

ADRENERGIC PROTECTION OF THE FISH HEART UNDER
SIMULATED EXERCISE CONDITIONS

by

LINDA MARIE HANSON

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Abstract

Exercise is an integral part of the survival of vertebrates. However, intense exercise disrupts extracellular homeostasis, resulting in venous blood becoming hypoxic, hyperkalemic and acidotic. These changes challenge the heart, as the venous blood passing through the ventricular lumen is the sole source of myocardial support for most vertebrates. Consequently, during exercise when the heart is working its hardest it must ironically be supported by the hostile composition of venous blood.

Rainbow trout (*Oncorhynchus mykiss*) and African catfish (*Claris gariepinus*) were used to examine the role of adrenergic stimulation in protecting cardiac performance during exercise-induced changes in venous blood composition. As cardiac performance is ultimately limited by oxygen supply, *in situ* perfused rainbow trout hearts were first used to determine the effect of adrenergic stimulation on the venous hypoxic threshold during simulated exercise conditions (5.0 mM K^+ , pH 7.5); where the hypoxic threshold is defined as the lowest level of venous oxygen tension (P_{vo_2}) that can support maximum cardiac performance. Hearts were tested under these relevant conditions with both tonic (5 nM adrenaline, AD) and maximal (500 nM AD) adrenergic stimulation. With 5 nM AD maximum cardiac performance in hearts at 10°C was significantly reduced even at normoxia. In contrast, 500 nM AD fully protected cardiac performance under hyperkalemia and acidosis to hypoxia of 2.0 kPa, a P_{vo_2} close to routine values *in vivo*.

Temperature acclimation alters the response of the rainbow trout heart to adrenaline such that an increase in acclimation temperature is associated with a decrease in adrenergic sensitivity. To determine how this reduction in adrenergic effectiveness alters the protection

afforded to the heart during exercise the *in situ* hypoxic threshold was determined for perfused rainbow trout hearts at 18°C. The hypoxic threshold at 18°C under the combined hyperkalemic, acidotic exposure with 500 nM AD was 5.6 kPa.

In rainbow trout, the loss of adrenergic sensitivity at high temperature is attributed to a decrease in cell surface β -adrenoceptor density (B_{max}). This does not seem to be a universal mechanism among fish as no evidence of B_{max} modulation was found in the tropical African Catfish.

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List of Abbreviations

β -AR	β -adrenoceptor
B_{max}	β -adrenoceptor density
ATP	adenosine triphosphate
AD	adrenaline
P_{ao_2}	oxygen tension in arterial blood
P_{co_2}	carbon dioxide tension
Q	cardiac output
PO	cardiac power output
cAMP	cyclic adenosine monophosphate
Hb	haemoglobin
P_{50}	half-maximal haemoglobin-oxygen saturation
f_H	heart rate
P_{in}	input pressure
K_d	ligand binding affinity
Q_{max}	maximum cardiac output
PO_{max}	maximum cardiac power output
Mo_2	myocardial oxygen consumption
nAD	noradrenaline
P_{out}	output pressure
PO_2	partial pressure of oxygen
V_s	stroke volume
C_{vo_2}	oxygen content in venous blood
P_{vo_2}	oxygen tension in venous blood
P_{va}	ventral aortic blood pressure

Preface

This thesis is comprised of five chapters. Chapter one is an introduction to the current body of literature where the effects of exercise-induced conditions on cardiac performance and the role of adrenergic stimulation in protecting performance under otherwise detrimental conditions are both thoroughly discussed. Emphasis is placed on cardiac function and its regulation in rainbow trout, the model species used for this thesis. The hypotheses under study and overall objectives of the thesis are outlined in the concluding elements of Chapter one. The following three chapters specifically address the thesis objectives and are written as individual manuscripts. This format was preferred, as Chapters 2 and 4 have already been published as individual studies in peer-reviewed journals, and I intend to submit the study presented as Chapter 3 for publication as well. The final chapter is a concise summary of the major findings and conclusions of the thesis, as a whole.

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Co-authorship Statement

Two of the manuscripts contained in this thesis are co-authored. Nevertheless, the vast majority of the research and data analysis was conducted independently. Two research assistants (Shannon Obradovich and Janet Mouniargi) assisted with portions of the perfused heart experiments in Chapter 2. In addition, Dr. Yuen (Alex) Ip obtained the African Catfish used in Chapter 4 and acclimated them in his laboratory in Singapore before shipping the hearts to me for further analysis. Following the conclusion of individual experiments, all manuscripts were written solely by me in consultation with my senior supervisor.

Chapter 1: Introduction and literature review

Rainbow trout cardiac physiology

Exercise is necessary for survival in most vertebrates. Activities such as migration, reproduction, prey capture and predator avoidance all depend on some form of exercise. In most animals, exercise in working muscles is fuelled by aerobic respiration, and hence exercise success is reliant on oxygen delivery. The heart pumps blood throughout the body and therefore facilitates oxygen delivery and removal of metabolic waste products. During exercise, the heart must work harder in order to increase blood flow, and thus oxygen delivery, to working muscles. As the heart is also a muscle, its oxygen needs also increase during exercise in proportion to the increased workload.

Intense exercise, however, disrupts extracellular homeostasis in an effort to maintain intracellular homeostasis, and therefore affects oxygen delivery and metabolic waste removal. Depending on an animal's aerobic threshold – the point at which anaerobic energy pathways start to operate – intense exercise can result in increases in plasma potassium (hyperkalemia), sodium, glucose, lactate, and osmolarity as well as a decrease in pH (acidosis) and oxygen level (hypoxia, especially in the venous blood) (Wood *et al.* 1983; Boutilier *et al.* 1986; Tufts *et al.* 1991; Wang *et al.* 1994; Brauner *et al.* 2000). These changes not only challenge the skeletal muscles that are metabolically active during this bout of exercise but also pose a great challenge to the cardiac muscle. Because the myocardium of most vertebrates is avascular, the venous blood passing through the ventricular lumen is the sole source of myocardial support. During exercise, when the heart is working its hardest, it must contend with, and ironically be supported by, the hostile composition of the

venous blood. Nowhere is this more pronounced than in teleost fish; ~80% of teleosts possess hearts that rely solely on venous blood (Santer, 1985).

The rainbow trout (*Oncorhynchus mykiss* Walbaum), whose heart utilizes both coronary (arterial) and venous blood, but relies mainly (60-70%) on venous blood (Santer and Greer Walker, 1980; Farrell *et al.*, 1988b) is the model species used for the experiments in this thesis. Because so much information is known regarding both exercise performance and cardiovascular morphology and physiology of rainbow trout, this species makes an ideal model species for investigating cardiovascular performance during exercise. The rainbow trout is also a member of the economically important salmonid family, thus research on rainbow trout may provide insight into the physiology of other salmonid species. For example, it has been proposed that recent declines in the number of successful migrating salmonids may be related to the deleterious effects of rising temperatures (along migratory routes) on cardiovascular performance. Understanding the physiology and potential limitations on cardiovascular performance in rainbow trout under similar conditions could provide important implications for wild migrating salmonids and other teleosts. The primary objective of this thesis is to examine the role of adrenaline in maintaining cardiovascular performance by combating the adverse extracellular environment generated during intense exercise.

The rainbow trout ventricle and its muscle composition

The teleost heart, a series of four chambers, is first perfused via the hepatic veins and ducts of Cuvier, which empty into the sinus venosus, venous blood then passes sequentially through the remaining three chambers of the heart: the atria, the ventricle and the bulbus

arteriosus. Teleost hearts share a common architecture but can differ greatly in structure, especially in the muscle composition of the ventricle, which may consist of two distinct myocardial layers, an inner, spongy myocardium (endocardium), and an outer, compact myocardium (epicardium). As the name implies, the former is characterized by a loose, trabecular arrangement of myocytes and multiple invaginations into the lumen. Conversely, compact myocardium possesses myocytes arranged into closely-packed, overlapping sheets that encircle the outside of the ventricle. Teleost ventricles are classified into four types (I-IV) based on proportion and structure of the two tissue types (Tota *et al.*, 1983). Rainbow trout possess a type II ventricle, which consists of an inner layer of spongy myocardium, bathed solely in blood from the cardiac circulation (venous blood), and an outer layer of compact myocardium, perfused by arterial blood via coronary circulation (Tota, 1983). Spongy myocardium accounts for approximately 60-70% of the total myocardium (Santer and Greer Walker, 1980; Farrell *et al.*, 1988b). Consequently, oxygen needs are met largely via diffusion from venous blood that has already oxygenated other tissues, a potential problem when venous oxygen tensions (P_{vo_2}) are reduced due to exercise or environmental hypoxia.

Cardiovascular control and cardiac work during exercise

During exercise, cardiac performance increases substantially. Maximum cardiac output (Q_{max}), the product of heart rate (f_H) and stroke volume (V_s), increases 2-3 fold (from 17.6-26.6 ml min⁻¹ kg⁻¹ to 43.9-62.5 ml min⁻¹ kg⁻¹) (Stevens and Randall, 1967b; Kiceniuk and Jones, 1977; Thorarensen *et al.*, 1996). Stroke volume, the volume of blood ejected by each heartbeat, is mainly influenced by ventricular filling time, atrial contractility, and,

because it is inversely related to ventricular filling, f_H . In rainbow trout, pacemaker cells located at the sinoatrial junction generate the intrinsic f_H (Santer, 1985); regulation is both cholinergic and adrenergic. A tonic, cholinergic tone from the cardiac branches of the vagus nerve (Laurent *et al.*, 1983) depresses f_H below the intrinsic pacemaker rate, while varying degrees of neural and humoral adrenergic stimulation act to offset cholinergic inhibition. Adrenergic innervation (neural control) is present in the sinus venosus, atrium and ventricle of the trout heart, although ventricular innervation may be confined to compact myocardium and coronary vasculature (Gannon and Burnstock, 1969). Ventricular adrenergic stimulation increases contractility and potentially mediates increases in coronary blood flow observed during exercise (Axelsson and Farrell, 1993; Gamperl *et al.*, 1994b; Gamperl *et al.*, 1994a; Gamperl *et al.*, 1995). Additionally, tonic levels of circulating catecholamines (humoral control), both adrenaline (AD) and noradrenaline (nAD), are present in rainbow trout plasma (Milligan *et al.*, 1989) and, during intense exercise, can increase from 5-11 nM to over 200 nM (Butler *et al.*, 1986; Milligan *et al.*, 1989) to compensate for the detrimental consequences of exercise-induced changes in venous blood composition. Thus, one of the objectives of this thesis is to determine the degree to which adrenergic stimulation can maintain maximum cardiac performance during hypoxia, hyperkalemia, and acidosis combined, an experimental setting meant to simulate the venous blood composition following intense exercise. This will be assessed by determining the hypoxic threshold for maximum cardiac performance during relevant hyperkalemic, acidotic conditions, both with and without maximal adrenergic stimulation.

