0.5)
Chronic Hypoxic	0.43 (0.02)	39.9 (0.5)	0.38 (0.02)	0.088 (0.002)	0.67 (0.02)	46.70 (1.6)	0.57 (0.05)	26.2 (2.0)	3.9 (0.2)

Values shown are means ± SEM (N = 11-12 per group). Resting values of each group were measured in normoxic water and before any injection of either saline or epinephrine. Mass was determined prior to surgery.
*Indicates significantly different values ($P < 0.05$) between groups (normoxic- vs hypoxic-acclimated).
Condition factor was calculated as $K = (mass/length^3) \times 100$, and RVM was calculated as (mass/ventricular mass) x 100

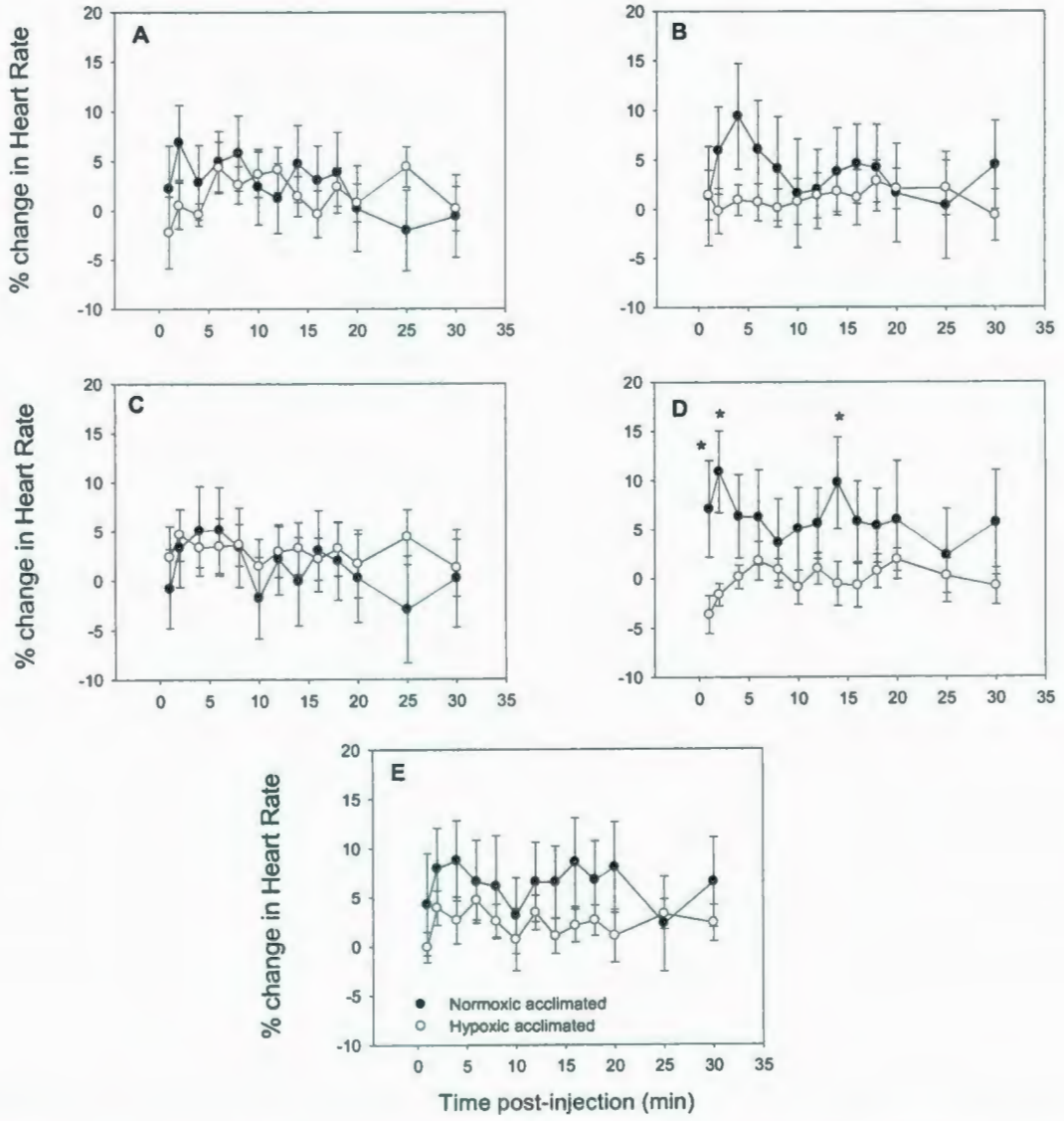

Figure 4.2. Percentage change in heart rate in normoxic and hypoxic acclimated Atlantic cod. Each group was exposed to increasing doses of epinephrine. (A) 0.2 μg kg^{-1}, (B) 0.5 μg kg^{-1}, (C), 1.0 μg kg^{-1}, (D) 2.0 μg kg^{-1} and (E) 4.0 μg kg^{-1}. Values are means ± SEM (N = 11-12) and are relative to pre-injection values after correcting for any changes in heart rate associated with saline injection. * Indicates a significantly different ($P<0.05$) value between normoxic and hypoxic acclimated groups.

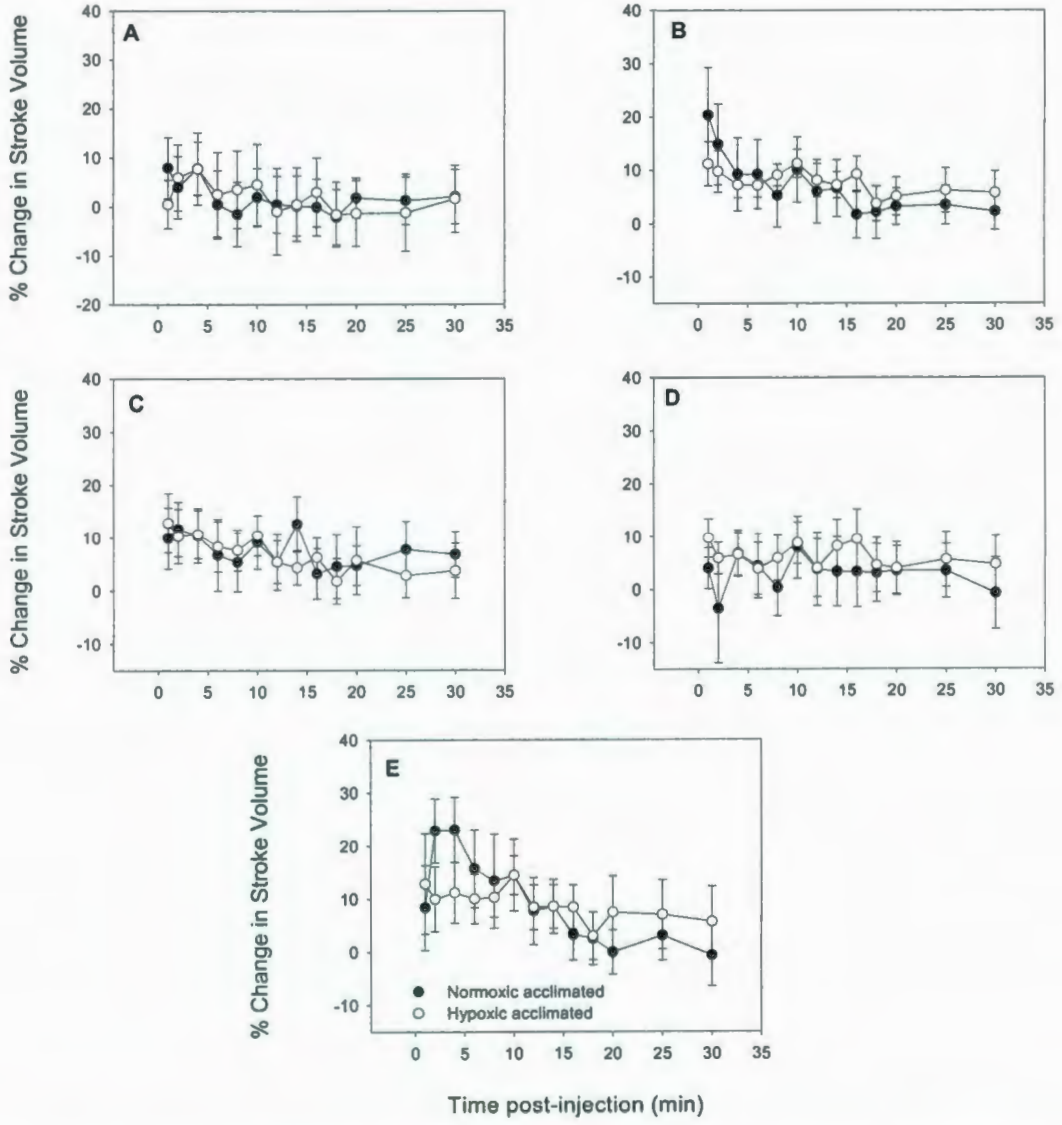

Figure 4.3. Percentage change in stroke volume in normoxic and hypoxic acclimated Atlantic cod. Each group was exposed to increasing doses of epinephrine. (A) 0.2 μg kg^{-1}, (B) 0.5 μg kg^{-1}, (C), 1.0 μg kg^{-1}, (D) 2.0 μg kg^{-1} and (E) 4.0 μg kg^{-1}. Values are means ± SEM (N = 11-12) and are relative to pre-injection values after correcting for any changes in stroke volume associated with saline injection. * Indicates a significantly different ($P<0.05$) value between normoxic and hypoxic acclimated groups.

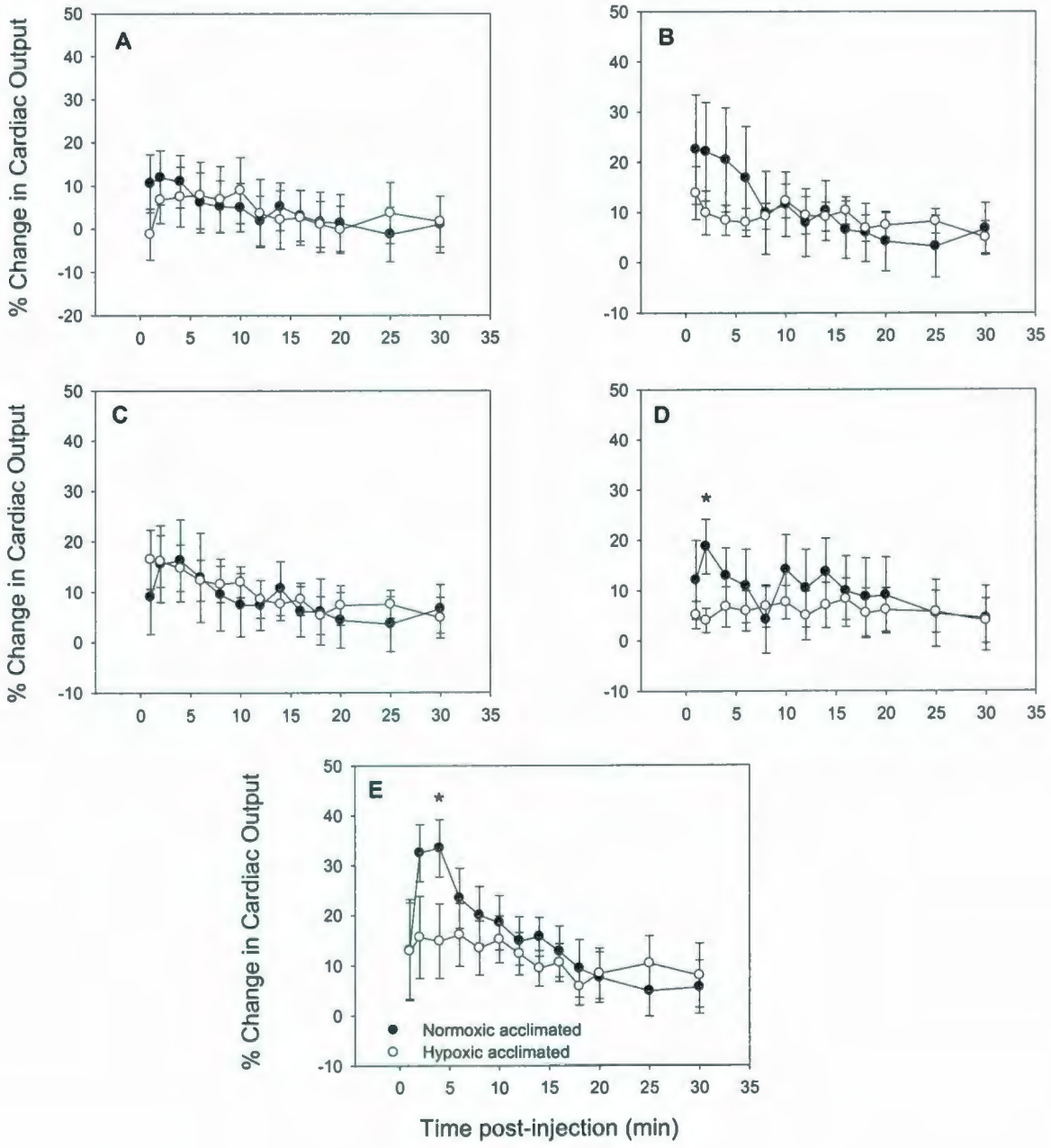

Figure 4.4. Percentage change in cardiac output in normoxic and hypoxic acclimated Atlantic cod. Each group was exposed to increasing doses of epinephrine. (A) 0.2 μg kg^{-1}, (B) 0.5 μg kg^{-1}, (C), 1.0 μg kg^{-1}, (D) 2.0 μg kg^{-1} and (E) 4.0 μg kg^{-1}. Values are means ± SEM (N = 11-12) and are relative to pre-injection values after correcting for any changes in cardiac output associated with saline injection. * Indicates a significantly different ($P<0.05$) value between normoxic and hypoxic acclimated groups.

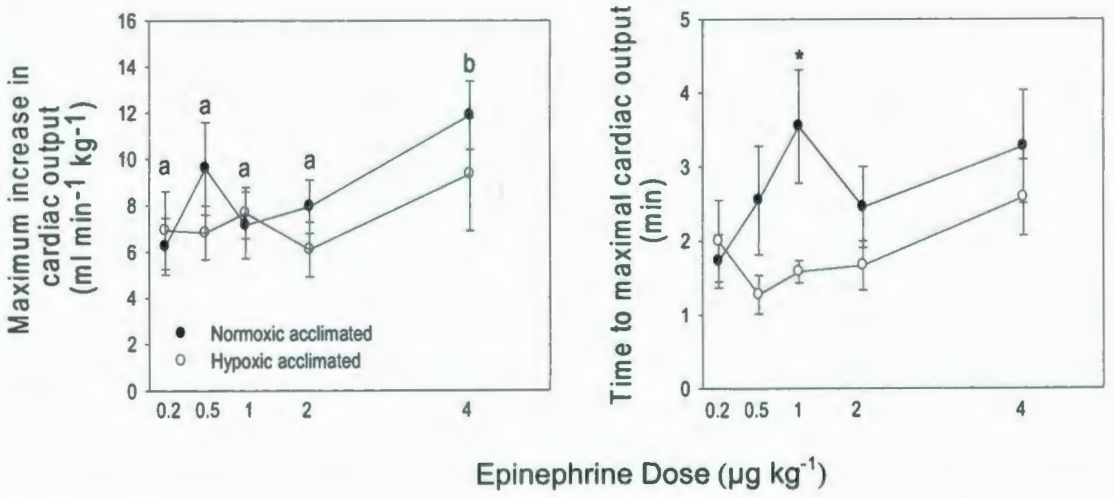

Figure 4.5. (A) Maximum increase in cardiac output and (B) time to maximum cardiac output when normoxic and hypoxic acclimated Atlantic cod were injected with increasing doses of epinephrine. Values are means ± SEM (N=11-12 for each group). *GLM repeated measures showed that chronic hypoxia significantly ($P<0.05$) decreased the time to maximum cardiac output. Dissimilar letters indicate significant difference in the normoxic group.