Oxygen supply to the myocardium during exercise

Myocardial oxygen consumption

Myocardial oxygen supply, in most teleosts, depends on the amount of oxygen available via cardiac circulation and is therefore a product of cardiac output (Q) and venous oxygen content (C_{vo_2}). In rainbow trout, and several other teleosts, arterial blood from the coronary circulation supplements myocardial oxygen supply but may be limited to the compact myocardium leaving C_{vo_2} to satisfy the oxygen demand of the spongy myocardium (~70% of the heart). Even though myocardial oxygen demand can vary greatly, Farrell *et al.* (1985) calculated that the heart uses less than 4% of C_{vo_2} and thus C_{vo_2} is likely more than adequate to support myocardial oxygen consumption (Mo_2). Seemingly, Mo_2 would depend on cardiac mass, i.e. larger hearts require more oxygen, but this idea may be countered if larger hearts have lower mass-specific Mo_2 (Graham and Farrell, 1989). It is clear, however, that Mo_2 increases in proportion with cardiac workload (Farrell *et al.*, 1985; Graham and Farrell, 1990) and can increase over 4-fold during intense exercise (Farrell and Jones, 1992).

Venous oxygen tension vs. venous oxygen content

During intense exercise, cardiac workload increases, and Q is amplified to meet increasing oxygen demand. In skeletal muscle, this means an 80% decrease in C_{vo_2} (Kiceniuk and Jones, 1977), but in myocardial cells, even during intense exercise, oxygen demand is still met via venous blood (Farrell *et al.*, 1985; Farrell and Jones, 1992). Myocardial cells will continuously utilize oxygen, leaving the venous oxygen tension (P_{vo_2}) directly adjacent to the myocardium lower than the P_{vo_2} in the more centrally located luminal

blood. This reduces oxygen diffusion to the spongy myocardium, despite the fact that there is still plenty of oxygen remaining in the luminal blood; therefore, myocardial oxygen supply is limited by diffusion, which is directly related to P_{vo_2} . Intense exercise can reduce P_{vo_2} by ~50% (Kiceniuk and Jones, 1977) but also increases f_H . As a result, not only is P_{vo_2} low, but the blood residence time in the lumen is decreased, subsequently reducing the time available for oxygen diffusion, all of which make oxygen supply to the spongy myocardium more difficult during intense exercise, a time when it is most imperative.

Venous oxygen tensions and threshold levels

Few studies have measured P_{vo_2} in fishes (Stevens and Randall, 1967a; Eddy *et al.*, 1977; Kiceniuk and Jones, 1977; Perry and Reid, 1994; Steffensen and Farrell, 1998; Farrell and Clutterham, 2003) and some of the earlier studies may have been done using stressed fish as resting P_{vo_2} values of 2.5 kPa (Stevens and Randall, 1967a) and 2.7 kPa (Thomas *et al.*, 1994) represent less than 50% haemoglobin-oxygen saturation at the particular temperatures under study (Nikinmaa and Soivio, 1979; Perry and Reid, 1992a). In addition, all studies, save one, have measured P_{vo_2} using intermittent blood sampling, which can result in misleading data if samples are drawn during non steady-state swimming. Erratic swimming behaviours can create abrupt changes in P_{vo_2} (Farrell and Clutterham, 2003).

Farrell and Clutterham (2003) used a fibreoptic micro-optode to measure the P_{vo_2} of maximally exercising rainbow trout and discovered that even at the most severe exercise intensity, P_{vo_2} did not drop below 2.0 kPa, the estimate obtained in another study via intermittent blood samples from exercising fish (Kiceniuk and Jones, 1977). Environmental

hypoxia induces more drastic changes in P_{vo_2} , (< 1 kPa at 8°C) (Thomas *et al.*, 1994), however, especially when combined with exercise (1.3 kPa) (Steffensen and Farrell 1998).

Existing evidence suggests that venous oxygen thresholds are influenced by both absolute cardiac workloads (van Raaij *et al.*, 1996) and the extracellular conditions experienced by the heart. This is further emphasized by the finding that isolated perfused hearts exposed to hypoxic saline (3.3 kPa) decreased Q_{max} and maximum cardiac power output (PO_{max}) by $\sim 50\%$ and $\sim 80\%$ respectively at routine physiological workloads (Farrell *et al.*, 1989), while isolated trout hearts at sub-physiological workloads could perform under near anoxic conditions with no effects on cardiac performance (Arthur *et al.*, 1992). It follows that the minimum P_{vo_2} for hypoxia will be lower than that with exercise. Nevertheless, the minimum P_{vo_2} will be one at which diffusion rates match myocardial oxygen demand; below this P_{vo_2} , the diffusion gradient will be insufficient to support myocardial function and survival of the heart will be compromised. A venous oxygen threshold, proposed to range from 0.8 to 2.1 kPa (Jones, 1986; Davie and Farrell, 1991; Perry and Reid, 1994; Steffensen and Farrell, 1998; Farrell and Clutterham, 2003), must exist to preserve the necessary oxygen diffusion gradient, but because of contrasting experimental conditions in existing literature, comparisons between environmental and exercise-induced hypoxia thresholds are difficult. Fittingly, another goal of this thesis is to establish the venous oxygen threshold for maximum cardiac performance under both hypoxia alone, and in conjunction with other exercise-induced changes in venous blood composition.

The coronary circulation

Approximately 20% of all teleosts, including the rainbow trout, utilize a combination of coronary and cardiac circulation (Santer and Greer Walker, 1980). Increased oxygen demand of the compact myocardium may be offset by increasing coronary perfusion; as coronary blood flow can increase by up to two-fold (Axelsson and Farrell, 1993; Gamperl *et al.*, 1994b; Gamperl *et al.*, 1994a; Gamperl *et al.*, 1995). Coronary ablation experiments have demonstrated that the coronary circulation is not necessary to maintain routine cardiac performance (Daxboeck, 1982; Steffensen and Farrell, 1998), despite the fact that the coronary artery has a routine flow (Axelsson and Farrell, 1993; Gamperl *et al.*, 1994b; Gamperl *et al.*, 1995). This suggests, that during routine conditions, oxygen diffusion from venous blood is presumably sufficient to meet the oxygen needs of both compact and spongy myocardium for resting cardiac power output, likely reflected in the routine P_{vO_2} (3-4 kPa) measured in rainbow trout. However, during exercise when P_{vO_2} of the cardiac circulation is reduced, the increase in coronary blood flow potentially accommodates oxygen diffusion from venous blood across the spongy myocardium to the compact myocardium. In fact, without this coronary supply, Steffensen and Farrell (1998) found that coronary-ligated rainbow trout reduced cardiac workloads by an estimated 37% during a hypoxic swimming challenge, a percent change very similar to the percentage of compact myocardium. Hence, the importance of coronary circulation may increase during exercise, but the majority of myocardial oxygen needs can still be supplied via diffusion from venous blood.

Effects of hypoxia on performance

Hypoxia exposure elicits compensatory ventilatory, cardiovascular and humoral responses in teleost fish. During environmental hypoxia, increases in the volume of water moved (Kinkead and Perry, 1991; Perry and Gilmour, 1996; Perry, 1999) and frequency of gill ventilation (Jones, 1952; Høle and Randall, 1967a; Powell *et al.*, 1998) occur in fish, all to ensure arterial oxygen tension (P_{ao_2}) approaches that of the inhaled water. Regardless, P_{ao_2} , arterial oxygen content (C_{ao_2}), P_{vo_2} and C_{vo_2} all decrease with progressive hypoxia. During exercise-induced hypoxia, similar increases in ventilation volume and frequency occur (Saunders, 1962; Smith *et al.*, 1967; Stevens and Randall, 1967b; Stevens and Randall, 1967a) to maintain P_{ao_2} and C_{ao_2} , but P_{vo_2} and C_{vo_2} decrease due to increased oxygen extraction by the tissues.

In fish, the major cardiovascular adjustments to environmental hypoxia include a reduction in heart rate (bradycardia or negative chronotropy) (Høle and Randall, 1967a; Høle and Randall, 1967b; Farrell, 1982; Perry, 1999) and a reduction in myocardial contraction force (negative inotropy) (Gesser, 1977; Gesser *et al.*, 1982; Farrell *et al.*, 1989; Arthur *et al.*, 1992; Overgaard and Gesser, 2004). Hypoxia can decrease heart rate by almost 60% (Farrell, 1982), which can be linked to an increased cholinergic inhibitory tone from the cardiac branches of the vagus nerve (Laurent *et al.*, 1983). Bradycardia will increase the residence time of venous blood in the heart, allowing more time for oxygen to diffuse from venous blood into myocardial tissues (Farrell, 1984), and decrease the rate of blood flow through the gills, resulting in increased O_2 uptake (Høle and Randall, 1967b). Severe hypoxia (≤ 1.6 kPa) decreases the isometric force of isolated cardiac muscle strips by 60-90% (Gesser, 1977; Gesser *et al.*, 1982; Overgaard and Gesser, 2004) and has similar

negative inotropic consequences for perfused hearts (Farrell *et al.*, 1989; Arthur *et al.*, 1992). This negative inotropy is likely due to an inability of myocardial anaerobic ATP production to overcome the deficit between oxygen supply and oxygen demand as well as metabolic acidosis.

Limitations in myocardial oxygen supply may restrict exercise performance (Farrell, 2002), an idea that has been well studied *in vivo* (Kiceniuk and Jones, 1977; Gamperl *et al.*, 1995; Steffensen and Farrell, 1998; Perry *et al.*, 2000); however, only a few *in situ* studies have directly examined the effects of hypoxia on maximum cardiac performance (Farrell *et al.*, 1989; Gamperl *et al.*, 1994b; Overgaard *et al.*, 2004b). For this reason, a secondary objective of this study is to further quantify the effects of various levels of venous hypoxia on maximum cardiac performance *in situ*.

Plasma acidosis

The dual nature of exercise-induced acidosis

Aerobic cellular respiration generates CO₂ as a by-product of ATP production, while anaerobic ATP production generates lactic acid, both of which are released during strenuous exercise from working muscles (including the heart; Overgaard *et al.*, 2004a) into the plasma. Venous blood pH may decrease from 7.9 to 7.5 or even 7.3, an acidotic condition that can persist for up to 8 hours during recovery (Kiceniuk and Jones, 1977; Wood *et al.*, 1977; Graham *et al.*, 1982; Holetton *et al.*, 1983; Turner *et al.*, 1983). Respiratory acidosis, when CO₂ is hydrated at a non-catalyzed rate to form (pK_a = 6.37) bicarbonate and protons, is different from a metabolic acidosis where lactic acid dissociates (pK_a = 3.86) into lactate and protons. Metabolic and respiratory factors contribute equally to post-exercise acidosis

(Turner *et al.*, 1983; Milligan and Wood, 1986b); however, the metabolic component is prolonged but slow to develop, while the respiratory component is quick to develop and short lived. The respiratory acidosis begins to disappear immediately following exhaustive exercise and by ~2 hours post-exercise, a metabolic acidosis dominates (Turner *et al.*, 1983; Milligan and Wood, 1986b; 1986a; McDonald *et al.*, 1989); nevertheless, both similarly reduce blood pH. Due to the more rapid onset of, and recovery from, hypercapnic acidosis, all experiments in the following studies utilized CO₂ to reduce pH.

The effects of venous acidosis on cardiac performance

The negative inotropic and chronotropic effects of hypercapnic acidosis have been well studied on isolated cardiac muscle strips (Gesser and Jorgensen, 1982; Gesser *et al.*, 1982; Kalinin and Gesser, 2002) and working perfused heart preparations (Farrell *et al.*, 1986; Farrell and Milligan, 1986; Farrell *et al.*, 1988a). Kalinin and Gesser (2002) observed a 60% decrease in isometric force of rainbow trout ventricular strips exposed to hypercapnic acidosis (pH 6.9), while Farrell *et al.* (1986) observed a ~10% decrease in maximum cardiac power output (PO_{max}), with hypercapnic acidosis (pH 7.4) in perfused rainbow trout hearts, a consequence of decreasing f_H and contractile force. Potentially, an acidosis competes with calcium-troponin binding (Williamson *et al.*, 1976; Fabiato and Fabiato, 1978; Gesser and Poupa, 1978; Gesser and Jorgensen, 1982) and therefore effectively reduces myocardial intracellular Ca²⁺ concentration, the major requirement for cardiac muscle contraction. Specifically, cardiac contraction in the teleost heart is induced by an influx of Ca²⁺ across the sarcolemma (Tibbits *et al.*, 1992). Once inside the cell, Ca²⁺ binds to troponin-C inducing muscle contraction, and if an acidosis affects Ca²⁺-troponin binding, myocardial contraction

can be adversely altered. Experiments with perfused canine hearts suggest that the negative chronotropic effects of acidosis are directly related to pH effects on pacemaker cells, possibly via decreased rates and strength of firing, and prolonged action potentials (Satoh and Hashimoto 1983).

Exercise-induced hyperkalemia

The source of excess plasma K^+

Another major exercise-induced change in venous blood composition is hyperkalemia, an increase in plasma $[K^+]$, which is normally tightly regulated because of its effects on membrane potential. During intense exercise and recovery, venous plasma $[K^+]$ increases from 2.5 mM to 3.4-4.7 mM (Holeton *et al.*, 1983; Turner *et al.*, 1983; Perry *et al.*, 1987; Thomas *et al.*, 1987; Nielsen and Lykkeboe, 1992; Holk and Lykkeboe, 1998) as skeletal myocytes rapidly and repeatedly contract. Repeated stimulation means that Na^+/K^+ ATPase cannot fully restore intracellular K^+ between successive contractions. Excess K^+ accumulates in the skeletal muscle interstitial fluid and diffuses into the plasma leading to venous hyperkalemia. Post-exercise, skeletal muscle and liver uptake of K^+ (Elfellah and Reid, 1990; Kes, 2001) and elimination via the kidneys (Ahmed and Weisberg, 2001) restores plasma $[K^+]$ to normal levels.

Effects of hyperkalemia on cardiac performance

The detrimental effects of hyperkalemia on cardiac performance in rainbow trout are not thoroughly studied, but it is known that hyperkalemia in isolated cardiac strips reduces

the contractive force by ~50% at 10 mM (Kalinin and Gesser, 2002) and is lethal at 12.5 mM (Nielsen and Gesser, 2001). When myocardial K^+ gradients are disrupted, resting myocyte membrane potentials are reduced (Miura *et al.*, 1977; Chapman and Rodrigo, 1987; Hove-Madsen and Gesser, 1989; Bouchard *et al.*, 2004). Depolarization may reduce the strength of ventricular contractions by inactivating a proportion of ventricular Na^+ channels and therefore slowing cardiac conduction and reducing the action potential strength (Chapman and Rodrigo, 1987; Fozzard and Shorofsky, 1992). In mammals, hyperkalemia ($K^+ > 5.5$ mM) results in ventricular arrhythmia (Paterson *et al.*, 1992; Kes, 2001). Hyperkalemia-induced depolarization can also indirectly affect myocardial contractility by affecting voltage-gated Ca^{2+} channels (Bouchard *et al.*, 2004). Since the effects of hyperkalemia on cardiac performance have only been examined on paced, isolated cardiac muscle strips, little is known regarding the chronotropic effects of hyperkalemic exposure. Consequently, another aim of this research is to quantify both inotropic and chronotropic effects of hyperkalemia in a perfused heart preparation.

The importance of adrenaline

Adrenergic stimulation of cardiac tissue

In rainbow trout, the predominant source of catecholamine synthesis and storage is the chromaffin tissue located near the head kidney in the posterior cardinal vein (Nandi, 1961; Reid *et al.*, 1998). Cholinergic nerve fibres innervate the chromaffin tissue, and thus catecholamine release is controlled by the sympathetic nervous system. The primary circulating catecholamines are AD and noradrenaline (nAD), with resting plasma concentrations of approximately 5-11 nM and 14-16 nM, respectively (Milligan *et al.*, 1989).

Under stressful conditions, including intense exercise, stored catecholamines (mostly AD) are released into the plasma (Reid and Perry, 1994). As a consequence of intense activity, nAD concentrations increase 2-15 fold, while studies in rainbow trout have reported post-exercise [AD] as high as $212 \text{ nM} \pm 89 \text{ nM}$ (Butler *et al.*, 1986), a 40-fold increase. This discussion will focus on the humoral role of AD, as the rainbow trout heart is ~10 times more sensitive to AD than nAD (Gannon and Burnstock, 1969; Ask *et al.*, 1981; Farrell *et al.*, 1986), and AD is the principal catecholamine released by rainbow trout during intense exercise and recovery.

The β -adrenoceptor signal transduction system

Adrenaline is derived from the amino acid tyrosine, as such, it does not readily cross lipid membranes, and hence it must bind to specific cell-surface receptors, known as adrenoceptors, in order to exert an effect on target cells. There are two distinct categories of adrenoceptors, α and β (Ahlquist, 1948), with each category being further divided into subtypes. As each category of adrenoceptors activates a different signal transduction pathway, catecholamines can trigger diverse effects in a wide range of cell types. The β -adrenoceptor (β -AR) signalling pathway is known to mediate the important cardiac inotropic and chronotropic actions of AD and nAD in fish (Ask *et al.*, 1981; Temma *et al.*, 1986; Gamperl *et al.*, 1994c). Currently, it is believed that rainbow trout possess three distinct subtypes of β -AR receptors, β_1 , β_2 , and β_3 , although the main β -AR subtype mediating these cardiac actions in rainbow trout is a β_2 subtype (Ask *et al.*, 1980; 1981; Keen *et al.*, 1993; Gamperl *et al.*, 1994c). β -adrenoceptor subtypes differ in their relative affinities for AD and nAD. β_1 and β_3 -AR possess an almost equal affinity for AD and nAD, with only a slight preference for nAD, while β_2 -AR show a clear preference for AD (Hoffman and Lefkowitz,

1982; Nickerson *et al.*, 2003). Nevertheless, both AD and nAD are capable of stimulating all β -AR subtypes; consequently, as AD is the principal catecholamine released by rainbow trout during intense exercise and recovery, the following experiments utilized only AD to elicit β -AR stimulation in rainbow trout hearts.

Stimulation of myocardial cell-surface β_2 -AR activates a G-protein mediated signal transduction cascade. Briefly, ligand binding activates an intracellular stimulatory G-protein which stimulates adenylyl cyclase, adenylyl cyclase catalyzes the formation of cyclic adenosine monophosphate (cAMP) from ATP, and the resulting increase in [cAMP] activates the enzyme cAMP-dependant protein kinase A; which can phosphorylate a variety of intracellular proteins to produce a specific response depending on the cell type involved. For example, activation of the myocardial β -AR signal transduction cascade is thought to phosphorylate sarcolemmal L-type Ca^{2+} channels increasing myocardial calcium influx (Tibbits *et al.*, 1992; Shiels and Farrell, 1997; Vornanen, 1998). Additionally, stimulation of erythrocyte β -AR activates Na^+/H^+ exchange which results in erythrocyte alkalization (Nikinmaa and Huestis, 1984; Tang *et al.*, 1988; Perry and Reid, 1992b; Perry and Gilmour, 1996; Val *et al.* 1998) and an increase in haemoglobin-oxygen affinity. Secondary results of this cascade include cell swelling, a decrease in extracellular pH, an increase in intracellular Cl^- and K^+ , and an increase in extracellular Na^+ and bicarbonate. Both the [catecholamines] and the density of cell surface β_2 -AR (B_{max}) influence myocardial adrenergic responsiveness, and both of these can vary dependant upon conditions.

Hypoxia and adrenaline

Hypoxia elicits significant increases in circulating [AD] and [nAD], with the peak [AD] being 2-6 fold higher than that of nAD (Aota *et al.*, 1990; Perry and Gilmour, 1996). Catecholamine release appears to be triggered by arterial P_{O_2} levels ranging from 50 to 60% haemoglobin O_2 -saturation (Perry and Reid, 1992a; Thomas and Perry, 1992; Perry and Reid, 1994) and leading to changes in the β -AR signal transduction system. Hypoxia causes significant (29% to 62%) down-regulation in B_{max} in mammalian and avian ventricular tissue (Voelkel *et al.*, 1981; Blake *et al.*, 1982; Marsh and Sweeney, 1989; Rocha-Singh *et al.*, 1991), but prolonged (6 h) hypoxia ($P_{vO_2} \approx 1.1$ kPa) did not trigger β -AR down-regulation in rainbow trout ventricular tissue (Gamperl *et al.*, 1998). Adrenergic stimulation may be preserved in myocardial tissue that is reliant upon the hypoxia-prone cardiac circulation; this may be further supported with the information that spongy myocardium possesses 14% more β -AR than compact myocardium (Gamperl *et al.*, 1998). The increase in anaerobic respiration during hypoxic exposure results in an intracellular acidosis, which is counteracted via adrenergic stimulation. Adrenergic stimulation of pacemaker cells opposes hypoxic bradycardia by increasing pacemaker self-excitation rates (Tibbits *et al.*, 1992) and intracellular $[Ca^{2+}]$, which help offset the deleterious effects of hypoxia-induced acidosis.

In contrast to ventricular β -AR, trout erythrocytes show β -AR up-regulation and a corresponding increase in adrenergic responsiveness, following a 30-minute hypoxic exposure (Reid *et al.*, 1993). β -adrenergic stimulation of trout erythrocytes appears to increase Na^+/H^+ exchange (Perry and Reid, 1992b; Reid *et al.*, 1993), resulting in erythrocyte alkalization. Alkalization counteracts hypoxia-induced acidosis and increases haemoglobin-oxygen affinity, resulting in more effective O_2 uptake at the gills. Improving oxygen

transport and uptake at the gills is crucial in maintaining fully saturated arterial blood oxygen levels during environmental hypoxia; however, increased haemoglobin-oxygen affinity will have negative consequences for myocardial oxygen delivery.