4.5 Discussion

4.5.1. Resting Cardiovascular Parameters

Resting f_H (42.1 ± 1.2 beats min^{-1}), S_V (0.63 ± 0.04 ml kg^{-1}), Q (26.6 ± 1.6 ml min^{-1} kg^{-1}) and P_{va} (3.5 ± 0.5 kPa) in the normoxic cod were comparable to values reported by other authors (f_H: 30.5-43.2 beats min^{-1}; S_V 0.39-0.73 ml kg^{-1}; Q: 17.3-29.1 ml min^{-1} kg^{-1}; P_{va}: 4.39-5.5 kPa; Pettersson and Nilsson, 1980; Axelsson and Nilsson, 1986; Axelsson, 1988; Fritsche and Nilsson, 1990; Webber et al., 1998; Petersen and Gamperl, unpubl., Chapter 2). However, f_H in cod in the present study was higher than previously recorded in this population of Newfoundland cod (Petersen and Gamperl, unpubl.; Chapters 2 and 5). As the extent of surgery and the period of recovery can affect f_H in cod (Axelsson, 1988; Webber et al., 1998), it is possible that the implantation of an afferent branchial artery cannula, in addition to a Transonic® flow probe, could have been the cause of the higher heart rate in the present study. However, this does not seem a likely explanation as cod fitted with both a Transonic® flow probe and an afferent branchial artery cannula in another study (Petersen and Gamperl, unpubl.; Chapter 5) had a resting f_H of 32.6 ± 1.3 beats min^{-1}. It is therefore more plausible that the higher heart rate in the present study was because the cod were recovered in black-boxes, contrary to my other studies where fish were recovered in a swim tunnel at 0.25 body lengths s^{-1} (Petersen and Gamperl, unpublished; Chapters 2 and 5). In the swim tunnel, the cod were able to move more freely and to orientate themselves against a constant water current, and it has been shown that trout in black-box confinement have elevated cortisol and catecholamine levels (Gamperl et al, 1994c); an indication of stress.

Similar to the findings of Petersen and Gamperl (unpubl., Chapters 2, and 5), resting f_H was significantly higher in the hypoxic-acclimated group as compared with normoxic controls (Table 4.1). This higher f_H, however, was not associated with significantly lower values for S_V and Q. While the former result is in agreement with our other studies showing that cod chronically exposed to moderate hypoxia have a higher f_H, it is likely that stress associated with black-box confinement masked the reduced levels of Q and S_V that are normally concommitant with hypoxic acclimation (Petersen and Gamperl, submitted, unpubl; Chapters 2, 3 and 5). Resting ventral aortic blood pressure (P_{va}) during normoxia does not appear to be affected by hypoxic-acclimation as there was no significant difference in resting P_{va} between normoxic and hypoxic-acclimated cod (3.5 ± 0.5 vs 3.9 ± 0.2 kPa, present study; 4.4 ± 0.14 vs 4.6 ± 0.2 kPa, Petersen and Gamperl, unpubl., Chapter 5). Thus, our results suggest that: 1) hypoxic acclimation does not result in a significant hypoxic vasoconstriction as has been observed with acute hypoxic exposure (water oxygen PO_2 4.0-5.3 kPa; Fritsche and Nilsson, 1990); or 2) any effects of hypoxic-acclimation on blood pressure are short-lived once the cod is returned to normoxic water.

4.5.2. Importance of Catecholamines to Normoxic Heart Function

Mendonca and Gamperl (2009) showed that the injection of 0.4 μg kg^{-1} of epinephrine and 0.2 μg kg^{-1} of norepineprhine into 8°C acclimated winter flounder (*Pleuronectes americanus*) increased epinephrine levels from 5 to 11.5 nM and norepinephrine levels from 5 to 13.7 nM. Further, Gamperl et al. (1994c) measured an increase in epinephrine levels from 5 to 107 ± 26 nM in rainbow trout (*Oncorhynchus*

mykiss) at 3.3-5.8 °C following the injection of 4.0 μg kg^{-1} epinephrine. Assuming a 1:1 relationship between injected dose and realised plasma catecholamine levels, and that catecholamine clearance varies little between the 3 species (e.g. see below), I estimate that post-injection epinephrine values in this study averaged 10 to 100 nM over the range of administered doses (0.2 to 4.0 μg kg^{-1}). The estimated maximum circulating concentration was therefore approx. ½ of that measured in both cod and trout after a chase to exhaustion (Costa and Gamperl, unpubl; Milligan and Wood, 1987; Tang and Boutilier, 1988; VanDijk and Wood, 1988), but approx. 1/3-1/5 of that measured during a severe hypoxic stress (Petersen and Gamperl, unpubl.; Chapter 5).

Gamperl et al. (1994b) recorded increases in S_V and Q of 35 and 30%, respectively, following the injection of only 0.2 μg kg^{-1} epinephrine into rainbow trout, and maximum increases (i.e. following the injection of 2.0 μg kg^{-1}) of approx. 60%. In contrast, when given the same doses of epinephrine, the cod in my study only showed increases in these parameters of 5-10 % and 5-20%, respectively (Figures 4.3 and 4.4). These results show that the cod heart has a diminished sensitivity to adrenergic stimulation as compared with salmonids, and correspond well with previous results obtained using the *in situ* cod heart or isolated atrial preparations. For instance, Gamperl and Genge (unpubl.) report no observable differences in resting or maximum f_H, Q, or S_V in cod hearts treated with 7 nM adrenaline vs. hearts perfused with adrenaline-free saline. The addition of 200 nM epinephrine only resulted in minor enhancements in maximum or resting cardiac parameters in *in situ* cod hearts exposed to temperatures from 10 to 0°C (Lurman et al., unpubl.). Finally, Axelsson (1988) found that epinephrine only has positive inotropic effects on the cod atrium at concentrations seen during severe stress or

exhaustive exercise (> 100 nM: Axelsson and Nilsson, 1986). However, the cod does not appear to be unique in exhibiting a diminished capacity to elevate cardiac performance in response to increases in circulating catecholamines. For example, Mendonca and Gamperl (2009) showed that the winter flounder heart is not dependent upon adrenergic stimulation at rest, and only report increases of 6% in S_V and 10% in Q following the simultaneous injection of 0.4 and 0.2 μg kg^{-1} of epinephrine and norepinephrine, respectively (these doses of epinephrine resulting in circulating catecholamine levels similar to those measured following an exhaustive chase).

While this study did not attempt to elucidate the mechanisms mediating the diminished sensitivity of cod heart function to adrenergic stimulation (epinephrine injection), there are several potential explanations. First of all, the muted response could be due to a higher clearance rate for catecholamines in cod as compared with salmonids, or a difference related to the injection sites used between Gamperl et al. (1994b; dorsal aorta) and the present study (afferent branchial artery). However, neither of these explanations seems likely. This is because >80-90% of epinephrine is cleared by 20 min. post-injection in the rainbow trout (Nekvasil and Olson, 1986a; Gamperl and Boutiler, 1994), a value very similar to the 16 min. reported by Ungell and Nilsson (1979) after cod were injected with labelled epinephrine via the afferent branchial artery of the 3rd gill arch. Further, the fish gill has a greater capacity to metabolise norepinephrine than epinephrine (Nekvasil and Olson, 1986b).

Second, while the results of Gamperl et al. (1998) make it highly unlikely that the lower adrenergic sensitivity of the cod heart is due to the fact that this species' heart is comprised entirely of spongy myocardium (these authors showing that β-adrenoreceptor

density is 14% higher in the spongy vs. compact myocardium of trout), recent findings suggest that β-receptor sub-type/functionality may be responsible for the observed muted response of the cod heart to adrenergic stimulation. For example, Mendonca and Gamperl (2009) showed that although cardiac β-adrenoreceptor density in the flounder heart (B_{max}, 252.8 fmol mg protein^{-1}) was the highest ever reported for a teleost species, myocardial β-adrenoreceptor affinity (K_d, 1.02 nM) was very low (Mendonca and Gamperl, 2009) compared to other teleosts (e.g., K_d between 0.13-0.25 nM for the rainbow trout: Gamperl, et al. 1994a; Olsson, et al. 2000; Hanson, et al. 2005). Recent molecular and pharmacological studies on the fish heart (Nikinmaa, 2003; Nickerson et al., 2003; Imbrogno et al., 2006) have revealed the presence of β_3-adrenoreceptors, and in mammals these receptors are activated at higher catecholamines concentrations than β_1/β_2-adrenoreceptors (Gauthier et al., 2007). Finally, the stimulation of β_3-ARs occurs via a Gi/o-NO-cGMP-PKG signal transduction pathway (Imbrogno et al., 2006; Angelone et al., 2008), and their stimulation has negative, not positive, inotropic effects (Post et al., 1999; Santos and Spadari-Bratfisch, 2006; Angelone et al., 2008). Thus, it is possible that a significant population of β_3-adrenoreceptors exists in the cod heart, and that they play a "protective role" by preventing excessive β_1/β_2-stimulation of the myocardium (Gauthier et al., 2007; Angelone et al., 2008). However, this hypothesis requires experimental validation, as does the involvement of these receptors in the ability of adrenergic stimulation to counteract the negative chronotropic and inotropic effects of hypoxia, hyperkalemia and acidosis on fish cardiac function (see Hanson et al., 2006; Hanson and Farrell, 2007).

Third, it is possible that myocardial β-adrenoceptors in the cod are functionally uncoupled from adenylate cyclase (e.g. see Hausdorff et al., 1990). β-adrenergic receptors act through the G-proteins: Gs and Gi, which stimulate and inhibit adenylyl cyclase (AC), respectively, and thus increase or decrease the level of cyclic adenosine monophosphate (cAMP) in the cytoplasm. A major function of the cAMP is to activate cAMP-dependent kinases (PKA), which in turn phosphorylate a variety of target proteins involved in cellular responses (Vornanen, 1997, 1998; Chakraborti et al. 2000). Thus, any change in the activity of G-proteins or adenylate cyclase will reduce the response to epinephrine. For instance, adaptation of rats to intermittent high-altitude hypoxia (PO_2 8.7 kPa) resulted in increased localization of Gsα-L in the cytosolic fraction, and this was associated with reduced bioactivity of membrane-bound Gsα protein and lower AC activity (Hrbasova et al., 2003). Chronic hypoxia in rats (5 days-4 weeks) has been shown to decrease the activity of AC by impairing Gs function and increasing membrane activity of Gi (Mardon et al., 1988; Pei et al., 2000; Leon-Valarde et al., 2001), both changes leading to decreased epinephrine sensitivity in the rat myocardium. Finally, Hrbasova and co-workers (2003) suggested that these changes may explain decreased sensitivity to β-adrenergic stimulation and may be considered as cardio-protective under conditions of acute myocardial ischemia. As the cod myocardium is functional adapted to a low PO_2 microenvironment (given that it has no coronary circulation) it is also possible that the diminished sensitivity of the cod heart to epinephrine may be the result of reduced activities of AC and Gs and/or increased Gi activity as compared with other teleost species.

Fourth, one of the effects of β-adrenoreceptor stimulation, is the phosphorylation of sarcolemma (SL) L-type Ca^{2+} channels *via* cyclic AMP and protein kinase A pathways (Tibbits et al., 1992). This phosphorylation increases the open probability of these channels (Bers, 1991), allowing for greater trans-sarcolemmal Ca^{2+} influx with each depolarization and producing, in part, the positive inotropic effect of catecholamines. Interestingly, however, catecholamine induced SL Ca^{2+} influx varies between species, and may be somewhat independent of Ca^{2+} channel density. For example, Vornanen (1998) showed that isoproterenol increased basal Ca^{2+} current (Ica) by approximately 2.3-fold in trout myocytes but only 1.4-fold in crucian carp (*Carassius carassius*) cardiac cells, despite the fact that there is a higher density of myocardial Ca^{2+} channels in the latter species. Based on these results, Vornanen (1998) suggested that the phosphorylation of sarcolemmal Ca^{2+} channels is regulated differently in these two species, and that adenylate cyclase in crucian carp cardiac cells is under tonic activation due to a low proportion of Gi-proteins relative to Gs-proteins. As the cod heart, like the crucian carp heart (Satchell, 1991; Vornanen and Tuomennoro, 1999), is comprised solely of spongy myocardium, it is possible that elevated basal Ca^{2+} current, caused by a increased tonic activation, may be sufficient to sustain cardiac performance in cod hearts, thus largely precluding the need for adrenergic stimulation.

Finally, changes in cod cardiac function are much more dependent on alterations in cholinergic than adrenergic tonus (Axelsson, 1988; Altimiras et al., 1997), and several authors (Laurent et al., 1983; Axelsson and Nilsson, 1986; Fritsche and Nilsson, 1990; Altimiras et al., 1997) suggest that the teleost heart is also controlled by a non-adrenergic non-cholinergic (NANC) tonus which could be more important in the cod heart than in

other teleosts. For instance, although NO generally results in negative chronotropy and inotropy, NO has also been identified as an important NANC regulator of cardiac performance in teleosts (Imbrogno et al., 2001; Tota et al., 2005) and mammals (Prendergast et al., 1997; Maisson et al., 2003). Thus, this research also raises the possibility that the cod heart has a diminished adrenergic sensitivity to catecholamines because other systems play a predominant role in controlling cardiac function in this species.

4.5.3. Decreased Adrenergic Responsiveness Following Hypoxic Acclimation

Following the injection of 2.0 and 4.0 $\mu g\ kg^{-1}$ of epinephrine, the percentage increases in S_V and Q were significantly lower (by 10-25 and 20-35%, respectively) in hypoxic- as compared to normoxic-acclimated fish (Figures 4.2 and 4.4). These results clearly demonstrate that hypoxic-acclimation diminishes the adrenergic sensitivity of the cod heart; an important and novel finding. The reduced cardiac adrenergic responsiveness of hypoxic-acclimated cod could be due to changes in the density of β-adrenoreceptors, in the proportion of β_3 vs. β_1/β_2 – adrenoreceptors, in β-adrenoreceptor coupling to adenylate cyclase, in the adrenergic signal transduction system, or in myocardial calcium channel density and/or regulation (see above). However, which of these potential mechanisms alone, or in combination, is associated with the reported decrease in cod cardiac adrenergic sensitivity following hypoxic-acclimation awaits further study. This is because little work has been conducted on this topic (especially in fish), and even in areas where a considerable amount of work has been done (e.g. the effects of hypoxia on myocardial β-adrenoreceptor density) the results are variable or difficult to interpret. For instance, a

reduction in β-adrenoreceptor density has been reported following 2 hrs of hypoxia in chick embryo ventricular cells (PO_2 < 0.2 kPa; March and Sweeney, 1989) and neonatal rat myocytes (PO_2 ~8 kPa; Rocher-Singh et al., 1991), whereas trout myocardial β-adrenoreceptor density is unaffected by 6h of hypoxia (P_wO_2 6 kPa) (Gamperl et al., 1998). While some studies have shown that decreases in myocardial β-adrenoreceptor density in mammals are dependent on the duration of hypoxic exposure (5 days to three weeks: Voelkel et al., 1981; Bernstein et al., 1990, 1992; Mardon et al., 1998; Leon-Valarde et al., 2001), others (Kacimi et al, 1992) show that even three weeks of hypoxia only causes a mild reduction in myocardial β-adrenoreceptor density. Despite lower receptor numbers after exposure to 19 days of chronic hypoxia, the contractile response of isolated ventricular strips to epinephrine was unaltered in broiler chick fetuses (Altimiras and Lindgren, 2007). Finally, the spongy myocardium of the trout heart, which is continuously perfused with hypoxic (venous) blood, has a 14% greater β-adrenoreceptor density than the compact myocardium, despite β-adrenoreceptor binding affinity (K_d) being the same in both tissues. Further, it appears that whether β-adrenoreceptor downregulation is associated with hypoxic exposure depends, at least in part, on whether there is a concomitant increase in circulating catecholamine levels. For example, moderate hypoxia (water PO_2 ~ 7–9 kPa) only causes a decrease in trout erythrocyte cell-surface β-adrenoreceptor density when accompanied by significant elevations in plasma epinephrine levels (e.g. compare Thomas et al., 1991 vs. Reid and Perry, 1995). Thus, based on these latter results, I would predict that myocardial β-adrenoreceptor levels were not affected if plasma catecholamine concentrations remained low in my cod when acclimated to a P_wO_2 of 8 kPa. However, a definitive conclusion as to whether chronic

hypoxia directly or indirectly (i.e. through increases in plasma catecholamines) affects myocardial β-adrenergic density in the cod heart will require further research.

4.5.4. *In Vivo* vs *In Situ* Studies

To this point I have assumed that the diminished response of cod cardiac function to epinephrine injection and the loss of cardiac adrenergic sensitivity following acclimation to chronic hypoxia were due to factors associated with the myocardium. This is largely because of *in situ* and *in vitro* studies have also reported diminished cardiac adrenergic sensitivity in this species (Axelsson, 1988; Lurman et al., unpubl.; Gamperl and Genge, unpubl.). However, it is also possible that alterations in control of the venous circulation played a role in the observed responses. This is because: 1) venous filling pressure is one of the most important factors controlling filling, and consequently S_V of the teleostean heart; 2) active increases in venous tone are responsible for the mobilization of blood to the central venous compartment during mild hypoxia, and this increases cardiac preload and hence S_V (Sandblom and Axelsson, 2005); and 3) although pharmacological pre-treatment with the α-adrenoreceptor blocker prazosin did not conclusively reveal the underlying mechanisms elevating venous pressure during hypoxia (Sandblom and Axelsson, 2005), the α-adrenergic system normally plays an important role in the control of venous function in trout during rest and exercise (Zhang et al.1998; Sandblom et al., 2006).