Acidosis and adrenaline

Studies with perfused hearts (Farrell *et al.*, 1983; Farrell *et al.*, 1986; Farrell and Milligan, 1986) and isolated cardiac muscle strips (Gesser and Jorgensen, 1982; Gesser *et al.*, 1982) reveal that adrenergic stimulation can counteract acidosis-induced chronotropic and inotropic effects. Acidosis (pH 7.4) can significantly reduce cardiac performance in perfused sea raven and ocean pout hearts; however, performance was fully restored at pH 7.4 with 1 μ M AD (Farrell *et al.*, 1983). Likewise, concurrent adrenergic stimulation (100 μ M AD) at pH 7.4 also maintained cardiac performance in isolated cardiac muscle strips, while acidosis alone reduced contractile force by 30% (Gesser *et al.*, 1982). Adrenergic β -AR stimulation counteracts the acidotic impairment of calcium-troponin binding by increasing intracellular $[Ca^{2+}]$. Adrenaline also restores plasma and erythrocyte pH during exercise-induced acidosis (McDonald *et al.*, 1989) via β_{3b} -AR-mediated erythrocyte Na^+/H^+ exchange (Tang *et al.*, 1988; Perry and Gilmour, 1996; Nickerson *et al.*, 2003), which results in erythrocyte alkalization and enhances haemoglobin-oxygen affinity (Thomas and Perry, 1992). Fish exposed to the β -AR antagonist propranolol required 4 times as long (4 hr vs. 1 hr) to restore plasma pH as opposed to untreated fish (Tang *et al.*, 1988). It is believed that an acidosis-induced hypoxia is what triggers catecholamine release (Perry *et al.*, 1989; Aota *et al.*, 1990) and, at least at the erythrocyte, is in place to prevent the Root effect from occurring and therefore compromising oxygen uptake at the respiratory surface. Additionally, while

plasma [AD] was shown to increase almost 5-fold during hypercapnic exposure, there were no significant changes in [nAD] (Perry and Gilmour, 1996) implying that adrenergic stimulation is responsible for acidotic compensation.

Hyperkalemia and adrenaline

Similarly to acidosis, the negative inotropic effects of hyperkalemia can also be alleviated by adrenergic stimulation. Concurrent adrenergic stimulation (10 μM AD) of isolated cardiac muscle strips counteracted the 50% decrease in contraction force associated with 10 mM K^+ (Kalinin and Gesser, 2002) and the complete loss of contractile force associated with 12.5 mM K^+ (Nielsen and Gesser, 2001). Perhaps the ameliorating effects of adrenaline during hyperkalemia are likewise mediated by β -AR as the effects can be counteracted in mammals with propranolol (Todd and Vick, 1971; DeFronzo *et al.*, 1981). As mentioned previously, hyperkalemia-induced depolarization inactivates cardiac Na^+ channels, preventing the upstroke of the cardiac action potential (Chapman and Rodrigo, 1987; Fozzard and Shorofsky, 1992). Potentially, adrenergic stimulation restores the action potential upstroke by increasing Ca^{2+} influx (Paterson *et al.*, 1992) and activates skeletal muscle and liver K^+ uptake (Elfellah and Reid, 1990) (Kes, 2001) via Na^+/K^+ -ATPase, all to moderate plasma K^+ concentrations.

Temperature as a modifier of cardiac performance

Effects on oxygen availability

Rainbow trout can encounter a wide range of seasonal temperatures, ranging from 2°C to 19°C. Outside their preferred temperature range of 11 to 13°C (Brett, 1964; Taylor *et al.*, 1996), conditions become less favourable, necessitating compensatory changes in physiological systems. In particular, temperature-dependant changes in oxygen solubility limit oxygen availability at high temperatures. Oxygen availability is further restricted by a temperature-induced right-shift in the haemoglobin-oxygen dissociation curve (Perry and Reid, 1994), therefore reducing the O₂ carrying capacity of the blood (Eddy, 1971), which in addition to reducing O₂ uptake at the gills, favours unloading at the skeletal muscles and reduces C_{vo2}. Moreover, routine O₂ needs of the heart increase with temperature (Farrell *et al.*, 1985; Mortensen and Gesser, 1999), further exacerbating the potential imbalance between myocardial O₂ supply and demand. Exercise at high temperatures can pose additional challenges including larger increases in blood P_{co2} and resulting larger decreases in haemoglobin-oxygen affinity when compared to similar changes in P_{co2} at lower temperatures (Eddy, 1971). Consequently, one of the objectives of this thesis is to determine how the hypoxic threshold for maximum cardiac performance changes as acclimation temperature increases from 10°C to 18°C.

Direct effects on myocardial tissue

In rainbow trout, increased temperature tends to have positive chronotropic and negative inotropic effects on cardiac muscle. Accordingly, increases in acclimation temperature are

associated with increased f_H (Heath and Hughes, 1973; Farrell, 1984; Graham and Farrell, 1989; Farrell and Jones, 1992; Farrell *et al.*, 1996; Aho and Vornanen, 1999), which can be up to 2-fold (25.8 to 53.6 min⁻¹) when temperatures in perfused trout hearts are increased from 5°C to 15°C (Graham and Farrell, 1989). Similar results were seen *in vivo* with acclimation temperatures of 4°C and 17°C (Taylor *et al.*, 1996) and with isolated ventricular strips at acclimation temperatures of 4°C and 17°C (Aho and Vornanen, 1999). Chronotropic effects of temperature are believed to arise from direct effects on pacemaker cells; however, temperature compensation involves extrinsic factors such as circulating catecholamines. Increases in f_H are necessary to support temperature-induced increases in metabolic rate (i.e., body O₂ demand) but decrease the time available for O₂ diffusion from the cardiac circulation to the spongy myocardium, posing problems for myocardial O₂ delivery. The effects of increased temperature on V_s are not as straightforward. As temperature increases above the optimum temperature (11-13°C as mentioned previously), V_s decreases (Farrell *et al.*, 1996) but V_s increases at values below the optimum temperature. Taylor *et al.* (1996) found that when acclimation temp was increased from 11°C to 18°C, V_s decreased from 1.3 ml beat⁻¹ to 0.4 ml beat⁻¹, but when temperature was increased from 4°C to 11°C V_s increased over 6-fold from 0.2 ml beat⁻¹ to 1.3 ml beat⁻¹. In order to simplify matters, this discussion will focus on the effects of temperature increase above the preferred temperature, as this is representative of the temperature change utilized in Chapter 3 of this thesis.

The relationship between f_H and V_s may be the source of conflicting results seen for the effect of temperature on Q_{max} . Temperature increases above the optimum have been shown to result in a decrease in Q_{max} *in vivo* (Taylor *et al.*, 1996); however, Farrell *et al.* (1996) found no change in Q_{max} with increased temperature, but this was likely due to the extreme

temperatures being studied (15-22°C). In contrast to Q_{max} , temperature increases from 11°C to 18°C had no effect on routine Q (Taylor *et al.*, 1996), suggesting that the detrimental effects of temperature increase in proportion to cardiac workload. This negative inotropy may be due to temperature-induced changes in ventricular Ca^{2+} sensitivity (Harrison and Bers, 1989; 1990).

Temperature and adrenaline

Temperature compensation is accomplished through extrinsic factors, including circulating catecholamines. Temperature acclimation is known to alter the response of the rainbow trout heart to AD (Ask *et al.*, 1981; Graham and Farrell, 1989; Farrell *et al.*, 1996). Specifically, an increase in acclimation temperature decreases AD sensitivity (Graham and Farrell, 1989; Keen *et al.*, 1993), and some of this change has been attributed to a temperature-dependent change in ventricular cell surface β -AR density. Keen *et al.* (1993) found that increasing the acclimation temperature of rainbow trout from 8°C to 18°C resulted in a ~65% decrease in myocardial β_2 -AR density. A comparison between rainbow trout acclimated at 8°C and 14°C yielded similar results (Gamperl *et al.*, 1998). Both studies found B_{max} to decrease by as much as 11% per 1°C increase in temperature.

β -adrenergic stimulation is extremely important in preserving the L-type Ca^{2+} current in isolated trout myocytes (Shiels *et al.*, 2000; Shiels *et al.*, 2003). Studies suggest that despite reduced AD sensitivity at high temperature, the positive inotropic effect of β -adrenergic stimulation increases in importance at higher temperatures (Graham and Farrell, 1989). β -AR plasticity is critical for maintaining cardiac performance in temperate species such as rainbow trout that experience a wide range of seasonal environmental temperatures.

Accordingly, one of the objectives of this thesis is to determine if a similar relationship between β -AR density and acclimation temperature exists in tropical species where acclimation mechanisms, such as β -AR plasticity, may be unnecessary due to relatively stable environmental temperatures.

Research Objectives and Hypotheses:

1. To quantify the venous oxygen threshold for maximum cardiac performance using *in situ* perfused hearts. It is hypothesized that the venous oxygen threshold for maximum cardiac performance during exercise-induced hypoxia will be higher than that previously reported for environmental hypoxia.
2. To quantify the inotropic and chronotropic effects of venous hyperkalemia on *in situ* perfused hearts.
3. To simulate the effects of post-exercise levels of hyperkalemia and acidosis in combination with hypoxia *in situ* and determine the hypoxic threshold for maximum cardiac performance with and without concurrent adrenergic stimulation. It is hypothesized that adrenergic stimulation will alleviate the detrimental effects of a combined hyperkalemic, acidotic and hypoxic exposure on maximum cardiac performance. It is further hypothesized that under these conditions the threshold for cardiac collapse will approximate that found in exercising fish *in vivo*.
4. To determine the effect of elevated temperature on the *in situ* hypoxic threshold for cardiac collapse associated with a hyperkalemic, acidotic and hypoxic exposure at 18°C. It is hypothesized that the hypoxic threshold for cardiac collapse under

hyperkalemic, acidotic, hypoxic conditions, with maximal adrenergic stimulation, will be higher at 18°C than at 10°C because β -adrenoreceptor (β -AR) density is known to decrease with increasing acclimation temperature in rainbow trout.

5. To determine whether the inverse relationship between β -adrenoreceptor density and temperature found in rainbow trout, a temperate species, also exists in African Catfish, a tropical species. If so this would suggest the β -AR mediated response is similar across species. Accordingly, it is hypothesized that β -AR density will show an inverse relationship with acclimation temperature in African Catfish.

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Chapter 2: The role of adrenergic stimulation in maintaining maximum cardiac performance in rainbow trout (*Oncorhynchus mykiss*) during hypoxia, hyperkalemia and acidosis at 10°C

Introduction

The extracellular environment is constantly fluctuating, with the consequence that cells and organs, such as the heart, must be able to function under a variety of conditions. For instance, strenuous exercise in rainbow trout (*Oncorhynchus mykiss*), like many animals, greatly alters the composition of the venous blood. During strenuous exercise and recovery venous blood becomes acidotic, with pH decreasing from pH 7.9 to 7.3-7.5 (Kiceniuk and Jones, 1977; Graham *et al.*, 1982; Holeton *et al.*, 1983; Turner *et al.*, 1983), hypoxic, with oxygen tension decreasing from 4.9 kPa to 2.1-1.1 kPa (Kiceniuk and Jones, 1977; Steffensen and Farrell, 1998; Farrell and Clutterham, 2003); and hyperkalemic, with venous plasma $[K^+]$ increasing from 2.5 mM to 3.4-4.7 mM (Holeton *et al.*, 1983; Turner *et al.*, 1983; Perry *et al.*, 1987; Thomas *et al.*, 1987; Nielsen and Lykkeboe, 1992; Holk and Lykkeboe, 1998). Such changes can be particularly taxing because the rainbow trout heart is nourished predominantly by venous blood (Santer and Greer Walker, 1980) and, despite exposure to the detrimental effects of hypoxia, hyperkalemia and acidosis, rainbow trout must maintain a high cardiac output (Q) during and after intense exercise. Therefore, to properly evaluate the detrimental effects of such changes in the extracellular environment in the context of fish exercise, one needs to know their combined effects on maximum cardiac

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performance. To the best of our knowledge, no one has yet examined the combined effects of exercise-induced hypoxia, hyperkalemia and acidosis on maximum cardiac performance.