4.5.5. Perspectives and Future Research

These *in vivo* experiments confirm previous studies using *in situ* cod hearts showing that this species' cardiac function is not very sensitive to adrenergic stimulation/regulation, and demonstrate for the first time that chronic acclimation to moderate hypoxia (40% O_2 saturation, P_wO_2 8-9 kPa) results in a further reduction in the capacity of the cod heart to respond to increases in circulating catecholamines. The general lack of sensitivity of cod cardiac function to epinephrine is consistent with what has recently been reported for the flounder (Mendonca and Gamperl, 2009), and when combined, these findings demonstrate that circulating catecholamines do not play a predominant role in stimulating cardiac function in all fish species. The reduced adrenergic responsiveness of cardiac function in hypoxic-acclimated cod is a particularly novel finding that suggests that the heart's β-adrenergic system has been downregulated (i.e. through reductions in cell-surface β-adrenoreceptor density, diminished β-adrenoreceptor-adenylate cyclase coupling, reduced amplification through the signal transduction system, etc.). However, it is also feasible that β_3 adrenorerceptors, which have been identified in the eel heart (Imbrogno et al., 2006) and have been hypothesized to comprise a significant proportion of β-receptors in the flounder heart based on agonist binding affinity (K_d) (Mendonca and Gamperl, 2009), are upregulated to prevent overstimulation of the myocardium or that myocardial calcium channel density or current were affected. Further, I cannot rule out the possibility that hypoxic mediated alterations in the control of venous tone contributed to the diminished response of cardiac function to epinephrine following hypoxic acclimation.

Clearly, these experiments have provoked a number of important mechanistic questions, whose answers will provide novel insights into inter-specific plasticity in the adrenergic control of fish cardiac function, and the effects that chronic hypoxia has on the capacity of fish to make circulatory adjustments after prolonged exposure to reduced water oxygen levels.

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Chapter 5

Cardiac Performance and Haematological Responses of Normoxic- and Hypoxic-Acclimated Atlantic Cod (*Gadus morhua*) when Exposed to Graded Hypoxia.

5.1. Abstract

Previous experiments on Atlantic cod (*Gadus morhua*) showed that hypoxia-acclimated (6-12 weeks at 10 °C; P_wO_2 ~8-9 kPa) fish had significantly reduced cardiac function, but could consume more oxygen for a given cardiac output (Q) as compared with their normoxic-acclimated conspecifics. However, what was not known from these studies is: 1) whether chronic exposure to hypoxia improves the hypoxia tolerance of cod; and 2) what physiological adaptations allowed hypoxia-acclimated cod to elevate oxygen consumption (MO_2) to the same extent as normoxic-acclimated fish despite reduced cardiac function. Thus, I exposed normoxic- and hypoxic-acclimated cod to a graded hypoxic challenge until loss of equilibrium while recording several cardiorespiratory variables [oxygen consumption (MO_2), ventilatory rate, cardiac function, (Q, f_H, Sv), ventral aortic blood pressure, venous PO_2 and C_vO_2, plasma catecholamines and blood haemoglobin ([Hb]) and hematocrit (Hct) levels]. In addition, I performed *in vitro* haemooglobin-oxygen binding curves to examine whether hypoxic-acclimation influences haemoglobin functional properties.

Several physiological adjustments occurred during the 6-12 weeks of hypoxic acclimation: increased f_H; decreased S_v; elevated Hct (by 11 %) and [Hb] (by 14 %); enhanced tissue oxygen extraction efficiency (by 15% at both P_wO_2 21 kPa and 5.3 kPa); a more robust stress response as seen by 2-8 fold higher levels of catecholamines at P_wO_2s of 5.3 and 2.7 kPa. In contrast, chronic exposure had no significant effect on the affinity of haemoglobin for oxygen or haemoglobin-oxygen carrying capacity. Further, while the cod's P_{crit} was improved slightly by hypoxic-acclimation (6.6 ± 0.6 vs. 8.1 ± 0.5

kPa), there was no effect on the cod's hypoxia tolerance [PO_2 at which they lost equilibrium (H_{crit}) was 4.3 ± 0.2 and 4.8 ± 0.3 kPa in normoxic- and hypoxic-acclimated fish, respectively]. This study: 1) raises several questions regarding the mechanisms that mediate improved tissue oxygen extraction following hypoxic-acclimation and that determine the hypoxic level that is lethal for cod; and 2) provides additional evidence that this species may have difficulty recolonizing areas within its' historical distribution that are now chronically hypoxic.

5.2. Introduction

Oxygen is a key element in the metabolic processes of fish, and thus, fishes employ a number of strategies to survive if environmental oxygen is low (i.e. the water is hypoxic). The response of fish to hypoxic environments includes complex behavioural changes such as decreased locomotion (Dalla Via et al., 1998; Chabot and Dutil, 1999; Herbert and Steffensen, 2005) or movement away from/avoidance of areas of low dissolved oxygen (Pihl et al., 1991; Clarieaux et al., 1995). With regards to physiological responses, adjustments to the oxygen transport system are key if fish are to remain in the hypoxic environment, and can include modifications to: 1) ventilation (Kerstens et al., 1979; Burleson et al., 2002; Timmerman and Chapman, 2004a); 2) gas exchange across the respiratory surface (Randall, 1982; Petterson, 1983; Sundin, 1995; Sundin and Nilsson, 1997; Sollid and Nilsson, 2006); 3) cardiovascular function (Burleson et al., 2002; Petersen and Gamperl, unpubl.: Chapter 2); and 4) haemoglobin-oxygen binding

and blood oxygen carrying capacity (Wood and Johansen, 1972; Greaney et al., 1980; Soivio et al., 1980).

Recent studies on Atlantic cod (*Gadus morhua*) showed that hypoxia-acclimated (P_wO_2 ~ 8 kPa for 6–12 weeks) fish had significantly lower values for resting and maximum stroke volume (S_V) and cardiac output (Q), despite elevated heart rates (f_H), when swum under normoxic and hypoxic conditions as compared with normoxic-acclimated individuals (Petersen and Gamperl, unpubl., Chapter 2). Further, the hypoxic-acclimated cod were able to consume more oxygen for a given Q than their normoxic-acclimated counterparts at rest and during exercise. This latter result allowed the hypoxic-acclimated fish to reach similar values for maximum metabolic rate and metabolic scope, despite the reduced cardiac function. These results are very interesting and provide novel information on how fish physiology adapts to prolonged exposure to reduced water oxygen levels. However, the research of Petersen and Gamperl (unpubl., Chapter 2) only examined the response of normoxic- and hypoxic-acclimated cod to water O_2 levels of $\geq$ 8 kPa (40% air saturation), and thus it is not known whether chronic exposure to reduced environmental oxygen levels improves the hypoxia tolerance of cod. For example, hypoxic-mediated bradycardia has a number of important implications for the capacity of most fish hearts to perform during periods of moderate to severe oxygen shortage (see Farrell, 2007 for a review), and based on the results of McKenzie et al. (2009) it is probable that the 'resetting' of f_H in hypoxia-acclimated cod (Petersen and Gamperl, unpubl.; Chapter 2) affects the oxygen level at which bradycardia is initiated, and/or the degree of bradycardia. Further, it is unclear what physiological adaptations might have allowed hypoxia-acclimated cod to elevate oxygen consumption to the same extent as

normoxic-acclimated individuals despite a reduced cardiac function. This is because increases in ventilation, in blood haemoglobin and haematocrit levels, and in haemoglobin-oxygen affinity do not always accompany hypoxic acclimation (e.g. see Lomholt and Johansen, 1979; Chabot and Dutil, 1999; Pichivant et al., 2003). In addition, although the release of catecholamines from the chromaffin tissue triggers a variety of physiological responses that improve cardiorespiratory function and elevate metabolism (Randall and Perry, 1992; Fabbri et al., 1998), and Montpetit and Perry (1998) report that basal and carbachol-induced catecholamine secretion are enhanced in rainbow trout exposed to prolonged hypoxia (5 days), Petersen and Gamperl (unpubl.; Chapter 4) showed that hypoxic-acclimation for 6 weeks reduced the sensitivity of the cod heart to adrenergic stimulation.

Given the limited information on how fish adapt to chronic (weeks of) hypoxia, and the presently unexplained ability of hypoxic-acclimated cod to maintain oxygen consumption in the face of reduced cardiac performance, I performed a comprehensive study of the cod's cardiorespiratory response to 6-12 weeks of chronic hypoxia (water oxygen level of 8 kPa) at 10°C. The main part of this work involved exposing both normoxic- and hypoxic-acclimated cod to a graded hypoxic challenge until loss of equilibrium, and the recording of numerous cardiorespiratory variables [oxygen consumption, ventilation, cardiac function (Q, f_H and S_V), ventral aortic blood pressure, venous PO_2 and C_vO_2, plasma catecholamines, and blood haemoglobin and haematocrit levels] at normoxia (100% air saturation; P_wO_2 21 kPa) and up to 8 different water oxygen levels. In addition, blood was collected from normoxic and hypoxic-acclimated

cod so that *in vitro* haemoblogin-oxygen binding characteristics could be determined in the two groups over a wide range of blood PO_2 levels.

5.3. Materials and Methods

5.3.1. *In Vivo* Cardiorespiratory Function and Hypoxia Tolerance

5.3.1.1. Experimental Animals

The Atlantic cod (*Gadus morhua*) used in this study (0.56 ± 0.02 kg; range = 0.37 - 0.83 kg) were transported from a sea-cage site at Hermitage Bay, Newfoundland, Canada to the Ocean Sciences Centre (OSC, Memorial University of Newfoundland, St. John`s, Newfoundland). At the OSC, they were maintained in a 20,000 litre tank supplied with aerated seawater at 10 ± 1°C for at least 6 months prior to experimentation. The fish were fed a commercial cod diet 3 times a week, and kept at a seasonally ambient photoperiod.

Prior to experimentation, 40 fish from the holding tank were acclimated at 10 ± 0.5 °C to either normoxia [water oxygen partial pressure (P_wO_2) 19.2 ± 0.2 kPa] or hypoxia (P_wO_2 8.2 ± 0.1 kPa) for at least 6 weeks in 3 m^3 tanks. Each acclimation tank held 20 fish, and oxygen levels in both tanks were controlled as previously described by Petersen and Gamperl (unpubl.; Chapter 2).

5.3.1.2. Surgical Procedures

Fish were netted and placed in aerated seawater containing tricaine methane sulfonate (MS-222, Finquel: 0.2 g L^{-1}) until ventilatory movements ceased. The fish were

then weighed and measured before being transferred to a surgery table where oxygenated seawater containing MS-222 (0.05 g L^{-1}) continuously irrigated their gills. *In vivo* cardiac function was determined by placing a 2S or 2.5S Transonic flow probe® around the ventral aorta of the cod as previously described by Gollock et al. (2006). After the flow probe had been carefully placed around the vessel, it was connected to a flow meter (Model TS420, Transonic Systems Inc., Ithaca, NY) to check the probes signal strength and the blood flow profile. The cable of the flow probe was then secured to the animal with 3 skin sutures (3-0 silk thread, American Cyanamid Company, Pearl River, NY); one close to the incision, a second under the pectoral fin, and a third close to the dorsal fin. Finally, a polyethylene cannula (PE 50, Clay Adams) filled with heparinised (100 u. ml^{-1}) physiological marine teleost saline was occlusively inserted into the afferent branchial artery of the third gill arch for measurement of ventral aortic blood pressure and blood sampling (Wahlqvist and Nilsson, 1977; Sundin and Nilsson, 1992; Axelsson and Fritsche, 1994) (See Plate 2.1A,B; chap 2, pg. 38 for further detail).

Once surgery had been completed, fish from both groups (hypoxic- and normoxic-acclimated) were transferred to a 81 litre Blazka-type swim-tunnel respirometer (University of Waterloo, Biotelemetry Institute, Waterloo, ON) designed for round-bodied fish [see Deitch et al. (2006) for details] with water velocity set at ~ 0.25 body length per second (BL s^{-1}): a velocity at which the fish did not have to swim constantly, but had no trouble orienting themselves in the tunnel. All fish commenced ventilation within 5 min. after being transferred to the swim tunnel, and were allowed at least 20 hours of recovery at a P_wO_2 of 21 kPa before experiments began.

5.3.1.3. Experimental Protocol

Following 20 hrs of recovery, measurements of normoxic (P_wO_2 21 kPa) oxygen consumption (MO_2), ventilation rate, cardiac function and blood pressure were made on resting, quiescent fish. Thereafter, two blood samples were taken. An initial blood sample (~200 µl) was taken using a gas-tight Hamilton syringe for the immediate measurement of venous oxygen partial pressure (P_vO_2), venous oxygen content (C_vO_2) and haematocrit (Hct), with 50 µl of this blood sample frozen in liquid nitrogen for the measurement of blood haemoglobin concentration ([Hb]). A further 500 µl of blood was then drawn from each fish, placed in an Eppendorf® tube, and spun for 1 min. at 10,000 *g* to separate the plasma and erythrocytes. Approximately 300 µl of plasma was then pipetted into a cryovial (containing 15 µl of 0.2 M EDTA and 15 µl of 0.2 M glutathione) and quickly frozen in liquid nitrogen for later analysis of resting catecholamine levels.

After all measurements had been made on normoxic individuals, P_wO_2 was reduced from 21 kPa to 13.33 kPa, and then in 1.33 kPa (10 mm Hg) intervals until a kPa of ~ 2.7 was reached or the fish lost equilibrium. The time interval between changes in water PO_2 was 50 min., with water PO_2 lowered during the first 20 min. of each interval and then held constant for the remaining 30 min. All of the measurements taken on normoxic fish, were also made at each water oxygen level, with one exception. To reduce the effects of repeated blood sampling, only two additional blood samples were taken for the measurement of plasma catecholamines. One at 5.33 kPa where cardiovascular variables were maximum, and a second one just before the fish lost equilibrium; these two samples above and below, respectively, the P_{50} of cod blood as determined by Gollock et al. (2006) (~ 4 kPa when temperature is adjusted to 10°C). To maintain the fish's blood

volume and avoid anaemia, any erythrocytes remaining from blood sampling were resuspended in marine teleost saline (Driedzic et al., 1985) and injected back into the fish. The volume re-injected equal to that of the initial blood sample. At the end of the experiment, the respirometer was quickly opened, and the fish euthanized in seawater containing 0.2 g l^{-1} MS-222. Thereafter, the flow probe and heart were removed, and the heart dissected so that ventricular mass (g), relative ventricular mass [(RVM; ventricle mass / body mass * 100)] and relative atrial mass [(RAM: atrial mass / body mass *100)] could be calculated.

5.3.1.4. Measurement of Cardiorespiratory Variables

Water temperature and oxygen concentration (mg O_2 L^{-1}) in the swim tunnel were continuously measured via an external circuit. This circuit consisted of tubing with extremely low gas permeability (Tygon Food, ser. 6-419, Cole Parmer) and contained a D201 flow cell (WTW Inc.: Weilheim, Germany) that housed a galvanic oxygen electrode (model CellOx 325, WTW Inc.), which was connected to an oxygen meter (model Oxi 340, WTW Inc.). MO_2 was measured by closing the swim tunnel for 10 min. at each water PO_2 level, and was calculated as:

$$MO_2 \text{ (mg } O_2 \text{ kg}^{-1} \text{ h}^{-1}) = [((C_i - C_f) \times V_c)/M] /(T \times 1/60)$$

Where C_i = water oxygen concentration (mg O_2 L^{-1}) at the start of MO_2 measurement; C_f = oxygen concentration (mg O_2 L^{-1}) at the end of MO_2 measurement; V_c = volume of the respirometer and external circuit (81 L); M = animal mass (kg); and T = time (min.)

required to make MO_2 measurements (10 min.). Ventilation rate (beats min^{-1}) was measured by observing the cod from underneath, and counting the number of opercular movements in a 30 – 45 sec. interval.

Cardiac output (Q) was continuously measured by connecting the flow probe to a flow meter (Model TS-420, Transonic Systems Inc., Itaca, NY) that was interfaced with a MP100A-CE data acquisition system (BIOPAC Systems Inc., Santa Barbara, CA) and a laptop running AcqKnowledge software (BIOPAC Systems Inc.). Data were recorded at a frequency of 20 Hz, and records of cardiac output were obtained during the last 5 min. of each oxygen consumption measurement. Cardiac output (ml min^{-1} kg^{-1}) was calculated in AcqKnowledge by dividing absolute blood flow (ml min^{-1}) by the body mass of the fish (kg). Heart rate (f_H, beats min^{-1}) was calculated by counting 20 systolic peaks in the blood flow trace, dividing by the time frame (sec) over which the peaks were measured, and multiplying by 60 (sec./min.). Stroke volume (S_V, ml kg^{-1}) was calculated as Q/f_H. Maximum values of Q, S_V and f_H were measured as the highest value that each individual fish achieved during graded hypoxia. Absolute scope was then calculated by subtracting normoxic values for Q, S_V and f_H from Q_{max}, S_{Vmax} and f_{Hmax}, respectively. Ventral aortic blood pressure (P_{VA}) was measured by attaching the afferent branchial artery cannula to a Gould Statham (model P23-10) pressure transducer that was interfaced with the BIOPAC MP-100 data acquisition system. Pressure was calibrated daily against a static water column.