What has been well studied in this regard are the negative inotropic and chronotropic effects of acidosis alone on both isolated cardiac muscle strips (Gesser and Jorgensen, 1982; Gesser *et al.*, 1982; Kalinin and Gesser, 2002) and working perfused heart preparations (Farrell and Milligan, 1986; Farrell *et al.*, 1986; Farrell *et al.*, 1988). Kalinin and Gesser (2002) observed a 60% decrease in isometric force of rainbow trout ventricular strips exposed to hypercapnic acidosis (pH 6.9), while Farrell *et al.* (1986) observed a ~10% decrease in maximum cardiac power output (PO_{max}), with hypercapnic acidosis (pH 7.4) in perfused rainbow trout hearts, a consequence of declines in both heart rate (f_H) and contractile force. It is thought that acidosis exerts its detrimental effects by competitively interfering with calcium-troponin binding (Williamson *et al.*, 1976; Fabiato and Fabiato, 1978; Gesser and Jorgensen, 1982) and effectively reducing the myocardial intracellular Ca^{2+} concentration.

Adrenergic stimulation can protect cardiac performance and counteract the acidosis-induced chronotropic and inotropic effects in both perfused hearts (Farrell *et al.*, 1983; Farrell and Milligan, 1986; Farrell *et al.*, 1986) and isolated cardiac muscle strips (Gesser and Jorgensen, 1982; Gesser *et al.*, 1982). An acidosis of pH 7.4 significantly reduced both Q and PO of perfused sea raven and ocean pout hearts; however, cardiac performance was fully restored when the acidosis was given in conjunction with 1 μ M adrenaline (AD) (Farrell *et al.*, 1983). Concurrent adrenergic stimulation (100 μ M AD) also maintained cardiac performance in isolated cardiac muscle strips, while acidotic cardiac muscle strips not exposed to AD exhibited a 30% decline in contractile force (Gesser *et al.*, 1982).

Adrenergic stimulation counteracts the acidotic impairment of calcium-troponin binding by increasing myocardial Ca^{2+} influx via the L-type Ca^{2+} channels (Shiels and Farrell, 1997; Vornanen, 1998). Adrenergic stimulation also activates erythrocyte Na^+/H^+ exchange (Tang *et al.*, 1988; Perry and Gilmour, 1996) which helps restore plasma and erythrocyte pH during exercise-induced acidosis (McDonald *et al.*, 1989).

The detrimental effects of hyperkalemia on cardiac performance in rainbow trout are known but less thoroughly studied. Hyperkalemia (10 mM and 12.5 mM K^+) reduces the contractive force of isolated heart strips by ~50% (Kalinin and Gesser, 2002) to ~100% (Nielsen and Gesser, 2001) respectively. Hyperkalemia reduces the resting membrane potential of myocardial cells (Chapman and Rodrigo, 1987; Hove-Madsen and Gesser, 1989), which in turn decreases the duration of the myocardial action potential, and thus the strength of myocardial contractions (Chapman and Rodrigo, 1987). Additionally, hyperkalemia in mammals ($\text{K}^+ > 5.5$ mM) has been shown to result in ventricular arrhythmia (Kes, 2001).

Similarly to acidosis, the negative inotropic effects of hyperkalemia can be alleviated by adrenergic stimulation. Concurrent adrenergic stimulation (10 μM AD) of isolated cardiac muscle completely eliminated a 50% decrease in contraction force associated with 10 mM K^+ (Kalinin and Gesser, 2002), and the complete loss of contractile force associated with 12.5 mM K^+ (Nielsen and Gesser, 2001). Since the effects of hyperkalemia on cardiac performance have only been examined on paced, isolated cardiac muscle strips, little is known about the chronotropic effects of hyperkalemic exposure. Consequently, one of the aims of this study is to quantify both inotropic and chronotropic effects of hyperkalemia in a perfused heart preparation.

The third important change in the extracellular environment of the trout heart during exercise is the venous oxygen tension (P_{vo_2}). During intense exercise, and as skeletal muscle oxygen demand increases, venous oxygen tension is reduced by ~50% (Kiceniuk and Jones, 1977). This reduces the oxygen gradient (driving diffusion of oxygen from the cardiac circulation to the myocardial tissues) at a time when myocardial oxygen consumption is concurrently increasing in proportion to the increased cardiac work during swimming (Farrell, 1985; Graham and Farrell, 1990). Hence, oxygen supply to approximately 70% of the ventricle becomes precarious at a time when it is most needed, despite the fact that coronary blood flow increases during exercise to the remaining myocardium (Axelsson and Farrell, 1993; Gamperl *et al.*, 1994a; Gamperl *et al.*, 1995). Severe hypoxia (≤ 1.6 kPa) decreases the isometric force of isolated cardiac muscle strips by 60-90% (Gesser, 1977; Gesser *et al.*, 1982; Overgaard and Gesser, 2004). Isolated perfused hearts can perform routine physiological workloads at 3.3 kPa, but this level of hypoxia decreases Q_{max} and PO_{max} by ~50% and ~80% respectively (Farrell *et al.*, 1989), which seems a rather high threshold given that P_{vo_2} decreases to around 2 kPa during prolonged swimming (Farrell and Clutterham, 2003). Clearly, the exact threshold will be determined by both the cardiac workload and the extracellular conditions experienced by the heart. With regard to the former, perfused trout hearts can generate a routine cardiac output even under near anoxic conditions provided the workload is sub-physiological (Arthur *et al.*, 1992; Overgaard *et al.*, 2004a). Therefore, to properly evaluate the effects of hypoxia it is necessary to examine maximum cardiac performance, which is now possible in trout using *in situ* heart preparations. In addition, while it has been theorized that a limited myocardial oxygen supply restricts exercise performance (Farrell, 2002), no studies have yet considered the

combined effects of hypoxia, acidosis, and hyperkalemia that are known to occur *in vivo*. Consequently, this study was designed to examine the effects of various levels of hypoxia on maximum cardiac performance under conditions simulating exercise *in vivo*. Hypoxia was studied alone and in conjunction with hyperkalemia (5 mM), acidosis (pH 7.5) and elevated catecholamines. We tested the hypothesis that adrenaline is critical in maintaining maximum cardiac performance under these conditions, which were intended to simulate those during and immediately after intense activity.

Materials and Methods

Fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) of both sexes (mass = $483 \text{ g} \pm 12 \text{ g}$; relative ventricular mass = $0.087\% \pm 0.002\%$) were obtained from a local fish hatchery (Richard Henley Farm, Langley, BC, Canada) and held indoors in 2000 L fibreglass tanks continuously supplied with dechlorinated tap water. The fish were maintained under a natural photoperiod at a temperature of $10^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Water temperature was maintained throughout the experimental period by utilizing either an immersible chiller or by heating the inflowing water with a heat exchanger of local construction. Fish were acclimated a minimum of two weeks prior to experimentation during which time they were fed commercial trout pellets (Pro-form Aquaculture Feeds, Chilliwack, BC, Canada) *ad libitum* three times per week.

Surgical Procedures

Fish were anaesthetized in an oxygenated solution of buffered tricaine methane sulfonate (MS-222) (0.1 g L^{-1} MS222 & 0.1 g L^{-1} NaHCO_3), weighed and placed on an operating table where their gills were continuously irrigated with chilled, oxygenated anaesthetic (0.05 g L^{-1} MS-222) buffered with 0.05 g L^{-1} NaHCO_3 . They were then injected with 1 ml kg^{-1} of heparinized saline (100 IU ml^{-1}) *via* the caudal vessels. An *in situ* perfused heart preparation was prepared as described in Farrell *et al.* (1986) as modified by Farrell *et al.* (1989). Briefly, a shallow lengthwise incision was made from the anal opening to an area just posterior to the pectoral girdle and a stainless steel input cannula was introduced into the sinus venosus *via* a hepatic vein. Perfusion of the heart, *via* the input cannula, was immediately commenced with chilled freshwater trout saline (composition below) containing 5.0 nM adrenaline (adrenaline bitartrate salt = AD) and 10 IU heparin per millilitre. A stainless steel output cannula was then secured into the ventral aorta at a point confluent with the bulbus arteriosus, and purse string sutures were used to occlude both ducts of Cuvier and destroy the cardiac branches of the vagus nerve. In addition, the spine was severed. The total time to prepare the perfused heart preparation was 15-20 minutes. All experimental procedures complied with the policies of the University Animal Care Committees of both Simon Fraser University and the University of British Columbia.

Following surgery, the fish was transferred to a temperature-controlled, physiological saline bath (124.1 mM NaCl , 2.5 mM KCl , 11.9 mM NaHCO_3 , $2.0 \text{ mM CaCl}_2 \cdot 2\text{H}_2\text{O}$, $0.2 \text{ mM NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, $3.4 \text{ mM Na}_2\text{HPO}_4$, $0.9 \text{ mM MgSO}_4 \cdot 7\text{H}_2\text{O}$; all chemicals from Sigma-Aldrich, Oakville, ON, Canada). The input cannula was immediately connected to an adjustable, constant-pressure reservoir, and the output cannula was connected to a separate

constant pressure head set at 4.9 kPa to mimic resting *in vivo* ventral aortic blood pressure. The height of the input pressure reservoir was adjusted to set routine cardiac output (Q) at approximately $17.0 \text{ ml min}^{-1} \text{ kg}^{-1}$ (Kiceniuk and Jones, 1977). Input (P_{in}) and output (P_{out}) pressure were measured through saline filled side arms (PE50 tubing) connected to disposable pressure transducers (DPT 6100, Smiths Medical, Kirchseeon, Germany). Cardiac outflow was continuously measured through the output line with an in-line electromagnetic flow probe (SWF-4, Zepada Instruments, Seattle, WA, USA) that had been previously calibrated with known flow rates of perfusate. The experimental solutions (both the perfusate and the saline bath) were contained in water-jacketed glassware so that temperature could be maintained at 10°C (Brinkman Instruments Inc., Mississauga, ON, Canada). Hearts were allowed to equilibrate for 5-10 minutes while receiving normoxic perfusate (see below) before the experiment commenced. The coronary circulation was not perfused in this preparation.

Test Conditions

Cardiac performance was assessed under several different protocols using the test conditions defined below. A tonic level of adrenergic stimulation (5 nM AD), consistent with that found in resting rainbow trout (Milligan *et al.*, 1989), was used in all situations except when the protective effect of AD was being evaluated. In this latter situation, 500 nM of AD was used in order to ensure maximum adrenergic stimulation, as studies in rainbow trout have reported post-exercise [AD] as high as $212 \text{ nM} \pm 89 \text{ nM}$ (Butler *et al.*, 1986).

Control (normoxic) condition: All preparations started under this condition. Freshwater trout saline (124.1 mM NaCl, 2.5 mM KCl, 0.9 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, D-glucose 5.6 mM, 11.9 mM NaHCO_3) was gassed with 0.5% CO_2 (balance air) to achieve a pH of 7.9 and an oxygen level of 20.0 kPa. Therefore, preliminary experiments ($n = 8$, data not shown) were performed to show no significant difference in maximum cardiac performance between hyperoxic hearts (95.5% O_2 , 0.5% CO_2) and hearts perfused with air, which was the control level of oxygen for all experiments.