P_vO_2 was measured by injecting ~200 µl of blood into a small thermostatted (10°C) chamber containing a Clark-type oxygen electrode (Cameron Instrument Co., Port Aransas, TX, USA), while C_vO_2 was measured on 30 µl blood samples using the

methodology of Tucker (1967) and a custom designed, thermostatted, Tucker chamber (volume=1.66 ml) maintained at 32°C. The oxygen electrodes were connected to an OM 200 oxygen meter (Cameron Instrument Co.) that was interfaced with a MP100A-CE data acquisition system and a laptop running AcqKnowledge software. Hct was determined in duplicate by centrifugation of blood in micro-haematocrit tubes at 10,000 *g* for 3-5 min., and [Hb] was measured in duplicate on 10 µl blood samples using Drabkin`s reagent and bovine haemoglobin standards (Sigma Chemical Co., St. Louis, MO, USA) and a spectrophotometer (Beckman Coulter, Mississauga, ON, USA; model DU 640) set to a wavelength of 540 nm. Mean corpuscular haemoglobin content (MCHC) (g $100ml^{-1}$) was calculated as [Hb]/Hct x 100.

In preliminary experiments, I tried fitting cod with a ventral aortic flow probe, and both afferent and efferent branchial artery cannulae. However, this resulted in an extremely long surgery time (1.0 – 1.5 h), and it proved extremely difficult to maintain the patency of efferent branchial arterial cannulae. Thus, it was decided to only fit the cod with afferent branchial cannulae. However, this meant that C_aO_2 would have to estimated, rather than directly measured, in order to calculate tissue oxygen extraction. To calculate tissue O_2 extraction at P_wO_2's 21 kPa (~ 150 mm Hg) and 5.3 kPa (~ 40 mm Hg) in both groups, I first used P_aO_2 values reported by Perry et al. (1991) for 10°C acclimated cod exposed to these P_wO_2 levels to calculate a C_aO_2 value based on the *in vitro* binding curves performed in this study (see below, Figure 5.3B). I then multiplied this C_aO_2 value by the Hb concentration for each fish measured *in vivo,* and divided this value by the average *in vitro* Hb for each group at the appropriate PO_2 (see Figure 5.3). Finally, tissue oxygen extraction for each fish was calculated in % as:

$$\frac{(C_aO_2 - C_vO_2) \times 100}{C_aO_2}$$

One of the goals of these experiments was to examine whether hypoxic acclimation alters the hypoxia tolerance of Atlantic cod. In these experiments two indices of hypoxia tolerance were calculated. Most fish are classified as oxygen regulators, because they are able to maintain a relatively constant MO_2 until water O_2 levels reach a specific point after which MO_2 will be decreasing at a faster rate, and generally in proportion to water O_2 saturation level. The point at which the rate of drop in routine MO_2 increased substantially was used to estimate the fish's critical oxygen tension (P_{crit}; Schurmann and Steffensen, 1997), and was determined for individual fish by performing separate linear regressions on the MO_2 data before and after the change in slope for the relationship between P_wO_2 and MO_2, and determining the intersection point of these two regression lines. In these experiments I also recorded the water PO_2 at which the fish lost equilibrium (H_{crit}).

5.3.1.5. Catecholamine Analyses

The plasma catecholamines epinephrine (EPI) and norepinephrine (NE) were measured using high performance liquid chromatography (HPLC, Bioanalytical Systems Inc. Lafayette, IN, USA) with electrochemical detection (+650mV) after extraction with alumina (BAS LCEC Application Note 14). Separation of the catecholamines was performed on a reverse phase column (ODS, 3.0 mm i.d. x 10 cm long, 3 μm pore size; model MF 8954), using an aqueous mobile phase (containing per litre: 7.088 g of monochloroacetic acid, 186.1 mg $Na_2EDTA{\cdot}2H_20$, 15 ml acetonitrile and 32.3 mg sodium

octyl sulphate, pH 3.00-3.05) pumped at a flow rate of 1 ml min^{-1}. EPI and NE plasma concentrations were calculated relative to NE/EPI synthetic standards (75 ng ml^{-1} NE, 75 ng ml^{-1} EPI), and with DHBA (3, 4-dihydroxybenzylamine) as an internal standard. Recovery from the alumina, determined on samples to which DHBA was added, was 77.2 ± 1.0 %, and individual recovery values were used in the determination of all EPI and NE concentrations.

5.3.2. *In Vitro* Haemoglobin-Oxygen Binding Curves

In vitro haemoglobin-oxygen binding curves were constructed for Atlantic cod blood incubated at 10°C. This was done as only binding curves for venous blood were constructed in the *in vivo* experiment, and thus, the *in vivo* Hb-O_2 binding curve only defined the lower portion of this curve.

5.3.2.1. Experimental Conditions

Prior to experimentation 40 fish from the holding tank were acclimated at 9.5 ± 0.5 °C to either normoxia [water oxygen partial pressure (P_wO_2) 19.2 ± 0.5 kPa] or hypoxia (P_wO_2 8.6 ± 0.1 kPa) for at least 6 weeks. Each acclimation tank held 20 fish each and oxygen levels in both tanks were measured daily as previously described by Petersen and Gamperl (unpubl.; Chapter 2).

Fish were anaesthetized in seawater containing MS-222 (0.1 g l^{-1}), measured and weighed, and 5 ml of blood was quickly withdrawn in heparinised (100 IU ml^{-1}) syringes via the caudal vein. Haematocrit (Hct) was then determined in duplicate by centrifugation

of blood in micro-haematocrit tubes at 12,000 rpm for 3-5 min., and the remaining blood sample adjusted to 20% haematocit using marine teleost saline (Driedzic et al., 1985). After adjusting Hct, blood samples were placed in heparinized round-bottom flasks in a 10°C shaking water bath, and initially gassed with a humidified mix of 100% air/0.2% CO_2 (blood PO_2 ~ 16 kPa) and left to equilibrate for ~ 0.5 h. After equilibration, 10 different O_2 tensions ranging from 20 – 1.3 kPa were attained by adjusting the relative percentages of N_2 and air (CO_2 remaining constant at 0.2%) using flow meters and a Wösthoff gas-mixing pump (H. Wösthoff Co., Bochum, Germany). Blood was allowed to equilibrate at each PO_2 level for approx. 30 min. prior to sampling. Blood samples were taken using gas-tight Hamilton syringes, and blood oxygen partial pressure and oxygen content were determined as described in the *in vivo* experiments. The amount of O_2 bound to haemoglobin was determined by subtracting physically dissolved oxygen according to the solubility coefficient values of Christophorides and Hedley-Whyte (1969, i.e. 0.00444 vol% mm Hg^{-1} at 10°C). Oxygen equilibration curves were determined from these data for each fish, and were used to determine the PO_2 at which haemoglobin was 50% saturated (P_{50}; an estimation of haemoglobin-O_2 binding affinity), and to obtain the cooperativity (Hill) coefficient, n_H. These values were calculated according to the Hill equation: $\text{Log } Y(1 - Y)^{-1} = n_H \text{ x log}PO_2 + K$, where Y = fractional O_2 saturation, and $K = -n_H \text{ x log } P_{50}$.

Blood from 8-9 individuals was used to generate the mean haemoglobin-oxygen binding curve for each group, and all fish were overanesthetized in 0.2 g l^{-1} MS-222 prior to the removal of the heart so that RVM and RAM could be determined. At each PO_2

level, small (50µl) blood samples were also taken and immediately frozen in liquid nitrogen for the measurement of haemoglobin concentration ([Hb]). [Hb] was subsequently measured in duplicate on 10µl blood samples as previously described. Hct was determined at the end of the experiments (as above), and mean corpuscular haemoglobin content (MCHC) (g $100ml^{-1}$) calculated as [Hb]/Hct x 100.

5.3.3. Statistical Analyses

Statistical analyses were carried out using SPSS (v.13.0; SPSS, Chicago, IL, USA). GLM repeated measures analyses were carried out to determine if chronic hypoxia and P_wO_2 significantly affected f_H, S_V, Q, P_{va}, MO_2, ventilation, Hct and [Hb], and the effects of PO_2 and chronic hypoxia on *in vitro* [Hb]. These analyses were followed by: 1) one-way ANOVAs to examine differences between groups at each P_wO_2 or PO_2: 2) one-way ANOVAs followed by Dunnett's post-hoc tests within each acclimation group to determine at what P_wO_2 (Figure 5.1) values for f_H, S_V, Q, P_{va}, MO_2 ventilation, and [Hct] and Hb were significantly different from initial (P_wO_2 21 kPa) levels. One-way ANOVA`s were carried out to evaluate the effect of chronic hypoxia on cardiac morphometrics, cardiorespiratory parameters (resting and maximum values, and scope for change), mean *in vivo* haematological parameters, and P_{crit} and H_{crit}, while one-way repeated measures ANOVAs were used to compare resting and maximum cardiorespiratory parameters within each acclimation group. Finally, as the assumption of equal variances was not fulfilled, Wilcoxson`s signed rank test and Mann Witney U tests were used to examine differences in EPI, NE and EPI:NE between and within groups. A result was considered significant when $P < 0.05$, but differences where $P < 0.10$ are also

reported. All data presented in the text, figures and tables are means ± standard error of the mean (SEM).

5.4. Results

There were no significant differences in animal mass, ventricular mass or RVM between the normoxic- and hypoxic-acclimated groups in either study (Table 5.1). For example, relative ventricular (RVM) and atrial masses (RAM) were approx. 0.90 and 0.29%, respectively, and condition factor was approx. 0.70 in the graded hypoxia experiment and 0.77 in fish used to generate the *in vitro* haemoglobin – oxygen binding curves.

5.4.1. Respiratory and Metabolic Responses, and Hypoxia Tolerance

Resting (at 21 kPa) ventilation rate was significantly higher in the chronic hypoxic group as compared with the normoxic group (45 vs. 32 min^{-1}; Figure 5.1A, Table 5.2). However, this difference was eliminated by the first level of hypoxia (13.3 kPa) (as ventilatory rate only increased significantly in the normoxic acclimated group), and similar increases in ventilatory rate (of ~ 20%) were observed in both groups as P_wO_2 was lowered to ~ 6 kPa. Thereafter, however, differences between the two groups again became apparent. The hypoxic-acclimated group was able to maintain ventilatory rate at levels above or equal to those measured under normoxia just before the loss of lost equilibrium, whereas ventilatory rate fell to 27 min^{-1} in normoxic-acclimated fish (Figure. 5.1F).

In agreement with the higher ventilation rate in hypoxic- vs. normoxic-acclimated fish, resting (P_wO_2 of 21 kPa) oxygen consumption was also significantly higher in the hypoxic group (by ~ 25%; Table 5.2). When both groups were exposed to graded hypoxia, there was a significant decrease in oxygen consumption from resting (normoxic) values by 11 kPa, and oxygen consumption continued to fall until the fish lost equilibrium (Figure 5.1B). However, there was a difference between groups in the P_wO_2 at which water oxygen tension – oxygen consumption became decidedly steeper (i.e. the point at which the fish switched from being a regulator to a conformer). This resulted in a P_{crit} value in hypoxic-acclimated fish (6.6 ± 0.6 kPa) that was significantly lower than measured in normoxic-acclimated cod (8.1 ± 0.5 kPa). Interestingly, although ventilatory rate was maintained at low P_wO_2 values and oxygen consumption was slightly higher in hypoxic-acclimated cod at all water oxygen levels, there was no difference in the P_wO_2 at which the two groups lost equilibrium (H_{crit} = 4.3 ± 0.2 vs. 4.8 ± 0.3 kPa).

Table 5.1. *Body and cardiac morphometrics for the normoxic (21 kPa) and hypoxic-acclimated (6-12 weeks at a water PO_2 of 8-9 kPa) Atlantic cod used in the* in vivo *and* in vitro *studies. Values are means ± SEM (in brackets), N = 9 - 12. RVM = Relative Ventricular Mass. RAM = Relative Atrial Mass. Condition Factor = (Mass/Length^3) x 100. Mass was determined prior to surgery.*

	Morphometrics					
	Animal Mass (kg)	**Length (cm)**	**Ventricular mass (g)**	**(RVM)**	**(RAM)**	**Condition Factor**
In Vivo Experiment						
Normoxia	0.55 (0.03)	42.62 (0.4)	0.51 (0.02)	0.09 (0.002)	0.03 (0.001)	0.71 (0.02)
Chronic hypoxia	0.57 (0.04)	43.8 (0.7)	0.51 (0.04)	0.09 (0.006)	0.03 (0.004)	0.67 (0.03)
In Vitro Experiment						
Normoxia	0.85 (0.05)	47.44 (0.8)	0.76 (0.04)	0.09 (0.002)	0.03 (0.001)	0.79 (0.02)
Chronic hypoxia	0.73 (0.07)	45.44 (1.1)	0.63 (0.05)	0.09 (0.005)	0.03 (0.002)	0.75 (0.03)

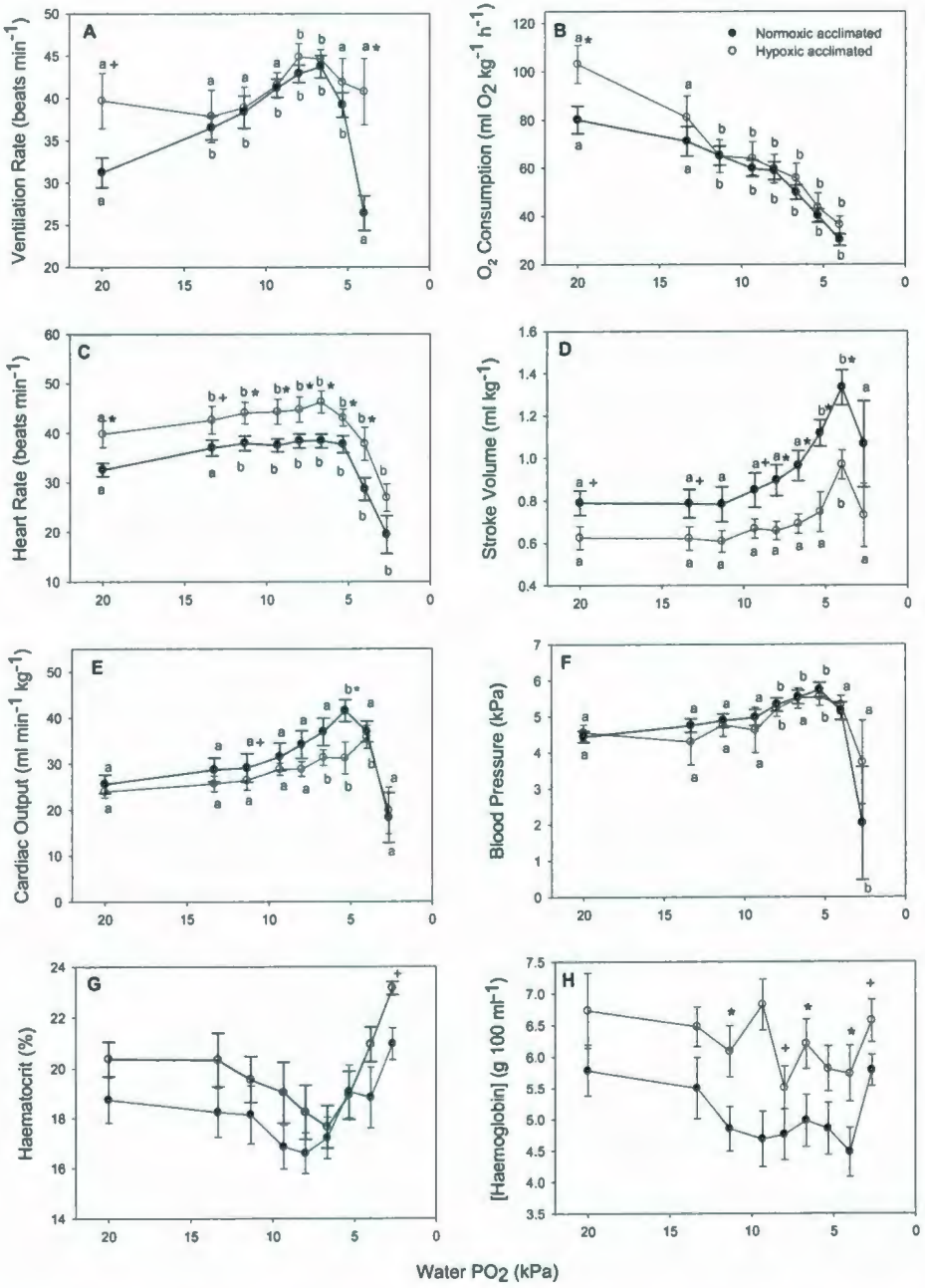

Figure 5.1. Changes in (A) ventilation rate; (B) oxygen consumption; (C) heart rate; (D) stroke volume; (E) cardiac output; (F) blood pressure; (G) haematocrit and (H) haemoglobin when normoxic- and hypoxic-acclimated (P_wO_2 8 kPa) cod were exposed to a graded hypoxic challenge until loss of equlibrium. Values are means ± SEM (N = 9-12 for each group), except for measurements at 2.7 kPa where N = 4 for both groups.. * Indicates a value significantly different ($P<0.05$) between normoxic and hypoxic groups. + Indicates values different between groups at $P< 0.10$. Dissimilar letters indicate significant differences ($P < 0.05$) from initial (normoxic) values within each acclimation group.

5.4.2. Cardiovascular Responses

Heart rate and S_V were decidedly higher (by ~ 7 beats min^{-1}) and lower (by 0.16 ml kg^{-1}), respectively, in hypoxic- vs. normoxic-acclimated cod in fully oxygenated water, and these differences were maintained as P_wO_2 was lowered towards the point of loss of equilibrium (Figures 5.1C, D; Table 5.2). However, the pattern of change in these two parameters was quite different. In both groups, heart rate increased slightly from normoxia to approx. 6 kPa (i.e. by approx. 8 beats min^{-1}), and then fell by 18 – 20 beats min^{-1}. In contrast, stroke volume increased in a curvilinear fashion after 12 kPa, and although S_V in both groups reached maximum values at ~ 4 kPa before declining, the maximum value for S_V and the scope for S_V were significantly lower in the hypoxic-acclimated fish (0.88 and 0.25 ml kg^{-1}, respectively) than measured in normoxic-acclimated individuals (1.36 and 0.57 ml kg^{-1}, respectively) (Table 5.2). Due to the opposite effects of hypoxic acclimation on f_H and S_V, there was no significant difference in cardiac output in resting (normoxic) fish, with both groups having a Q of ~ 25 ml min^{-1} kg^{-1}. During graded hypoxia, changes in cardiac output followed the same general pattern as for S_V, with Q peaking in both groups at approx. 5 kPa (Figure 5.1E; Table 5.2) and falling considerably (by approx. 40 - 45%) when P_wO_2 was lowered to 2.7 kPa. Cardiac output during graded hypoxia was generally lower in hypoxic-acclimated fish, and this difference reached statistical significance at a P_wO_2 of 5.3 kPa. Further, maximum Q for hypoxic-acclimated fish (35.6 $\pm$ 2.1 ml min^{-1} kg^{-1}) was lower (by approx. 6 ml min^{-1} kg^{-1}) than measured in normoxic-acclimated fish ($P < 0.05$). Blood pressure (Figure 5.1 F) was not significantly different between the groups at any P_wO_2. However, it did increase in

both groups by approx. 1.5 kPa before falling sharply when water PO_2 was lowered to 2.7 kPa.

5.4.3. Haematology and Tissue Oxygen Extraction

Haematocrit and blood haemoglobin concentration were 11 and 14% higher at the beginning of the experiments, respectively, in the hypoxic-acclimated cod. Although these differences were not significant at this P_wO_2 (21 kPa) due to variability in the measurements, both variables were consistently higher throughout the experiment in the hypoxic-acclimated fish (Figures 5.1G and H). Further, hypoxic acclimation had an overall effect on both Hct and [Hb] (i.e. main effects in both GLM analyses were significant at $P < 0.05$ and $P = 0.08$, respectively), and Hb was significantly ($P< 0.10$) greater in this group at several P_wO_2 values. Haematocrit fell slightly in both groups as P_wO_2 was decreased to 5 – 6 kPa. After this point, however, Hct in both groups increased sharply. Despite this increase in Hct, [Hb] changed little as P_wO_2 was lowered. This was likely because MCHC was approx. 28 g 100 ml^{-1} at the end of the experiment as compared to ~ 34 g 100 ml^{-1} at a P_wO_2 of 21 kPa (Table 5.3).

There was no difference in P_vO_2 between the groups in normoxia. However, the pattern of decrease in P_vO_2 with P_wO_2 was slightly different between hypoxic- and normoxic-acclimated fish. P_vO_2 fell in an almost linear fashion with P_wO_2 in the hypoxic-acclimated group, as compared with a sigmoidal relationship for the normoxic-acclimated fish, and this resulted in P_vO_2 being somewhat higher in the hypoxic-acclimated fish before a P_wO_2 of ~ 5.7 kPa, but slightly lower thereafter (Figure 5.2A). The *in vivo* haemoglobin-oxygen binding curves generated from the normoxic- and hypoxic-

acclimated cod also showed subtle differences between the groups (Figure 5.2B). For example, while C_vO_2 was lower for a given P_vO_2 in the hypoxic-acclimated group at higher P_vO_2 values (~ 3.5 - 6 kPa), the binding curves crossed at a P_vO_2 of approx. 3 kPa, and after this point C_vO_2 was either similar or slightly higher in the hypoxic- as compared to normoxic-acclimated individuals. Interestingly, tissue oxygen extraction was approx. 15% (~ 22 vs. 35%) higher in the hypoxic-acclimated cod at P_wO_2 levels of 21 and 5.3 kPa (this difference significant at the higher water oxygen level) (Table 5.3).

The *in vitro* haemoglobin-oxygen binding curves (Figure 5.3) generated using blood taken from normoxic- and hypoxic-acclimated cod, did not reveal any significant differences in the affinity of haemoglobin for oxygen or in haemoglobin-oxygen carrying capacity. For example there was no significant difference in the P_{50} values (5.8 $\pm$ 0.3 vs. 5.1 $\pm$ 0.4 kPa), or the Hill coefficient (nH) between the two groups (2.9 $\pm$ 0.2 vs. 2.2 $\pm$ 0.2). Further, the slightly higher blood oxygen values at all PO_2 values (i.e. the upward shift of the curve) can be explained by the slightly, or significantly, higher values for blood haemoglobin levels in the hypoxic-acclimated fish (Figure 5.4); this latter result consistent with what was found in the *in vivo* study (see Figure 5.1H).

In Figure 5.3C the *in vivo* and *in vitro* haemoglobin-oxygen binding curves are overlayed so that the data can be compared. Overall, there is good agreement between the two sets of data. However, it does appear that the *in vitro* curves are shifted slightly to the right as compared to the *in vivo* data, and thus that the P_{50} value determined *in vitro* is an overestimate of *in vivo* values (i.e. blood oxygen content *in vivo* would be higher at a given PO_2 than predicted from the *in vitro* curves).

5.4.4. Circulating Catecholamine Levels

At 21 kPa (i.e. normoxia) there was no significant difference in EPI levels between the two groups (both ~ 5 nM). In contrast, plasma NE levels in the hypoxic-acclimated fish were slightly ($P < 0.10$) higher than in the normoxic-acclimated group (approx. 4 vs. 3 nM, respectively), and this resulted in a significantly lower EPI:NE ratio in the former group (Figures 5.5D, E). Exposure to a P_wO_2 of 5.3 kPa resulted in a significant increase in both plasma EPI and NE levels in the two groups, and the levels of these two catecholamines were elevated even further when the fish were exposed to a P_wO_2 of 2.7 kPa. However, there were clear differences in the magnitude of the increases in EPI and NE, and between groups (Figures 5.5A, C). For example, plasma levels of EPI were approx. 6.5-fold greater than NE in the normoxic-acclimated group at 2.7 kPa (Figure 5.5E). Further, although the difference in catecholamine levels between the two groups was only significant for NE, both catecholamines were substantially higher in the hypoxic-acclimated fish: values for EPI and NE reaching 387 ± 66 nM and 59 ± 8 nM in normoxic-acclimated fish, but 565 ± 228 nM and 175 ± 42 nM in hypoxic-acclimated fish, at 2.7 kPa. This difference in catecholamine levels between groups at 2.7 kPa also resulted in a significantly lower EPI:NE ratio in the hypoxia-acclimated fish (Figure 5.5E).

Table 5.2. *Cardiorespiratory parameters in normoxic- (21 kPa) and hypoxic-acclimated (6-12 weeks at a water PO_2 of 8-9 kPa) cod (*Gadus morhua*) exposed to decreasing water PO_2 until loss of equilibrium. Resting values were measured at 21 kPa and maximum values were measured at the PO_2 indicated in the table.*

	Normoxic				*Chronic Hypoxic*			
	Resting	**Maximum**	**Scope**	**Water PO_2 at which max. value was measured (kPa)**	**Resting**	**Maximum**	**Scope**	**Water PO_2 at which max. value was measured (kPa)**
Ventilation Rate (beats min^{-1})	31.2 (1.8)^{a*}	44.8 (1.1)†	13.5 (1.4)	7.3 (0.4)	39.7 (3.3)^{a}	48.0 (1.3)	9.8 (2.8)	6.9 (0.7)
Oxygen Consumption (mg O_2 h^{-1} kg^{-1})	80.1 (5.7)*	-	-	-	103.2 (7.9)	-	-	-
Heart Rate (beats min^{-1})	32.6 (1.3)^{a*}	40.6 (1.3)*	8.0 (1.7)	7.8 (0.8)	39.6 (2.7)^{a}	47.6 (2.0)	7.8 (1.6)	6.1 (0.6)
Stroke Volume (ml kg^{-1})	0.79 (0.1)$^{a+}$	1.36 (0.1)*	0.57 (0.1)*	3.8 (0.1)	0.6 (0.1)^{a}	0.88 (0.1)	0.25 (0.1)	5.0 (0.9)
Cardiac Output (ml^{-1} min^{-1} kg^{-1})	25.6 (2.0)^{a}	43.3 (2.2)*	17.7 (1.3)$^{+}$	5.3 (0.3)	24.0 (1.4)^{a}	35.6 (2.1)	11.6 (2.1)	5.1 (0.6)
Blood Pressure (kPa)	4.4 (0.2)^{a}	6.0 (0.2)	1.6 (0.1)	5.6 (0.5)	4.6 (0.2)^{a}	5.9 (0.2)	1.4 (0.1)	4.9 (0.9)

Values shown are mean ± SEM (in brackets), N= 9-12 for each group. * Indicates a significant difference between groups (normoxic vs. hypoxic acclimation) within a particular test condition. ^{a} Indicates a significant difference ($P < 0.05$) between resting and maximum values within each acclimation condition. $^{+}$ Indicates a significant difference ($P < 0.10$) between groups.

Table 5.3. In vivo *haematological parameters in normoxic (21 kPa) and hypoxic-acclimated (6-12 weeks at a water PO_2 of 8-9 kPa) cod (*Gadus morhua*) exposed to decreasing water PO_2 levels until loss of equilibrium (~ 2.7 kPa; final). Values for venous blood oxygen content and tissue oxygen extraction are reported at normoxia (21 kPa; initial) and 5.3 kPa as the latter was the water PO_2 at which data for arterial oxygen partial pressure were available for cod at 10°C (Perry et al., 1991). This latter value was needed in the calculation of tissue oxygen extraction. Values shown are means ± SEM (N=9-12 for both groups). * and $^+$ Indicate a significant difference between acclimation groups at $P < 0.05$ and < 0.10.*

	Acclimation Condition	
	Normoxic	**Hypoxic**
Initial haematocrit (%)	18.7 (0.9)	20.4 (0.7)
Initial Hb (g 100 ml^{-1})	5.8 (0.4)	6.7 (0.6)
Initial MCHC (g 100 ml^{-1})	33.9 (1.2)	34.8 (1.8)
Final haematocrit (%)	21.0 (0.9)+	23.2 (0.4)
Final Hb (g 100 ml^{-1})	5.8 (0.3)	6.6 (0.3)
Final MCHC (g 100 ml^{-1})	27.2 (1.1)	28.4 (1.5)
C_vO_2 (ml O_2 100 ml blood^{-1}) at 21 kPa	3.2 (0.2)	2.7 (0.2)
C_vO_2 (ml O_2 100 ml blood^{-1}) at 5.3 kPa	0.7 (0.1)	0.9 (0.1)
Tissue Oxygen Extraction Efficiency (%) at 21 kPa	20.9 (5.5)*	35.9 (5.1)
Tissue Oxygen Extraction Efficiency (%) at 5.3 kPa	22.0 (18.8)	34.7 (11.3)

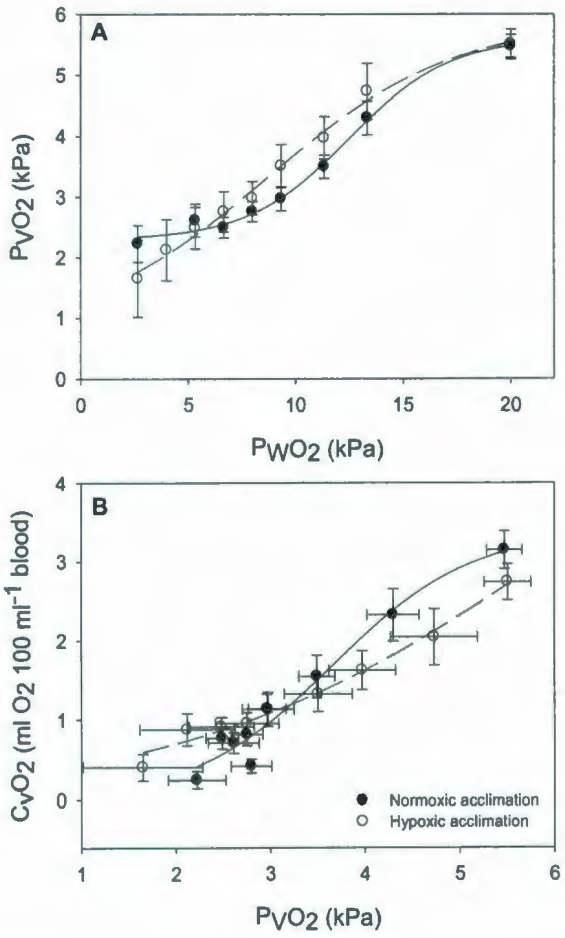

Figure 5.2. *In vivo* haemoglobin-oxygen binding curves for Atlantic cod (*Gadus morhua*) acclimated to normoxia (P_wO_2 ~ 21 kPa) and hypoxia (P_wO_2 8-9 kPa) for 6-12 weeks. Changes in water PO_2 were made every 50 min. and blood was collected via a cannula in the afferent branchial artery. Values are means ± SEM (N = 9-12 for each group). (A) Venous oxygen partial pressure (P_vO_2) vs. water PO_2 (P_wO_2): lines were fitted using a 4-parameter sigmoidal curve. (B) Venous oxygen content (C_vO_2) against venous oxygen partial pressure (P_vO_2): lines fitted to the data for each group using a 3-parameter sigmoidal function.

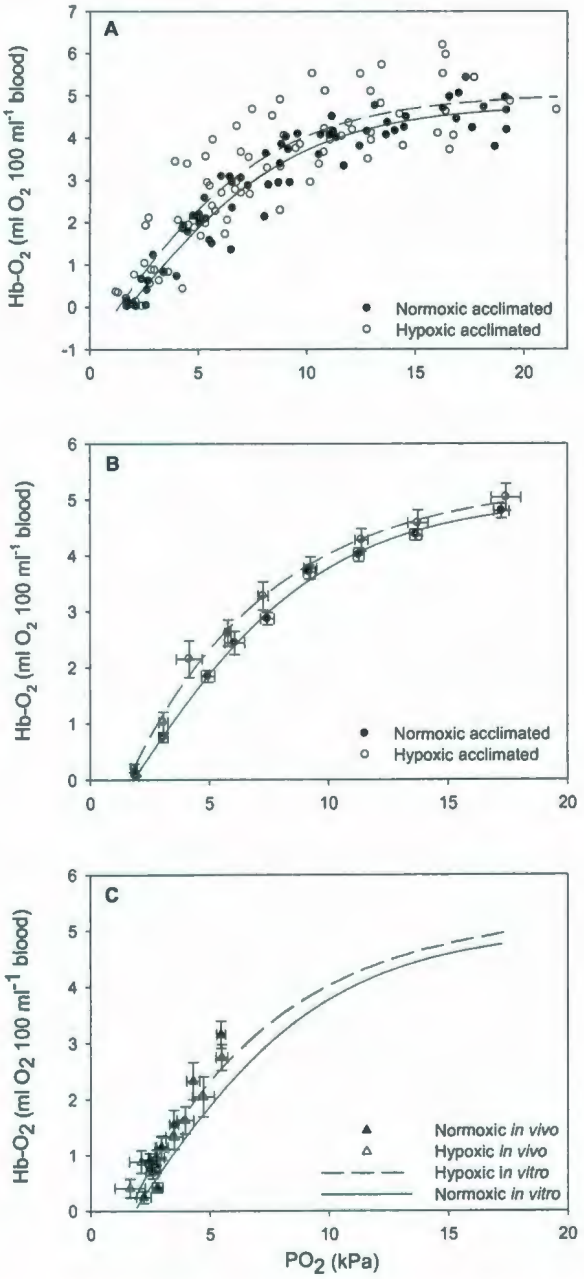

Figure 5.3. *In vitro* haemoglobin-oxygen binding curves for blood obtained from normoxic- (P_wO_2 ~ 21 kPa) and hypoxic-acclimated (P_wO_2 ~ 8-9 kPa) cod, and incubated at 10°C. Haematocrit was initially set at 20%, and changes in PO_2 were made every 30 min. (A) Data for individual fish (N = 8-9), lines fitted to the data using a 4-parameter sigmoidal function. (B) Mean data (± SEM) data for the two groups, lines fitted to the data using a 4-parameter sigmoidal function. (C) *In vitro* and *in vivo* (see Figure 5.2C) haemoglobin-oxygen binding curves for normoxic- and hypoxic acclimated cod overlayed. The *in vivo* values are means ± SEM (N= 9-12 for each group).