Hyperkalemia: The composition of the hyperkalemic perfusate was the same as the control perfusate except additional KCl was added to increase the $[\text{K}^+]$ to either 5.0 mM or 7.5 mM.

Acidosis: To achieve a pH of 7.5, the concentration of NaHCO_3 in the control perfusate was decreased to 10.1 mM and the solution was equilibrated with a gas mixture containing 1.0% CO_2 (balance air).

Hypoxia: Control perfusate was made hypoxic by equilibrating it with 0.5% CO_2 and an amount of oxygen corresponding to the desired level of hypoxia (balance nitrogen). The hypoxia levels used in kPa were 20, 12.6, 10, 6.7, 5.0, 3.3, 2.7, 2.0 and 1.3. Premixed, calibrated gases (Praxair, Vancouver, BC, Canada) and Wostoff gas pumps (M303/a-F, Bochum, Germany) were used to generate gas mixtures.

Hyperkalemia and acidosis: Acidotic perfusate (to achieve a pH of 7.5), was made hyperkalemic by increasing the $[\text{K}^+]$ to either 5.0 mM or 7.5 mM.

Hyperkalemia, acidosis and hypoxia: A hyperkalemic (5.0 mM), acidotic (pH 7.5) perfusate (as above) was gassed with a mixture of 1.0% CO_2 and various concentrations of O_2 (balance nitrogen) in order to achieve particular levels of hypoxia as specified for the hypoxic perfusate.

Experimental Protocols

Maximum cardiac performance was repeatedly assessed under 3-5 conditions. By initially measuring both maximum cardiac output (Q_{max}) and maximum cardiac power output (PO_{max}) under normoxic, control conditions each heart acted as its own control. To determine Q_{max} , P_{in} was gradually increased in increments of ~ 0.05 kPa until cardiac output reached a plateau (usually around 0.4 kPa). To assess PO_{max} , P_{in} was left at its maximum and P_{out} was increased in a stepwise fashion in ~ 0.5 kPa increments until PO reached a plateau. After PO_{max} was determined, P_{out} and P_{in} were returned to resting levels and the heart was allowed to recover (~ 5 min) before being exposed to the next perfusate. Hearts were exposed to each perfusate for a total of 15 minutes; this time period ensured continued viability of the photosensitive AD (which was renewed with each change in perfusate) while remaining physiologically relevant, as P_{vo_2} can take more than 20 minutes to return to normal following exhaustive exercise (Farrell and Clutterham, 2003). Under some extreme conditions individual hearts did not perform for 15 minutes, succumbing to a cardiac collapse (i.e. cardiac output approached zero). These hearts were terminated early, the duration noted and normoxic conditions restored. Hearts that were unable to complete both cardiac performance tests were considered to have failed under that test condition. The following sets of protocols were used, each with its own order and combination of test conditions.

Series I (Hyperkalemia alone)

The main purpose of this series ($n = 9$) was to define a level of hyperkalemia that was physiologically relevant but did not result in irreversible cardiac failure under normoxic

conditions. Exercise *in vivo* increases plasma K^+ to ~ 5.0 mM (Thomas *et al.*, 1987) but previous studies done on isolated cardiac muscle strips have tested higher concentrations of 5.0 – 12.5 mM. The order of the test conditions was: (1) control, (2) 5 mM K^+ (3) 7.5 mM K^+ (4) control, and (5) 7.5 mM K^+ with 500 nM AD.

Series II (Acidosis & Hyperkalemia)

The purpose of series II was to quantify under normoxic conditions the (a) effects of a combined hyperkalemic, acidotic exposure on maximum cardiac performance, and (b) the ameliorative effects of adrenaline. Several levels of hyperkalemia were tested in order to determine the tolerance threshold for these conditions. Individual hearts ($n = 8$) were tested under the following conditions (1) control, (2) 5.0 mM K^+ , pH 7.5, (3) control, (4) 5.0 mM K^+ and pH 7.5 with 500 nM AD, and (5) 7.5 mM K^+ and pH 7.5 with 500 nM AD. In addition, three preliminary preparations were tested using 7.5 mM K^+ and 5 nM AD at a pH of 7.5 directly after the first control step. However, this exposure resulted in an almost immediate decrease in cardiac output leading to a rapid (< 5 min), irrecoverable cardiac collapse. In view of this, 5.0 mM K^+ was used for all subsequent combined hyperkalemic exposures.

Series III

The purpose of series III was to determine the hypoxic thresholds for maximum cardiac performance for hypoxia alone and in conjunction with hyperkalemic (5.0 mM) acidosis (pH 7.5). The levels of respiratory acidosis and hyperkalemia chosen mimic those found in the plasma of exercising rainbow trout *in vivo* (Milligan and Wood, 1987; Nielsen

and Lykkeboe, 1992). Hearts were exposed to the following sequence of test conditions (1) control (normoxia), (2) hypoxia, (3) control, (4) hypoxia, 5.0 mM K^+ and pH 7.5 with 5 nM AD, and (5) hypoxia, 5.0 mM K^+ and pH 7.5 with 500 nM AD. The specific hypoxia levels used were 12.6 kPa (n = 6 fish), 10 kPa (n = 10 fish), 6.7 kPa (n = 6 fish) and 5.0 kPa (n = 3 fish). At lower oxygen tensions the combined hypoxic, hyperkalemic, acidotic exposure with 5.0 nM AD (step 4) resulted in myocardial failure. As this appeared to be specifically related to the absence of maximal adrenergic stimulation, the protocol was modified for series IV and V to permit further exploration of the hypoxic thresholds.

Series IV

To preclude the problem of a heart receiving tonic [AD] not being able to tolerate the hyperkalemic, acidotic test condition at P_{vo_2} levels below 6.7 kPa, series IV studied hypoxic thresholds with an abbreviated series of test conditions. The following sequence of perfusates was used: (1) control (normoxia), (2) hypoxia, (3) control, and (4) hypoxia, 5.0 mM K^+ and pH 7.5 with 500 nM AD. The specific oxygen tensions were 5.0 kPa (n = 7 fish), 3.3 kPa (n = 8 fish), 2.7 kPa (n = 8 fish) and 2.0 kPa (n = 3 fish).

Series V

Because series IV revealed hearts receiving only tonic adrenergic stimulation could not tolerate hypoxia alone below 2.7 kPa, hearts in series V were subjected to a further abbreviated experimental protocol: (1) control, (2) hypoxia, 5.0 mM K^+ and pH 7.5 with 500 nM AD, and (3) control. The hypoxia levels tested were 2.7 kPa (n = 6 fish), 2.0 kPa (n = 6

fish) and 1.3 kPa ($n = 4$ fish). These test conditions best simulate the changes in venous blood pH, $[K^+]$, P_{vo_2} and $[AD]$ seen *in vivo* during intense activity and recovery.

Calculations and Statistical Analysis

All experimental data was collected using data acquisition software (Labview version 5.1, National Instruments, Austin, TX, USA), which allowed for real-time measurements of f_H , P_{in} , P_{out} , Q , and PO . Statistical differences within test groups were determined by one-way repeated measures analysis of variance (ANOVA). When warranted, the Holm-Sidak procedure was used for *post hoc* multiple comparisons. Sigma Stat (3.0, SPSS Inc., San Rafael, CA, USA) was used for all statistical analysis. For statistical comparisons $\alpha = 0.05$.

Results

Hyperkalemia alone

Based on the experiments performed in series I, both levels of hyperkalemia significantly ($P < 0.05$) decreased cardiac performance in a dose-dependant manner when compared to control (Fig. 2.1). The 30% decrease in Q_{max} and PO_{max} with 5.0 mM K^+ was caused by a 25% decrease in f_H and a 10% decrease in maximum stroke volume (V_s). Similarly, the 60% reduction in Q_{max} and PO_{max} with 7.5 mM K^+ was caused by a 45% decrease in f_H and a 25% decrease in maximum V_s . A noticeable arrhythmia also developed near the end of the 7.5 mM K^+ exposure. Despite these effects of hyperkalemia, maximum cardiac performance was fully restored when hearts were returned to control conditions (Fig. 2.1). Maximal adrenergic stimulation significantly improved maximum cardiac performance

of hyperkalemic (7.5 mM K⁺) hearts, with increases in both Q_{max} and PO_{max} to within 20% ($P < 0.05$) of their original performance under control conditions (Fig. 2.1).

Hyperkalemia combined with acidosis

The results for 5 mM hyperkalemia alone, and in combination with acidosis (pH 7.5), are presented in figure 2. Hyperkalemia and acidosis significantly decreased ($P < 0.05$) both Q_{max} ($-65.9\% \pm 6.0\%$) and PO_{max} ($-55.2\% \pm 14.6\%$) from control, and to a greater (20-30%) degree when compared to 5.0 mM K⁺ alone. The additional 20-30% decrease in Q_{max} was mainly due to a further decrease ($-62.0\% \pm 6.6\%$, $P < 0.05$) in f_H as maximum V_s was still only depressed by 10% ($9.73\% \pm 4.8\%$, $P > 0.05$). The combined hyperkalemic, acidotic condition also resulted in a pronounced cardiac arrhythmia. Nevertheless, hearts fully recovered when returned to control conditions (not shown). In contrast, exposure to acidosis and 7.5 mM K⁺ resulted in rapid, irrecoverable cardiac collapse (associated with severe cardiac arrhythmia). Maximal adrenergic stimulation completely prevented the debilitating effect of 5 mM K⁺ and acidosis, allowing the hearts to perform at control levels of Q_{max} and PO_{max} (Fig. 2.2). Moreover, concurrent maximum adrenergic stimulation allowed hearts to perform under the previously lethal conditions of 7.5 mM K⁺ and acidosis, but with both Q_{max} and PO_{max} ~35% lower than control ($P < 0.05$; Fig. 2.2).

Hypoxic thresholds without hyperkalemia and acidosis

The first three test conditions in series III and IV provided an assessment of the effects of hypoxia alone and these data are summarized in figure 3. Hypoxia at 12.6 kPa and 10 kPa had no significant effect on Q_{max} and PO_{max} either during hypoxia or with subsequent

normoxic exposure (Fig. 2.3). However, hypoxic levels between 6.7 and 3.3 kPa not only significantly decreased Q_{max} and PO_{max} by 10-25% (Fig. 2.3), maximum performance did not show any immediate recovery during subsequent normoxia from the level seen under hypoxia. At 2.7 kPa, 3 out of 8 hearts failed during the hypoxia treatment, and all hearts failed during the 2.0 kPa ($n = 3$) and 1.3 kPa ($n = 3$) treatments. Based on these results, it appears the hypoxic threshold for impairment of Q_{max} is between 10 and 6.7 kPa, and the threshold for complete cardiac failure under these conditions is between 2.7 and 2.0 kPa.