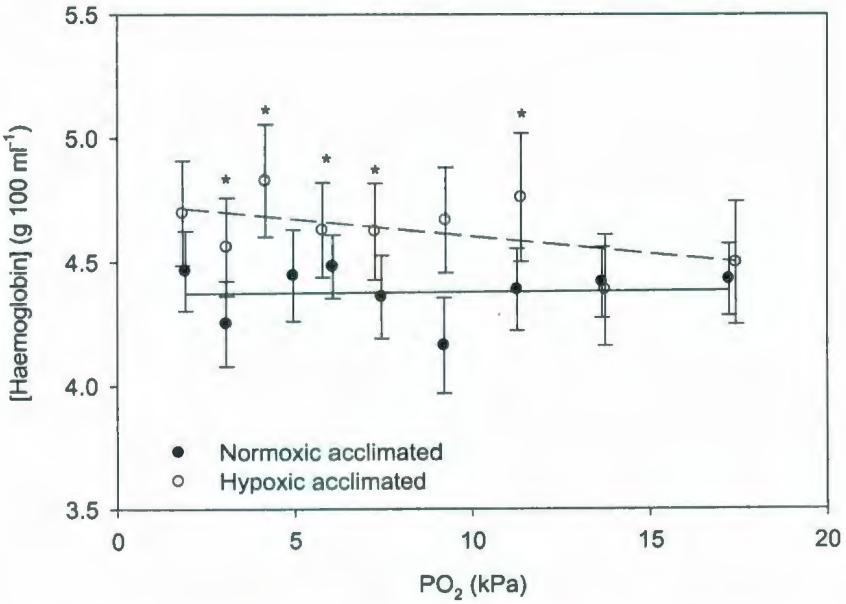

Figure 5.4. *In vitro* haemoglobin concentrations in the cod blood used to generate the haemoglobin-oxygen binding curves presented in Figure 5.3. Haematocrit was initially set at 20%, and changes in P_wO_2 were made every 30 min. Values are means ± SEM (N = 8-9 for each group). GLM repeated measures showed that haemoglobin levels were significantly higher ($P < 0.05$) in the hypoxic-acclimated group.

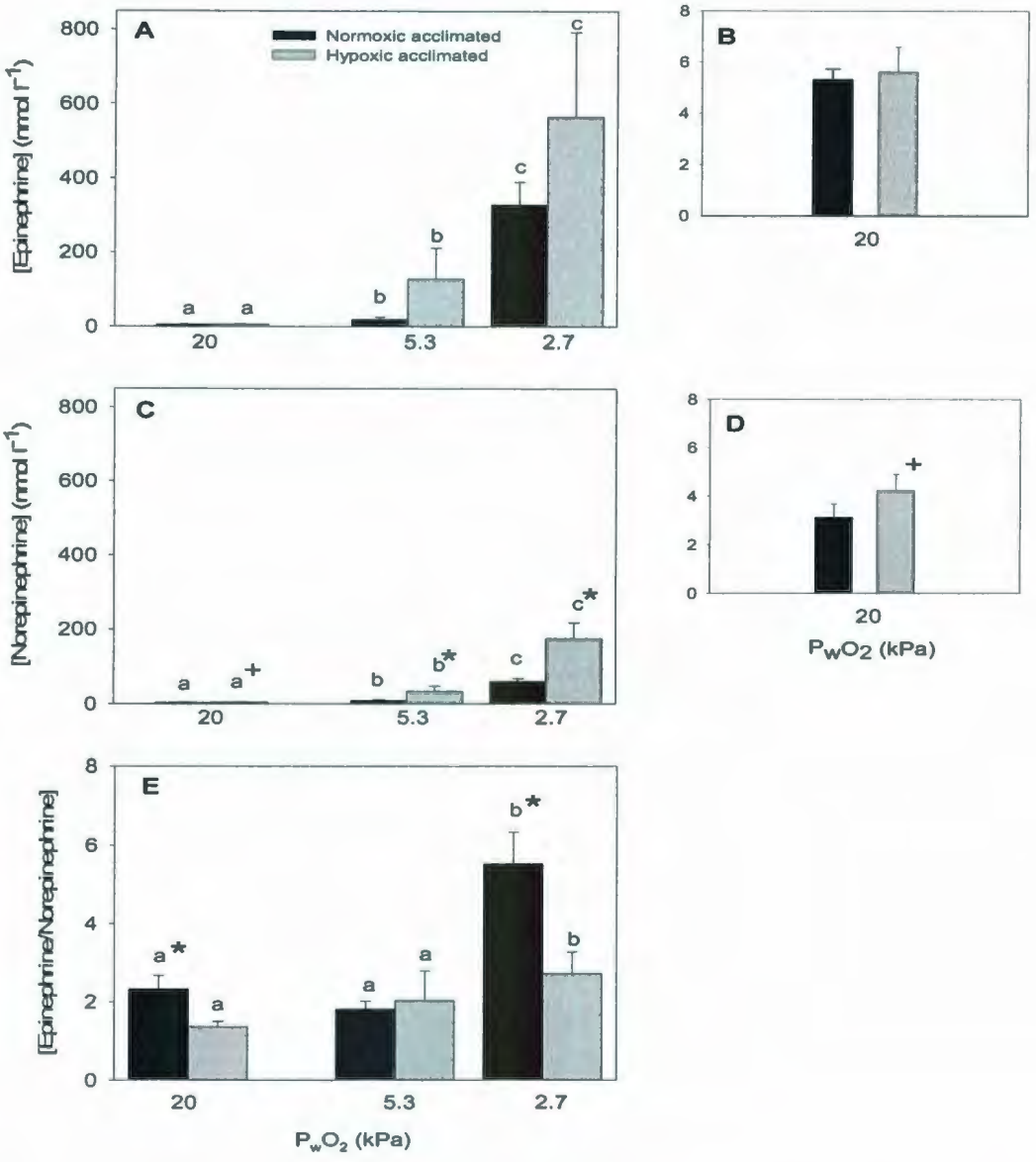

Figure 5.5. Plasma catecholamine levels in normoxic- and hypoxic-acclimated (P_wO_2 of 8-9 kPa for 6-12 weeks) cod when exposed to water PO_2 levels of 21, 5.3 and 2.7 kPa. (A) Epinephrine levels; (B) normoxic (21 kPa) epinephrine levels at an enlarged scale; (C) norepinephrine levels; (D) normoxic (21 kPa) norepinephrine levels at an enlarged scale; (E) ratio of epinephrine to norepinephrine. Values are means ± SEM (N = 6-12 for each group). *Indicates a significant difference ($P<0.05$) between acclimation groups at a particular water PO_2. $^+$ Indicates a difference ($P<0.10$) between acclimation groups at a particular water PO_2. Dissimilar letters indicate a significant ($P<0.05$) difference within each acclimation group.

5.5. Discussion

5.5.1. Cardiorespiratory Responses and Hypoxia Tolerance: Normoxic-Acclimated Fish

In the normoxic-acclimated group, ventilation increased gradually until approx. 6 kPa, and then declined steeply as water PO_2 was lowered further. This response pattern (hyperventilation followed by respiratory collapse) is similar to that observed by many other authors (e.g. Randall and Shelton, 1963; Marvin and Heath, 1968; Gehrke and Fielder, 1988; Mckenzie et al., 2009) and suggests that the ventilatory effort required to protect arterial O_2 saturation/PO_2 became too much for the normoxic cod to sustain as P_wO_2 was lowered further. Despite the increase in ventilation as P_wO_2 was reduced toward the cod's P_{crit}, routine MO_2 declined in the normoxic-acclimated cod by approx. 25%. This fall in routine MO_2 is in the range of values reported for Greenland (*Gadus ogac*: 24%; Corkum and Gamperl, 2009) and Atlantic cod (~ 16%, Schurmann and Steffensen, 1997; ~ 22%, McKenzie et al., 2009) and measured in other species at similar temperatures (~ 16% in *Gobius cobitis* Pallas; Berschick et al., 1987: ~ 40% in *Leiopotherappon unicolor*: Gehrke and Fielder, 1988). Nonetheless, it does not fit with the expectation that most fish are oxygen regulators (i.e. maintain MO_2 relatively constant until P_{crit}). It is likely that the cod in this study became quiescent (reduced their spontaneous activity) as water PO_2 was lowered, and that this led to the diminished MO_2 between normoxia and P_{crit}.

Using the point at which MO_2 began to decrease in proportion to water P_wO_2, I calculated the P_{crit} of normoxic-acclimated cod to be 8.1 kPa. This value corresponds very

well with the P_{crit} values reported for cod at 10°C (7.4 – 8 kPa) (Sundnes, 1957; Saunders, 1963; McKenzie et al. 2009) and with the P_wO_2 values that free-swimming cod begin avoidance behaviour (~ 9.0 kPa; Claireaux et al., 1995) or reduce their swimming speed significantly (8.4 kPa; Herbert and Steffensen, 2005). However, my value for P_{crit} is considerably above values obtained by Schurmann and Steffensen (1997; 4.8 kPa) using a similar protocol, and estimated by Claireaux et al. (2000) and Jordan and Steffensen (2007) using limiting oxygen concentration curves (4-5 kPa). Variability between studies is also observed when comparing O_2 levels that are lethal to cod; the value obtained in this study (H_{crit} = 4.3 $\pm$ 0.2 kPa) towards the high end of the range reported by other authors at similar temperatures (~ 2.0 – 5 kPa: Sundnes, 1957; Scholz and Waller, 1992; Schurmann and Steffensen, 1992; Plante et al., 1998; McKenzie et al., 2009). Collectively, these data suggest that hypoxia tolerance is highly dependent on the particular experimental conditions (e.g. rate of oxygen decrease, duration of exposure, closed vs. open respirometry, degree of surgical intervention) under which it is measured, and/or that it shows significant variation in Atlantic cod, even at similar temperatures. If the latter is the case, it is probable that this variability is due to seasonal, latitudinal and/or environmental effects on cod respiratory physiology (Nelson et al., 1994; Schurmann and Steffensen, 1997; Plante et al., 1998).

During graded hypoxia, heart rate increased slightly (by approx. 7 beats min^{-1}) until bradycardia was initiated at approx. 6 kPa (Figure 5.1C). This response pattern is very similar to that shown by McKenzie et al. (2009) who reported an oxygen partial pressure threshold of 6.1 kPa for the initiation of bradycardia. Further, my finding that the P_wO_2 at which bradycardia occurred was approx. 2 kPa below the cod's P_{crit} (see Figures 5.1B

and C) adds support to the prevailing theory that chemoreflexive bradycardia plays little or no role in the homeostatic regulation of oxygen uptake during hypoxia (Perry and Desforges, 2006; McKenzie et al., 2009). To my knowledge, this is the first study to directly measure Q and S_V in cod when exposed to graded hypoxia. I show that S_V begins to increase at approx. 10 kPa P_wO_2, and increases in a curvilinear fashion until approx. 4 kPa. Further, because the increase in S_V (60%) is much greater than that shown for f_H (13%), changes in Q follow a similar pattern to that observed for S_V. This pattern of change in Q with graded hypoxia is different from that displayed by many fish species, where S_V only begins to increase after bradycardia has been initiated, and Q is either maintained or falls as P_wO_2 is lowered further (see Gamperl and Driedzic, 2009). However, it is not unique, as the rainbow trout (*Oncorhynchus mykiss*) exhibits similar changes in S_V and Q with graded hypoxia (Wood and Shelton, 1980; Sandblom and Axelsson, 2005). I have a less than complete picture of what mechanisms enable fish to elevate S_V in response to aquatic hypoxia. Nonetheless, evidence has accumulated over the past decade that the active regulation of venous tone and cardiac filling are important in controlling S_V in fishes, including during hypoxia. For example, Sandblom and Axelsson (2005) showed that venous pressure and S_V increase in rainbow trout at water oxygen levels that do not elicit bradycardia. Sandblom and Axelsson (2006) showed, using venous capacitance curves, that some of the venous blood volume is actively shifted into the stressed vascular compartment by an increase in venous smooth muscle tonus during hypoxia, and that this results in an elevated mean circulatory filling pressure. In addition to venous tone, several other mechanisms may be involved in mediating the increase in S_V observed in fishes during hypoxia. These include: hypoxia-mediated

changes in gill vascular resistance, potentially leading to alterations in cardiac afterload and end-systolic volume; and local (regional) alterations in vascular tone resulting in reduced systemic vascular resistance (R_{sys}) and a decreased arterio-venous pressure gradient (Sandblom and Axelsson, 2005). Although my results cannot answer the extent to which alterations in R_{sys} contributed to the hypoxia-mediated increase in S_V in my cod, it is clear that cardiac afterload increases, not decreases during graded hypoxia. This is because, as shown for the rainbow trout (Holeton and Randall, 1967), P_{VA} increases in cod starting at a P_wO_2 of about 10 kPa and reaches a level approx. 1.6 kPa above normoxic values before falling just prior to the loss of equilibrium.

5.5.2. Cardiorespiratory Responses and Hypoxia Tolerance: Effects of Hypoxic Acclimation

In this study, hypoxic-acclimated cod had a significantly higher ventilation rate in normoxia (P_wO_2 21 kPa) as compared with normoxic-acclimated fish, and this difference was concomitant with higher oxygen consumption. The elevated ventilatory rate exhibited by hypoxic-acclimated cod was somewhat of a surprise, as although ventilatory effort has been reported to be higher in hypoxic-acclimated fish when measured under normoxic conditions, this increase in ventilation has mainly been associated with higher opercular pressure (e.g. ventilatory stroke volume) not ventilatory frequency (e.g. see Burleson et al., 2002). However, measurements of ventilatory frequency in hypoxia-acclimated fish at PO_2s approaching normoxia are rare, and the response of different species under these specific experimental conditions may be variable. The higher routine MO_2 in hypoxic-acclimated cod under normoxia is consistent with the data for ventilation

and with the findings of Petersen and Gamperl (unpubl.; Chapter 2). However, it is difficult to compare these data with those obtained in other studies due to methodological differences. For example, although Bushnell et al. (1984) reported that hypoxic acclimation did not influence normoxic MO_2 values in resting rainbow trout, these authors only acclimated their trout to hypoxia for 3 weeks, the level of hypoxia during acclimation (~ 5.6 kPa) was more severe than utilized in the present study, and the hypoxia-acclimated trout were only allowed a brief period at normoxic levels of oxygen prior to measurements of MO_2. Further, there does not appear to be a consistent pattern of how chronic hypoxia influences fish oxygen consumption when measured under normoxic conditions. Lomholt and Johansen (1979) report that carp (*Cyprinus carpio*) acclimated to a PO_2 of 4 kPa for 4 weeks had a reduced MO_2 when they were returned to high oxygen conditions. Whereas, the data of Del Toro-Silva et al. (2008) suggest that exposure to moderate (~ 13 kPa), but not severe (~ 5 kPa) hypoxia, for two weeks elevates routine MO_2 in southern flounder (*Paralichthys lethostigma*), and only at lower temperatures.

Although hypoxic-acclimated cod had significantly elevated metabolic and ventilatory rates under normoxic conditions, these differences were largely eliminated at lower PO_2 values (i.e. the difference was only approx. 5-10%, and not statistically significant; Figures 5.1 A and B). The generally similar metabolic and ventilatory rates between normoxic- and hypoxic-acclimated cod during graded hypoxia are in contrast to most other studies where these variables are elevated by 1.4 to 2 fold (Prosser et al., 1957; Lomholt and Johansen, 1979; Kerstens et al., 1979; Johnston and Bernard, 1982; Jonhston et al., 1983; Timmerman and Chapman 2004a). However, they are in agreement with

Bushnell et al. (1984) who showed that routine MO_2 was not different between hypoxic- and normoxic-acclimated rainbow trout when measured at a water PO_2 of 5.3 kPa. Despite the lack of a significant effect of hypoxic acclimation on MO_2 when measured during hypoxia, my results are consistent with Timmerman and Chapman (2004a, b) and Del Toro-Silva et al. (2008) who reported that hypoxic acclimation leads to a reduction in critical oxygen tension (P_{crit}). The fact that the cod's P_{crit} was significantly lower following hypoxic acclimation, despite no significant difference in MO_2 between the groups, suggests that hypoxic-acclimated cod are better able to balance metabolic demands with energy production when water oxygen levels become limiting. This hypothesis is directly supported by research which shows that although plasma lactate levels are initially elevated when cod are exposed to moderate to severe hypoxia, plasma and tissue lactate levels are similar to, or significantly lower than, measured in normoxic-acclimated conspecifics when hypoxia is prolonged (> 4 weeks) (Plante et al., 1998; Chabot and Dutil, 1999; Hall et al., 2009).