Hypoxic thresholds with hyperkalemia and acidosis

As expected (based on the results of series III for normoxic hearts) the combined effects of acidosis and hyperkalemia in series IV impaired Q_{max} and PO_{max} by 38% to 66% ($P < 0.05$) when $P_{vo_2} \geq 6.7$ kPa (Fig. 2.4). Thus, hypoxia ≥ 6.7 kPa had no additive effect on maximum performance when compared to hyperkalemia and acidosis alone. Similar to normoxia, adrenergic stimulation fully restored Q_{max} and PO_{max} with 5 mM K^+ and acidosis at 12.6 kPa and 10 kPa, while the protective effect of AD was apparently lost at 6.7 kPa (Fig. 2.4), this result could have been due to poor recovery from prior exposures (hypoxia alone or hypoxia, hyperkalemia, and acidosis with tonic [AD]) in this series of experiments. Therefore, series V was designed to eliminate this possibility.

In series V, concurrent adrenergic stimulation protected maximum cardiac performance under hyperkalemia and acidosis down to hypoxia levels of 2.0 kPa, because neither Q_{max} nor PO_{max} were significantly different from control (Fig. 2.4). At hypoxia levels of 1.3 kPa, however, maximal adrenergic stimulation only partially protected cardiac performance, as Q_{max} and PO_{max} were reduced by $29.4 \pm 3.3\%$ and $43.6\% \pm 2.8\%$

respectively (Fig. 2.4). Moreover, following the hypoxic, hyperkalemic, acidotic exposure, with 500 nM AD, hearts exposed to 2.7, 2.0, and 1.3 kPa did not recover from these effects when returned to normoxic conditions (data not shown, $P < 0.05$). Therefore, this suggests that under these hyperkalemic, acidotic conditions with adrenergic stimulation the hypoxic threshold for maximum cardiac performance is 2.0 kPa, but in the absence of adrenergic stimulation, there is no refuge from cardiac impairment.

Discussion

The *in situ* perfused heart preparation utilized in this study can perform at levels approximating maximum *in vivo* cardiac performance, and remain stable for several hours (Farrell *et al.*, 1986; Farrell, 2002; Overgaard *et al.*, 2004b). Correspondingly, our values for maximum cardiac performance under normoxic conditions ($Q_{max} = 51.3 \pm 1.7 \text{ ml min}^{-1} \text{ kg}^{-1}$; $PO_{max} = 5.9 \pm 0.2 \text{ mW g ventricle}^{-1}$) are similar to previous *in vivo* and *in situ* studies done in rainbow trout at 10°C. Reported maximum cardiac output values in previous studies ranged from 43.9 to 62.5 $\text{ml kg}^{-1} \text{ min}^{-1}$, while maximum power output values ranged from 5.1 to 6.9 $\text{mW g ventricle}^{-1}$ (Kiceniuk and Jones, 1977; Farrell *et al.*, 1991; Faust *et al.*, 2004; Gamperl *et al.*, 2004; Overgaard *et al.*, 2004b). Potential variations among studies due to fish stocks, fish size and level of adrenergic stimulation are not accounted for in these comparisons. As our longest experiments lasted for only 100 minutes, we are confident that time-induced deterioration was not significant.

The goal of this experiment was to examine consequences on maximum cardiac performance of the venous extracellular conditions experienced during and after exhaustive exercise because the combination of factors has not been studied previously. The

hyperkalemia, acidosis and hypoxia conditions that we used were intended to simulate those seen in the plasma of maximally exercising rainbow trout *in vivo* (Milligan and Wood, 1987; Nielsen and Lykkeboe, 1992). Our results confirmed that acidosis and hypoxia decrease both the force (Farrell *et al.*, 1983; Driedzic and Gesser, 1994) and frequency (Gesser and Poupa, 1983) of myocardial contractions. In addition, we confirmed that hyperkalemia has a detrimental effect on contraction force (Kalinin and Gesser, 2002) and negatively affects contraction frequency (this study). A novel finding is that without the chronotropic and inotropic protection provided by maximum adrenergic stimulation, physiologically relevant acidosis and hyperkalemia prevent maximum cardiac performance even under normoxic conditions. In fact, with only tonic adrenergic stimulation, complete cardiac collapse occurred at a P_{vO_2} level below 6.7 kPa. However, when hearts were maximally stimulated with adrenaline, the hypoxic threshold for maximum cardiac performance under physiologically relevant hyperkalemic and acidotic conditions was lowered to less than 2.0 kPa. This finding is consistent with earlier work that had shown that isolated perfused hearts with tonic adrenergic stimulation performed routine physiological workloads to 3.3 kPa, but this level of hypoxia decreased Q_{max} and PO_{max} by ~50% and 80% respectively (Farrell *et al.*, 1989). Consequently, this study has clearly demonstrated for the first time that maximum adrenergic stimulation is necessary for maximum cardiac performance to occur at the levels of venous hypoxia, hyperkalemia and acidosis seen during intense activity and recovery *in vivo*.

The importance of adrenergic stimulation for the hypoxic myocardium corresponds well with what is known about the role of hypoxia in releasing catecholamines into the circulation of rainbow trout. Perry and Reid (1992) found that rainbow trout exposed to a

graded hypoxia challenge released adrenaline when arterial blood oxygen tension (arterial P_{O_2}) fell below 3.4 kPa. Here we found that hearts exposed to hypoxia ≤ 3.3 kPa with only tonic adrenergic stimulation lasted less than 5 minutes before undergoing a catastrophic cardiac collapse. In contrast, hearts exposed to a hyperkalemic, acidotic perfusate in conjunction with maximal adrenergic stimulation were able to function maximally at levels of hypoxia as low as 2.0 kPa.

We have shown that adrenergic stimulation can counteract the negative chronotropic and inotropic effects of hypoxia, hyperkalemia and acidosis. In fish adrenergic stimulation of cardiac tissue is mediated by the β -adrenoceptor (β -AR) signalling pathway (Ask *et al.*, 1981; Temma *et al.*, 1986; Gamperl *et al.*, 1994c). β -AR mediated increases in myocardial Ca^{2+} influx help offset the deleterious effects of both hyperkalemia and acidosis. Ca^{2+} influx restores the action potential upstroke lost during hyperkalemia (Paterson *et al.*, 1992), and counteracts the acidotic impairment of calcium-troponin binding. β -AR stimulation also helps restore plasma and erythrocyte pH by activating erythrocyte Na^+/H^+ exchange (Tang *et al.*, 1988; Perry and Gilmour, 1996). In addition, direct adrenergic stimulation of pacemaker cells opposes hypoxic bradycardia by increasing pacemaker self-excitation rate (Tibbits *et al.*, 1992).

One major difference between the present study and *in vivo* is the lack of a coronary circulation. The coronary circulation provides arterial blood to the 30% of the ventricle that comprises the compact myocardium; spongy myocardium which receives oxygen solely from the cardiac circulation (venous blood) comprises the remaining 70% (Santer and Greer Walker, 1980; Tota, 1983; Davie and Farrell, 1991). *In vivo*, the coronary circulation is not necessary to maintain routine cardiac performance, as demonstrated by coronary ablation

experiments (Daxboeck, 1982; Steffensen and Farrell, 1998), although routine flow does occur in the coronary arteries (Axelsson and Farrell, 1993; Gamperl *et al.*, 1994a; Gamperl *et al.*, 1995). Thus, during routine conditions, *in vivo* oxygen diffusion from venous blood is presumably sufficient to meet the needs of both compact and spongy myocardium, and presumably this would reflect the routine P_{vo_2} s found in trout of around 3-4 kPa. When P_{vo_2} of the cardiac circulation is reduced, as happens during exercise or environmental hypoxia, coronary blood flow increases (Gamperl *et al.*, 1994a; Gamperl *et al.*, 1994b) up to 2 fold (Gamperl *et al.*, 1995), reflecting the increased oxygen needs of the compact myocardium and the fact that oxygen diffusion from the lumen to the compacta becomes limited relative to this demand. Indeed, without this coronary supply, Steffensen and Farrell (1998) found that coronary-ligated rainbow trout reduced cardiac workloads by an estimated 37% during a hypoxic swimming challenge. Hence, while the coronary circulation increases in importance during exercise, the majority of the ventricular myocardial oxygen supply comes from venous blood, and so a venous oxygen threshold must exist below which the spongy myocardium fails. Thus, the lack of a coronary circulation in the present study will tend to overestimate the hypoxic thresholds for maximum cardiac performance. However, the coronary artery is difficult to cannulate while maintaining the integrity of the pericardium, which is integral to maximizing PO of the heart (Farrell *et al.*, 1988). Agnisola *et al.* (2003) found that coronary perfusion in isolated trout hearts at 10°C can increase cardiac stroke work by 12%, from 3.36 to 3.77 mJ g⁻¹. Even with coronary perfusion, the PO_{max} was only 56% of the control value obtained here (we assumed $f_H = 60 \text{ min}^{-1}$ since the information was not provided).

An additional factor that sustains lower P_{vo_2} thresholds *in vivo* is the oxygen buffering capacity of haemoglobin. Specifically, unloading of oxygen that occurs on the steep portion of the dissociation curve will have little effect on P_{vo_2} , and thus the oxygen diffusion gradient to the myocardium can remain high. In contrast, the linear nature of oxygen solubility in saline and its lower oxygen capacitance means that cardiac oxygen removal from saline decreases P_{o_2} more so than oxygen extraction from blood. Although the oxygen buffering capacity of haemoglobin and the presence of a coronary circulation allow for lower P_{o_2} thresholds *in vivo* than we measured here, neither of these factors are likely to affect the main finding that adrenaline protected the heart under adverse conditions.

Farrell and Clutterham (2003) used a fibreoptic micro-optode to measure the P_{vo_2} *in vivo* during maximal exercise in rainbow trout and discovered that at even the most severe exercise intensity P_{vo_2} did not drop below 2.1 kPa, a value that corresponds closely to the present study in which maximum cardiac performance at 2.0 kPa was not significantly different from that observed under control conditions. At the next lowest tested hypoxia value in this study (1.3 kPa), PO_{max} decreased by 43.6%. The correspondence of these findings suggests that although the *in vivo* venous oxygen threshold may be lower than that determined here, the difference may not be that great.

Any hypoxic threshold will be influenced by the absolute level of cardiac work (van Raaij *et al.*, 1996), as shown by the ability of rainbow trout hearts to perform sub-physiological workloads under near anoxic conditions (Arthur *et al.*, 1992). Therefore, comparisons of hypoxic thresholds *in vivo* need to incorporate the work load of the heart, as shown below. A situation comparable to the absence of coronary perfusion of the perfused heart is coronary ligation *in vivo*. Steffensen and Farrell (1998) swam coronary-ligated

rainbow trout in hypoxic water (5.2 kPa) and this resulted in a P_{vo_2} threshold of 10 torr (1.3 kPa). From their data, we can estimate that PO_{max} was 4.1 mW g⁻¹ ventricle for coronary-ligated fish at this P_{vo_2} threshold. [Ventral aortic blood pressure (P_{va}) was ~50 cm H₂O (~4.9 kPa). We assume that Q_{max} was 50 ml min⁻¹ kg⁻¹ (as above) in coronary-ligated and non-ligated fish (as suggested by the work of Gamperl *et al.*, 1994a), and that rainbow trout have a ventricular mass of ~1 g kg⁻¹] By comparison, perfused hearts subjected to an acidotic, hyperkalemic challenge at a comparable P_{vo_2} level of 1.3 kPa generated a PO_{max} of only 2.6 mW g⁻¹ ventricle. Since PO_{max} was 5.9 mW g⁻¹ ventricle at 2.0 kPa, this comparison not only reemphasizes the importance of the coronary circulation in maintaining maximum cardiac performance during intense exercise, but reemphasise that the difference between the *in vivo* and *in vitro* hypoxic thresholds may not be that great.