While the P_{crit} of hypoxic-acclimated cod was approx. 1.5 kPa lower than their normoxic-acclimated counterparts, there was no difference in the P_wO_2 at which they lost equilibrium (H_{crit}). This result indicates that hypoxic acclimation does not affect the P_wO_2 that is lethal for this species, and is consistent with the similar MO_2 values for hypoxic- and normoxic-acclimated fish during severe hypoxia, and the findings of Hall et al. (2009). These authors report that increases in the expression of genes related to glucose transport, uptake and metabolism are short-lived when cod are acclimated to hypoxia ($P_wO_2 \sim 9.0$ kPa) (i.e. hypoxia-acclimated cod do not appear to have an enhanced capacity for anaerobic metabolism). However, it is somewhat surprising given that: 1) hypoxic-

acclimated cod in this study were able to maintain ventilation rate at normoxic values (~ 41 breaths min^{-1}) at a P_wO_2 of 4.0 kPa whereas this variable fell to ~ 27 breaths min^{-1} before normoxic-acclimated fish succumbed; and 2) McKenzie et al. (2009) suggest that the inability of cod to maintain an effective ventilatory response is associated with a diminished capacity to maintain equilibrium when exposed to severe hypoxia. The discrepancy between these studies probably lies in the fact that the study of McKenzie et al. (2009) compared the cardiorespiratory responses of vagotomised and sham-operated cod to graded hypoxia. Adrenergic tone on the cod heart is predominantly of nervous origin (Axelsson and Nilsson, 1986; Axelsson, 1988), and it is likely that the lack of appropriate adrenergic-mediated support of cardiac function, not the diminished hyperventilatory response to severe hypoxia, caused vagotomised fish to be less tolerant of low oxygen conditions. This hypothesis is supported by the fact that vagotomised cod showed a much greater tendency to 'lose their ECG signal'. What factor(s) allowed ventilatory effort to be maintained in hypoxia-acclimated cod at P_wO_2 values close to their H_{crit} is not obvious as both groups appeared to be in a similar overall metabolic state. However, Q was higher in normoxic-acclimated cod just prior to the loss of equilibrium, and thus our results are consistent with the suggestion put forward by McKenzie et al. (2009) that the inability to maintain gill ventilation occurs when elevations in cardiac work compromise the allocation of limited oxygen resources to the ventilatory muscles. Clearly, more studies are needed in this area, and of the potential involvement of cardio-respiratory interactions in modulating ventilatory function below the cod's P_{crit}.

In this study, Q was not different between normoxic- and hypoxic-acclimated fish when measured at 21 kPa P_wO_2. While this is in contrast to the slightly reduced Q

reported for hypoxic-acclimated cod in Chapter 2 under normoxic conditions (Petersen and Gamperl, unpubl; Chapter 2), the reduced normoxic values for S_V and elevated $f_{H,}$ and reduced maximum Q as the fish were made progressively hypoxic (Figure 5.1; Table 5.2), are very much in agreement with the findings of Petersen and Gamperl (unpubl.; Chapter 2) and Burleson et al. (2002). While the mechanism(s) responsible for the reduced pumping capacity (S_V) of hearts from hypoxic-acclimated cod have yet to be fully resolved (see Petersen and Gamperl, Chapter 2, 3; Gamperl and Driedzic, 2009), changes in adrenergic (increase) or cholinergic nervous tone (decrease) are the most likely explanation for the compensatory increase in f_H in hypoxic-acclimated fish *in vivo*. This conclusion is based on two pieces of evidence. First, *in situ* heart rate in hypoxic-acclimated cod was comparable to, or slightly lower than, measured for normoxic-acclimated individuals (Petersen and Gamperl, unpubl; Chapter 3); a finding which indicates that the heart's intrinsic rate was not affected by hypoxic acclimation. Second, the Atlantic cod heart is minimally responsive to increases in circulating catecholamines (Axelsson, 1988; Lurman et al., unpubl; Petersen and Gamperl, unpubl; Chapter 4) and chronic hypoxia reduces the adrenergic sensitivity of the cod heart further (Petersen and Gamperl, unpubl.; Chapter 4). Despite heart rate being significantly higher in chronically hypoxic cod compared with normoxic-acclimated individuals, the response pattern to decreasing oxygen levels and the onset of bradycardia (~5 - 6 kPa) were similar in both groups (Figure 5.1). In Atlantic cod and other fishes, hypoxic bradycardia is mediated via externally orientated oxygen-sensitive chemoreceptors in the gills (Burleson and

Smatresk, 1990; Sundin et al., 2000). The similar onset of bradycardia in both groups suggests that hypoxic-acclimation does not affect the sensitivity of these chemoreceptors.

5.5.3. Haematology and Tissue Oxygen Extraction

Hct and [Hb] in normoxic-acclimated fish were 18.7 ± 0.9 % and 5.8 ± 0.4 g 100 ml^{-1} prior to hypoxic exposure, both values within the range reported by other authors (Claireaux and Dutil, 1992; Nelson et al., 1996; Plante et al., 1998; Chabot and Dutil, 1999). During graded hypoxia, there was a slight (~ 2 %) decrease in Hct until a water oxygen tension of ~7 kPa which was probably due to repeated blood sampling. However, below a P_wO_2 of ~ 5-6 kPa, Hct began to increase and this trend continued until the fish lost equilibrium. This increase in Hct began at a P_wO_2 concurrent with the release of catecholamines into the circulation (see Figure 5.5), and is consistent with the release of stored erythrocytes from the spleen (Pearson et al., 1990; Kita and Itazawa, 1989) and the erythrocyte swelling that are induced by these hormones (Borgese et al., 1987; Berenbrink and Bridges, 1994). Indeed, it appears that erythrocyte swelling contributed greatly to the increased Hct as [Hb] levels were unchanged, while MCHC decreased from 33.9 to 27.2 g 100 ml^{-1} in this group over the course of the experiment.

The P_{50} value derived from the *in vitro* haemoglobin – oxygen binding curves was 5.8 ± 0.3 kPa (~ 44 mm Hg) for normoxic-acclimated fish. This value is within the range reported by other authors for erythrocyte suspensions at similar temperatures (~ 52 mm Hg, Karpov and Novikov, 1980; ~ 30 mm Hg at 10°C, Gollock et al. 2006; ~ 13 – 52 mm Hg depending on P_{CO2}, Herbert et al. 2006). Nonetheless, it appears that the haemoglobin-oxygen binding curve was shifted to the right (i.e. P_{50} was higher) as compared to the data

collected *in vivo*. Indeed, this observation would explain why the calculated tissue O_2 extraction value in resting normoxic-acclimated fish (~ 21%) was lower than previously reported for Atlantic cod (~ 42%, Perry et al., 1991) or other teleost species such as the rainbow trout (25.8%, Eddy, 1974; 31%, Kiceniuk and Jones, 1977), common carp (52%) or tench (60%) (Eddy, 1974). In this study, I used the P_aO_2 values in Perry et al. (1991) and the *in vitro* curves presented in Figure 5.3B to estimate C_aO_2 in my cod, and to calculate tissue O_2 extraction. A shift to the right of the haemoglobin-O_2 binding curve would have led to an underestimate of C_aO_2 at both 21 and 5.3 kPa, and subsequently lower O_2 extraction efficiencies.

Hb concentration was higher (by approx. 15%) in the cod exposed to chronic hypoxia as compared with the normoxic-acclimated group (Figure 5.1H; Table 5.3), while Hct was marginally (e.g. 20.4 vs. 18.7% at 21 kPa) higher (Figure 5.1G, Table 5.3). Since MCHC was not different between the two groups (Table 5.3), this indicates that the hypoxic-acclimated cod had an enhanced oxygen carrying capacity as compared with normoxic-acclimated fish. This result contrasts with previous research on chronic hypoxia in cod (Chabot and Dutil, 1999), rainbow trout (Bushnell et al., 1984), sea bass (*Dicentrarchus labrax*) and turbot (*Psetta maxima*) (Pichavant et al., 2000, 2001). This discrepancy probably reflects the fact that blood was obtained by caudal puncture in these studies (not from cannulated fish), and any small hypoxia-induced increases in blood oxygen carrying capacity (ie. Hct and [Hb]) may have been obscured by stress effects related to this procedure. Although increases in blood oxygen carrying capacity in cod appear to be minor following acclimation to chronic hypoxia, other authors have reported large increases in Hct and/or [Hb]. For example, RBC number and [Hb] increased by 21

and 27% respectively when the sailfin molly (*Poecilia latipinna*) was exposed to water with an oxygen concentration of 1.0 mg l^{-1} for 6 weeks (Timmerman and Chapman, 2004b), and thirty-five days of hypoxic acclimation at a P_wO_2 of 3.3-2.8 kPa increased Hct by 45 % in the gulf killifish (Greaney et al., 1980). Thus, it appears that the capacity to increase oxygen carrying capacity depends on the particular species in question, and probably also on their hypoxic tolerance and life history.

Tissue oxygen extraction was higher in the chronically hypoxic cod at P_wO_2 values of 20 and 5.3 kPa (~ 35 vs. 21%). This finding fits very well with the *in vivo* hemoglobin-oxygen dissociation data at higher P_vO_2 values where, despite enhanced oxygen carrying capacity, C_vO_2 values for hypoxic-acclimated cod were consistently (by approx. 20-25%) lower. Nonetheless, some caution must be taken when interpreting the data for oxygen extraction. First, it appears that the *in vitro* hemoglobin-oxygen binding curve was shifted to the right (i.e. P_{50} was higher) as compared to the data collected *in vivo*. A shift of the *in vitro* hemoglobin-O_2 binding curve to the right would have led to an underestimate of C_aO_2 values at both 20 and 5.3 kPa, and may explain why our value for tissue O_2 extraction (21%) in normoxic-acclimated cod is on the low end of those estimated for other species (26 – 65%; Eddy, 1974; Kiceniuk and Jones, 1977; Perry et al., 1991). Second, gill remodelling (Nilsson 2007; Matey et al. 2008) could have resulted in elevated P_aO_2 (and thus C_aO_2) values in the hypoxic-acclimated cod, and thus an underestimation of the increase in O_2 extraction efficiency with hypoxic-acclimation. However, we feel the latter is unlikely. This is because P_aO_2 did not change with hypoxic acclimation in the catfish (Burleson et al., 2002) and gill remodelling following hypoxic

acclimation has only been shown for fish in the families Cyprinidae and Cyprinodontidae (Nilsson, 2007; Matey et al., 2008).

In this study, I constructed *in vitro* haemoglobin-oxygen binding curves to examine whether chronic acclimation to low water O_2 levels changed blood-oxygen binding characteristics. Hypoxic acclimation did not change the *in vitro* values for P_{50} (haemoglobin-oxygen binding affinity) or the Hill coefficient; although the P value for this latter variable was 0.095. While the lack of a difference in n_H between normoxic and hypoxic-acclimated cod is consistent with other studies (e.g. Tetens and Lykkeboe, 1981; Bushnell et al., 1984; Pichavant et al., 2003), the similar P_{50} value following hypoxic acclimation is in contrast to studies on the rainbow trout (Soivio et al. 1980; Tetens and Lykkeboe, 1981; Bushnell et al., 1984; Montpetit and Perry, 1998) and eel (*Anguilla Anguilla*, Wood and Johansen, 1972). However, this may be related to the duration of hypoxic exposure. For example, the period of chronic hypoxia was relatively brief (< 1-3 weeks) in the above studies, and Pichavant et al. (2003) report that the P_{50} of sea bass and turbot blood is unchanged by 40 days of exposure to P_wO_2 levels as low as 5 kPa. Although the *in vitro* haemoglobin-oxygen dissociation curve was largely unaffected by hypoxic-acclimation, the *in vivo* curve (Figure 5.2B) for this group was shifted to the right above P_vO_2 values where plasma catecholamine levels began to increase. In the *in vitro* experiments I did not assess the effect of hypoxic acclimation on the haemoglobin's Bohr factor (change in P_{50} per unit change in pH), and thus it is possible that an enhanced haemoglobin pH sensitivity (Bohr and/or Root effects) explains this observation and the improved oxygen extraction efficiency found in hypoxia-acclimated cod. Nonetheless, I feel this is unlikely for two reasons. First, most studies show that the Bohr factor is

unaffected by hypoxic-acclimation (Tetens and Lykkeboe, 1981; Bushnell et al., 1984; Pichavant et al., 2003), whereas Wood and Johansen (1972) report that it actually decreases. Second, although at least nine genes code for haemoglobin proteins in the Atlantic cod (Borza et al., submitted), and studies on the rainbow trout have shown that hypoxic acclimation results in significant variation in the occurrence and abundance of specific isomorphs (Tun and Houston, 1986; Marinsky et al., 1990), the Bohr factor of the most commonly identified cod haemoglobin polymorphisms (Hb-1/1 and Hb-2/2) is similar at 12°C (-0.75 vs. -0.68 from pH 7.0 – 8.0) (Brix et al., 2004).

Unfortunately, the literature also fails to provide clear insights into whether improved oxygen diffusion between the capillaries and mitochondria might have mediated the observed improvement in O_2 extraction. For example, although myoglobin facilitates intracellular oxygen diffusion (Legate et al., 1998; Wittenberg and Wittenberg, 2003) hypoxic acclimation for prolonged periods does not lead to increased myoglobin levels in the eelpout (*Zoareces viviparous*) heart (Driedzic et al., 1985) and actually decreased myoglobin mRNA levels in the cod heart and liver (Hall et al. 2009). Further, while Gallaugher et al. (2001) proposed that increased capillary density was associated with improved oxygen extraction in exercise-trained Chinook salmon (*Oncorhynchus tshawytscha*), the effect of hypoxic acclimation on muscle capillary density is variable; from no change (Johnston and Bernard, 1983), to a decrease (Johnston and Bernard, 1982) to an increase (Johnston and Bernard, 1984; Sanger et al., 1990). Blood transit time has been identified as a major limitation to tissue oxygen extraction in mammals (Saltin, 1985), and Saldivar et al. (2003) showed that improved oxygen extraction by the 'window chamber preparation' of hypoxic-acclimated Syrian hamsters was associated with

increased functional capillary density and reduced blood flow velocity. This latter data suggests that the reduced Q of hypoxic-acclimated cod, combined with the dilation/recruitment of existing capillaries, slowed tissue blood flow and that this resulted in the improved oxygen extraction efficiency exhibited by this group. This is a hypothesis that warrants experimental verification.

5.5.4. Circulating Catecholamine Levels

Resting EPI and NE levels were 5.3 ± 0.4 and 3.2 ± 0.56 nM in normoxic-acclimated cod, values comparable to those reported for cod and other teleosts at similar temperatures (Axelsson and Nilsson, 1986; Butler et al., 1989; Fritsche and Nilsson, 1990; Axelsson and Fritche, 1991; Perry et al., 1991; Gamperl et al., 1994). When normoxic-acclimated cod were exposed to a water oxygen level of 5.3 kPa, EPI and NE increased to 18.3 ± 6.4 and 8.9 ± 1.9 nM, respectively, and the EPI:NE ratio remained at approx. 2:1 (Figure 5.5). It is very difficult to compare these data with the hypoxia literature for cod, because other studies on this species where plasma catecholamines were measured only exposed fish to very short durations of hypoxia (≤ 30 min.), and even these data are extremely variable. For example, there are three measurements of plasma catecholamines in cod exposed to a P_wO_2 of 5-6 kPa at 10°C for 30 min., and EPI and NE values range from ~ 11 – 50 and ~ 8 – 110 nM, respectively (Perry et al., 1991; Kinkead et al., 1991). However, it is not surprising that the cod in this study appeared to only be mildly stressed by a P_wO_2 of 5.3 kPa. This is because the fish were exposed to a gradual decrease in P_wO_2 over several hours. Further, Herbert and Steffesen (2005) showed using a protocol very similar to mine that plasma cortisol levels do not increase in cod until a

P_wO_2 of 4.0 is reached. In contrast to the low levels of catecholamines at 5.3 kPa, those measured just before the cod lost equilibrium were 387 ± 66 nM (EPI) and 59 ± 8 nM (NE). These are the highest post-stress values reported for cod to date, with the EPI level approx. twice that reported following a prolonged net stress (EPI 220 nM; NE 55 nM: Nilsson et al., 1976) or a chase to exhaustion (EPI 188 nM; NE 48 nM: Costa and Gamperl, unpubl).

Catecholamine levels in normoxic- and hypoxic-acclimated fish were similar in normoxic water. However, both catecholamines were much (2- to 8-fold) higher at both levels of hypoxia in hypoxic-acclimated fish, and at a P_wO_2 of ~ 2.7 kPa the EPI/NE ratio was significantly lower in this group (Figure 5.5). These results are consistent with data for the rainbow trout. For example, Perry et al. (1999) and Montpetit and Perry (1998) report that plasma catecholamine levels are not elevated following 5 days of hypoxia (8 kPa) or 7 days of severe anaemia (Hct 11%) when measured under normoxic conditions. Montpetit and Perry (1998) showed that total catecholamine levels in anaemic trout were 3-fold higher when exposed to acute severe hypoxia. Finally, both authors showed that while the *in situ* secretory response of the chromaffin tissue to cholinergic stimulation was enhanced in hypoxic/hypoxaemic fish, this response was most evident for noradrenaline secretion. Perry et al. (1999) suggested that a specific modification of the cholinergic (nicotinic) receptors, or alterations in the signalling pathways linked to cholinergic receptor activation, increased the sensitivity of the trout chromaffin tissue to cholinergic stimulation following prolonged hypoxia/hypoxaemia. While a similar mechanism may be responsible for the elevated catecholamine levels measured in hypoxic-acclimated cod in this study, other possibilities do exist. Catecholamine secretion

from the chromaffin tissue of cod can be directly stimulated by localized hypoxia (Perry et al., 1991), and these authors further suggest that a 'negative feedback mechanism' is involved in the control of plasma catecholamines in this species. Thus, an increase in the sensitivity of the chromaffin cells to hypoxaemia, or a decreased sensitivity to circulating catecholamine concentrations, could explain the difference in plasma EPI and NE levels in hypoxic- vs. normoxic-acclimated cod. Alternatively, it is possible that the higher catecholamine levels measured in hypoxic-acclimated cod reflect differences in metabolic clearance.

In fishes, elevations in circulating catecholamine levels have been shown to improve blood oxygen carrying capacity (Nikinnma, 1982, 1983; Berenbrink and Bridges, 1994; Jensen et al., 1998), to have positive inotropic and chronotropic effects on the heart (Farrell, 1986; Gamperl et al., 1994; Farrell et al., 1996), and to protect the heart from the negative effects of hypoxia, acidosis and hyperkalemia (Hanson et al., 2006; Hanson and Farrell, 2007). Blood oxygen content was elevated in hypoxic-acclimated fish at low P_vO_2 values (see Figure 5.2), and this was likely related, at least in part, to the higher catecholamine levels measured in this group. This conclusion is based on data showing that trout erythrocyte ß-adrenoreceptor characteristics are not altered by chronic hypoxia (P_wO_2 8 kPa) if plasma catecholamines are only elevated slightly (Reid and Perry, 1995), the strong concentration-dependent effects of EPI and NE on cod erythrocyte proton extrusion (Berenbrink and Bridges, 1994), and that mean corpuscular haemoglobin concentration in both groups decreased from ~ 34 g 100 ml^{-1} at a P_wO_2 of 21 kPa to ~ 28 g 100 ml^{-1} at a water oxygen level of 2.7 kPa. In contrast it is apparent, based on similar patterns of change for f_H and S_V, that the elevated catecholamine levels in

hypoxic-acclimated cod were of no benefit to cardiac function during severe hypoxia. This result may not be surprising given that hypoxic acclimation diminishes the *in vivo* response of cod cardiac performance to adrenergic stimulation (Petersen and Gamperl, unpubl.; Chapter 4). However, the inability of hypoxic-acclimated cod to sustain cardiac function longer when exposed to severe hypoxia does contrast with work presented in Chapter 3. In that experiment hearts from hypoxic-acclimated cod were better able to sustain maximum *in situ* Q under severe hypoxia. The most plausible explanation for this disparity between studies is that severe hypoxia compromises the ability of cod to regulate venous tone, and thus cardiac filling. This hypothesis is based on research showing that increases in venous tone during mild hypoxia are important for elevating cardiac preload and hence S_V (e.g. Sandblom and Axelsson, 2005), and that cardiac preload was maintained at maximum levels in the *in situ* studies presented in Chapter 3.

5.5.5. Perspectives and Future Research

In this study, I investigated how cod cardiorespiratory physiology responds to acute hypoxia, and provide the first comprehensive data set on how chronic acclimation to hypoxia influences a fishes' metabolic and cardiorespiratory responses to graded hypoxia. This research revealed that while there are several physiological adjustments associated with 6-12 weeks of hypoxic acclimation (increased f_H; decreased S_V; elevated Hct and [Hb]; enhanced tissue oxygen extraction efficiency; more robust stress response), they do little to improve the cod's hypoxia tolerance. When this information is combined with previous data showing that hypoxic acclimation does not improve the cod's metabolic capacity or scope (Petersen and Gamperl, unpubl.; Chapter 2) it is apparent that

this species may have difficulty recolonizing areas within its' historical distribution that are now chronically hypoxic (e.g. Gulf of St. Lawrence, Baltic Sea). Nonetheless, there are many examples of where fish have adapted to oxygen limited habitats (e.g. see Yang et al., 1992; Timmerman and Chapman, 2004a,b), and thus one might expect that over time strong selection pressure for hypoxia tolerance may enable cod populations to return to these areas. This research has also raised several questions with important implications for fish physiology. For example, what mechanisms mediated the improved oxygen extraction following exposure to chronic hypoxia, and determine the hypoxic level that is lethal for cod (i.e. the O_2 level at which they lose equilibrium). This latter question arises because both normoxic and hypoxic-acclimated cod lost equilibrium at the same P_wO_2 even though the hypoxic-acclimated cod were able to maintain ventilation down to a P_wO_2 of 2.7 kPa. The fact that they could maintain ventilation suggests that there was not a 'general' failure of nervous control.

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Chapter 6

Summary

6.1. Summary of Findings

In this thesis, I performed four experiments to investigate how exposure to chronic hypoxia (6-12 weeks at 10 °C; P_wO_2 8-9 kPa) influenced the physiology of adult Atlantic cod (*Gadus morhua*). In the first experiment, I assessed how chronic hypoxia affected the metabolic, cardiovascular and swimming capacity of this species under both normoxic and hypoxic conditions. Exposure to acute hypoxia lowered the U_{crit} of normoxic-acclimated cod by approx. 30% (from 1.50 to 1.02 BL s^{-1}), and this reduction in swimming performance was associated with large decreases in maximum MO_2 and metabolic scope ($\geq$ 50%), and maximum f_H and Q (by 16 and 22%). While hypoxic acclimation resulted in a significant elevation in normoxic standard and routine metabolic rates as compared with normoxic-acclimated fish (by 27 and 44%, respectively), it did not influence U_{crit}, maximum MO_2 or metabolic scope at either level of water oxygenation. Although this result indicates that there was no 'overall' adaptation of the cod to the imposed hypoxic conditions, this research did reveal several interesting physiological adjustments that had been made by the hypoxic-acclimated fishes. For example, although resting and maximum values for Q were significantly diminished in hypoxic-acclimated cod because of much lower values for S_V, increased heart rate (f_H) partially compensated for the latter, and hypoxic-acclimated cod were able to consume more oxygen for a given cardiac output.

This initial study raised several questions including, was the diminished cardiac function in hypoxic-acclimated cod due to altered nervous and/or hormonal regulation, or a direct result of a reduction in the heart's pumping capacity? This question was

addressed in the next two chapters of the thesis. In Chapter 3, I investigated how hypoxic acclimation affected resting and maximum *in situ* cardiac function during oxygenated conditions, during severe hypoxia ($PO_2 \sim 0.6$ kPa), and following recovery from severe hypoxia. This research showed that although hypoxic acclimation did not influence resting (basal) *in situ* cardiac performance during oxygenated or hypoxic conditions, it caused a decrease in maximum cardiac output (Q_{max}) under oxygenated conditions (from 49.5 to 40.3 ml min^{-1} kg^{-1}; by 19%) which was due to reduced values for maximum S_V and scope for S_V. Further, it showed that while severe hypoxia reduced Q_{max} in both groups to approx. 20 ml min^{-1} kg^{-1}, the hypoxic-acclimated fish were better able to sustain this level of Q under hypoxia, and that the recovery of Q_{max} (as compared to initial values under oxygenated conditions) was significantly improved (94 vs. 83%) as compared with normoxic-acclimated individuals.

In Chapter 4, I measured the *in vivo* sensitivity of cod cardiac function to adrenergic stimulation by injecting increasing doses of epinephrine (0.2 - 4.0 μg kg^{-1}), and examined whether the heart's response to adrenergic stimulation was affected by hypoxic acclimation. This research confirmed previous *in vitro* and *in situ* work (Axelsson, 1988; Lurman et al., unpubl.; Gamperl and Genge, unpubl.) showing that the cod heart is minimally responsive to adrenergic stimulation. Further, it showed that hypoxic acclimation reduced the already low sensitivity of the cod heart to adrenergic stimulation. This latter result raises the possibility that decreased myocardial adrenergic sensitivity contributed to the diminished *in vivo* S_V reported for hypoxic-acclimated cod (Chapter 2).

The above research provided a considerable amount of novel information about how fish cardiovascular physiology is affected by long-term hypoxic acclimation. However, several questions still remained. For example: 1) does acclimation to chronic hypoxia influence the cod's hypoxia tolerance?; and 2) what mechanism(s) is/are responsible for the increased oxygen pulse (oxygen uptake for a given cardiac output) reported for hypoxic-acclimated cod? Thus, in the last chapter of my thesis I fitted normoxic- and hypoxic-acclimated cod with afferent artery cannulae and a Transonic® flow probe for measuring cardiac output, and exposed them to a graded hypoxic challenge until loss of equilibrium. Further, I performed *in vitro* haemoglobin-oxygen dissociation curves to investigate whether hypoxic acclimation influences haemoglobin-oxygen binding characteristics. This comprehensive study showed that several physiological adjustments had taken place during the 6-12 weeks of hypoxic acclimation (increased f_H; reduced S_V; elevated Hct by 11 % and [Hb] by 14 %; enhanced tissue oxygen extraction efficiency by ~ 15% at P_wO_2's 21 kPa and 5.3 kPa; and a more robust stress response as indicated by 2-8 fold higher levels of plasma catecholamines at P_wO_2's of 5.3 and 2.7 kPa). However, these adjustments were only successful in improving the cod's critical oxygen tension (P_{crit} of normoxic and hypoxic-acclimated cod 8.1 ± 0.5 vs. 6.6 ± 0.6 kPa, respectively), not the cod's hypoxia tolerance (H_{crit} = 4.3 ± 0.2 vs. 4.8 ± 0.3 kPa).

6.2. Perspectives and Future Research

The research conducted in this thesis highlights the challenge that moderate to severe chronic hypoxia poses to marine fishes, and provides several examples of how the

cod's (fish's) cardiorespiratory system responds to this ecologically-relevant environmental threat. However, it also demonstrates that our knowledge of cardiovascular control and function is far from complete, and that more detailed research is required before we can fully understand the tissue, cellular, and molecular mechanisms that allow for cardiovascular plasticity and adaptation in fishes (vertebrates), and that protect the heart from environmental insults that might normally lead to cardiac dysfunction, myocardial damage, and eventually death. In particular there are four areas where this research has identified deficiencies in our knowledge: 1) what factors/mechanisms limit maximum cardiac function in fishes following hypoxic acclimation?; 2) which nervous/hormonal mechanisms are responsible for the resetting of f_H following chronic hypoxic exposure?; 3) how can hearts from hypoxic-acclimated cod maintain cardiac function longer during severe hypoxia, and recover better following a hypoxic insult?; and 4) what are the physiological adaptations responsible for the improved oxygen extraction efficiency observed in hypoxic-acclimated cod?

In chapter 3, several mechanisms were proposed which could explain both why the hearts of hypoxic-acclimated cod had a reduced pumping capacity, and their increased ability to recover following a bout of severe hypoxia. With regards to the former, these included myocardial necrosis, stunning, and remodeling. However, I feel that the last possibility is the most likely. This is because Marques et al. (2008) recently showed that acclimation of the zebrafish (*Danio rerio*) and the cichlid *Haplochromis piceatus* to 10% air saturation (P_wO_2 2 kPa) for 21 days increased cardiac myocyte density and such remodelling would have several benefits for the hearts of chronically hypoxic fish. For example, decreasing the size of the heart's lumen through hyperplastic myocardial growth

would diminish the workload of individual cardiomyocytes and thus limit apoptosis and/or myocardial necrosis when oxygen became limiting (Des Tombe et al., 2002; Laarse et al., 2005). Further, this type of myocardial growth would reduce the wall tension required for ventricular ejection, thus allowing for pressure generation at considerable mechanical advantage, and ultimately, reduced myocardial oxygen consumption (Farrell and Jones, 1992; Giordano, 2005). With regards to the ability of fish hearts to recover maximum cardiac function following a period of oxygenated perfusion, Overgaard et al. (2004a): 1) showed that functional impairment of trout hearts following anoxic exposure occurs even though energetic and biochemical status of the myocardium is not compromised (altered); and 2) suggested that increased levels of oxygen radicals were responsible for the stunning of trout hearts following recovery from severe hypoxia/anoxia. Based on this information, it could be hypothesized that the improved functional recovery shown by hearts of hypoxia-acclimated cod following 15 min. of severe hypoxia was due to an increased ability to protect the myocardium against the negative effects of reactive oxygen species (ROS). Indeed, this is a plausible explanation as Marques et al. (2008) showed that the expression of 6 genes important for protection against ROS were upregulated (by 2.1 to 6.5 fold) in zebrafish hearts following 21 days of acclimation to a P_wO_2 of 2 kPa.

As described in chapter 5, hypoxic-acclimated cod were able to compensate for the potential effects of reduced cardiac output on oxygen consumption and metabolic capacity through small increases in oxygen carrying capacity (Hct by 11 % and [Hb] by 14 %) and a significant improvement in tissue oxygen extraction efficiency. This higher tissue oxygen extraction efficiency could have been mediated by several mechanisms

including increased tissue myoglobin levels and/or capillary density. However, hypoxic acclimation for prolonged periods does not lead to increased myoglobin levels in eelpout (*Zoareces viviparous*) (Driedzic et al., 1985) or cod (Hall et al. 2009) hearts, and the effect of hypoxic acclimation on muscle capillary density is variable; from no change (Johnston and Bernard, 1983), to a decrease (Johnston and Bernard, 1982) to an increase (Johnston and Bernard, 1984; Sänger et al., 1990). However, blood transit time has been identified as a major limitation to tissue oxygen extraction in mammals (Saltin, 1985), and Saldivar et al. (2003) showed that improved oxygen extraction by the 'window chamber preparation' of hypoxic-acclimated Syrian hamsters was associated with increased functional capillary density and reduced blood flow velocity. This latter data suggests that the reduced Q of hypoxic-acclimated cod, combined with the dilation/recruitment of existing capillaries, slowed tissue blood flow and that this resulted in the improved oxygen extraction efficiency exhibited by this group. This is an interesting hypothesis that warrants experimental verification.

6.3. Ecological Implications of Current Research

My thesis' experiments provide some very important insights into the physiological adjustments made by cod when exposed to reduced oxygen levels. However, these physiological adjustments did not benefit swimming performance, metabolic capacity or improve hypoxia tolerance, and thus it is apparent that the Atlantic cod will have a difficult time adapting to, and/or re-colonizing, areas within its' historical distribution that are now chronically hypoxic (e.g Gulf of St. Lawrence, Baltic Sea;

Gerlach, 1988; Plante et al., 1998; Gilbert et al., 2005). This conclusion is based, for one thing, on the widely accepted theory that habitat selection by fish is primarily governed by the need to optimize metabolic scope (Evans, 1990; Neill and Bryan, 1991; Neill et al., 1994). My experiments clearly show that although metabolic scope is considerable in normoxic and hypoxic-acclimated cod in normoxic water, it is reduced greatly (by 80 %) in both groups in hypoxic water. As pointed out by Claireaux and LeFrancois (2007) any environmental condition that causes a decrease in scope for metabolism results in a conflict between the various energy demanding processes, and this results in energy allocation occurring at the expense of somatic and gonad growth (Claireaux and LeFrancois, 2007). Given that these two parameters are the primary determinants of reproductive success in cod (Kjesbu et al., 1991; Burton et al., 1997; Kraus et al., 2000), it is likely that the observed reduction in metabolic scope with hypoxia will have direct negative effects on cod reproduction and population strength. Further, there is strong evidence that hypoxia has a number of other effects on cod physiology/biology that will make it difficult for this species to survive/thrive in areas with reduced water O_2 levels: 1) hypoxia disrupts endocrine functions, which in turn affects gametogenesis, sexual maturity, gamete quality, fecundity, fertilization success, hatching, and larval viability (Wu et al., 2003); 2) fish may be forced to change diet as mobile marine organisms avoid hypoxic waters by relocating to more oxygenated areas (Pihl et al., 1991; Claireaux et al. 1995); 3) appetite and feeding are decreased in cod exposed to hypoxia (Chabot and Dutil, 1999); and 4) reduced O_2 availability results in a more than doubling of the duration of specific dynamic action (SDA; 212 vs. 95 hrs. in normoxia) and an increase in

the percentage of meal energy content taken up by SDA (i.e. an increased cost of digestion) (Jordan and Steffensen, 2007).

6.4. References

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