Rainbow trout hearts have a limited glycolytic potential and a PO of 1.5 mW g ventricle⁻¹ could be maintained for 20 minutes during anoxia at 10°C (Overgaard *et al.*, 2004a). While this PO is well below the PO_{max} here, the possibility still exists that a small component of maximum cardiac performance near the hypoxic threshold could have been supported by glycolysis during the short-term hypoxic exposures used here. If this is the case, we would have underestimated the hypoxic threshold. Implicit with this possibility is that if P_{vo_2} does fall below the hypoxic threshold *in vivo*, a component of post-exercise cardiac performance could be briefly fuelled by glycolysis.

Previously, Gamperl *et al.* (2001) found that rainbow trout hearts were stunned when exposed to extreme hypoxia ($PO_2 < 5$ mm Hg) at sub-physiological workloads, with Q_{max} decreasing by 23-38% upon return to control conditions. Similarly, we found that cardiac recovery was compromised by some of the hypoxic conditions. This was true for hypoxia

alone, as well as the combination of hypoxia, hyperkalemia, and acidosis. The majority of hearts exposed to hypoxia alone at levels below 10 kPa experienced impaired recovery, and this may have led to an underestimation of the protection afforded by the maximum adrenergic stimulation treatment that followed. The converse may also be true since prior exposure to hypoxia may confer a protective advantage. Hypoxic pre-conditioning has been indirectly shown to confer a protective advantage in some (Gamperl *et al.*, 2001) but not all strains of rainbow trout (Gamperl *et al.*, 2004; Overgaard *et al.*, 2004b). Nevertheless, hypoxia of 2.7 kPa did not result in preconditioning here as hearts pre-exposed to hypoxia experienced a larger reduction in Q_{max} compared to hearts with no previous hypoxic exposure.

In summary, we conclude that adrenaline is critical in maintaining maximum cardiac performance during conditions that simulate those observed in venous blood during and following intense activity. Adrenergic stimulation, when administered in conjunction with hypoxia, hyperkalemia and acidosis, was found to lower the hypoxic threshold for cardiac collapse from 5.0 kPa to less than 1.3 kPa, a value that corresponds closely to P_{vo_2} levels found in maximally exercising rainbow trout.

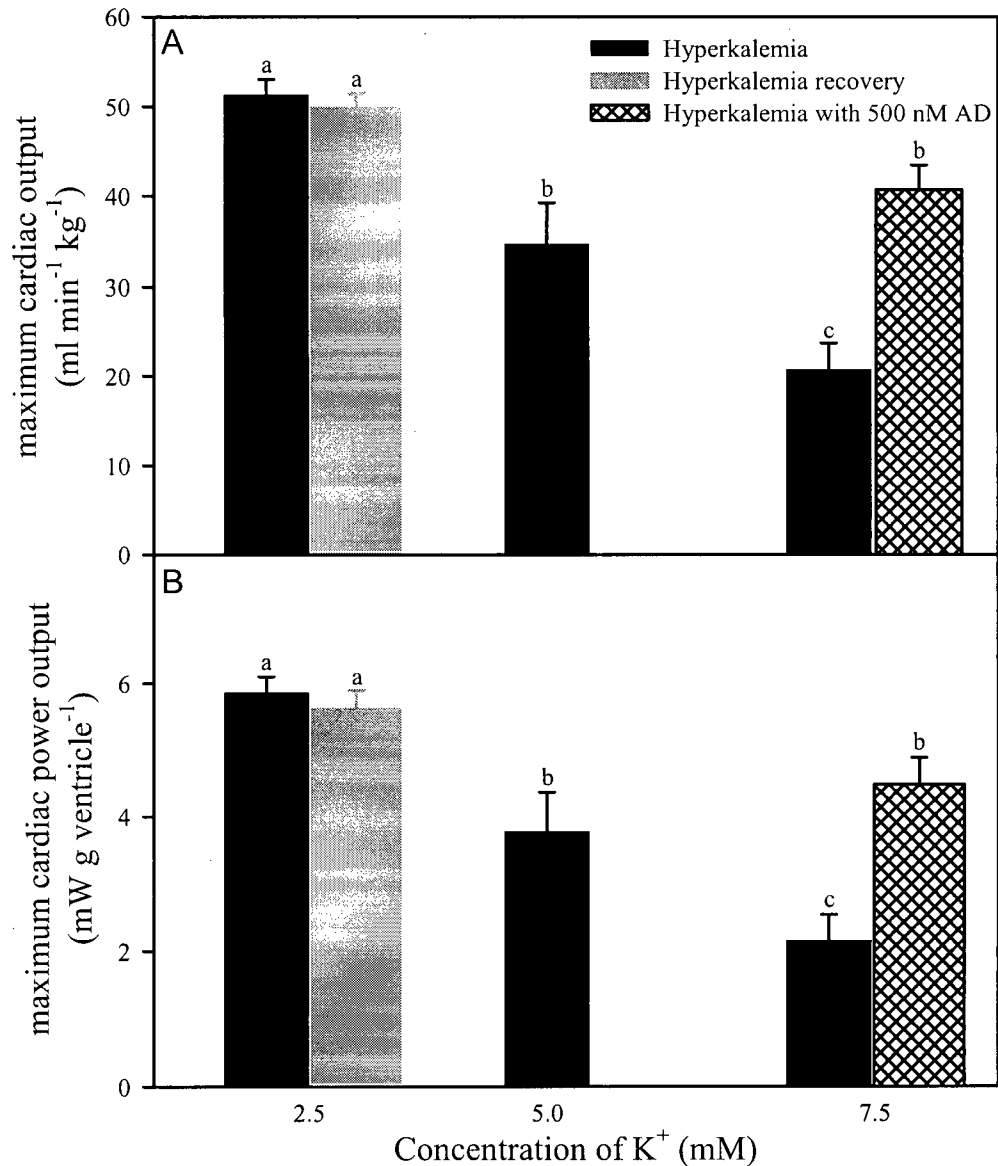


Figure 2.1: The effects of hyperkalemia on maximum cardiac output (A) and maximum cardiac power output (B) of perfused rainbow trout hearts at 10°C, pH 7.9. Values are reported as mean \pm SEM. Individual hearts ($n = 9$) were exposed to the following sequence of perfusates: (1) control (normoxia), (2) 5 mM K^+ , (3) 7.5 mM K^+ , (4) control (recovery), and (5) 7.5 mM K^+ with 500 nM epinephrine (AD). Repeated measures one-way ANOVA and a Holm-Sidak multiple comparisons test were used to compare treatment means. Dissimilar letters denote significant differences at $\alpha = 0.05$.

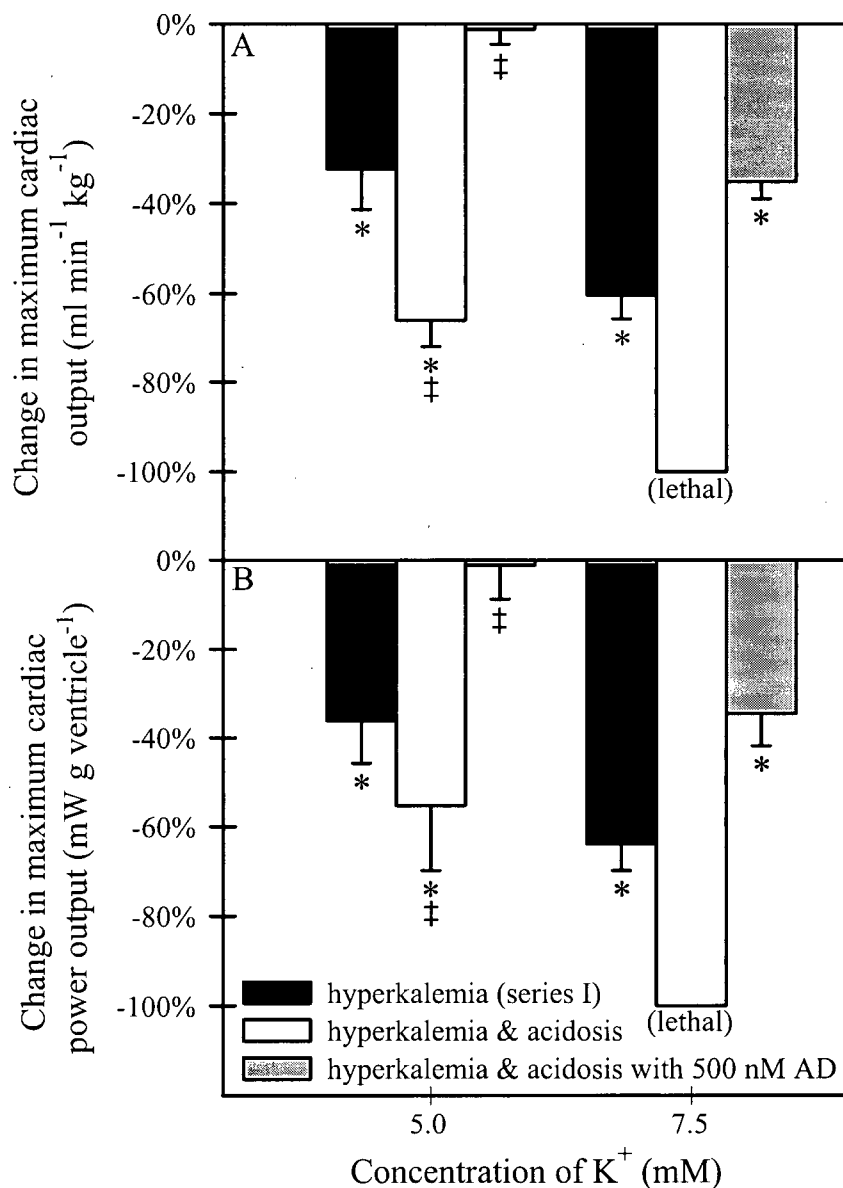


Figure 2.2: The effects of hyperkalemia and acidosis (pH = 7.5) on maximum cardiac output (A) and maximum cardiac power output (B) of perfused rainbow trout hearts at 10°C. Values are reported as mean \pm SEM. Individual hearts (n = 8) were exposed to the following sequence of perfusates: (1) control (normoxia), (2) 5.0 mM K⁺, pH 7.5, (3) control (recovery), (4) 5.0 mM K⁺ and pH 7.5 with 500 nM AD, and (5) 7.5 mM K⁺ and pH 7.5 with 500 nM AD. Values from series I are presented for comparison purposes (see Fig. 2.1). One-way ANOVA and a Holm-Sidak multiple comparisons test were used to compare treatment means. *denotes a significant difference from control while ‡ denotes a significant difference from pH 7.9 at that particular level of hyperkalemia, $\alpha = 0.05$. Three preliminary preparations were exposed to 7.5 mM K⁺ and 5 nM AD at a pH of 7.5 directly after the first normoxia step. However, this exposure resulted in an almost immediate decrease in cardiac output leading to a rapid (< 5 min), irrecoverable cardiac collapse.

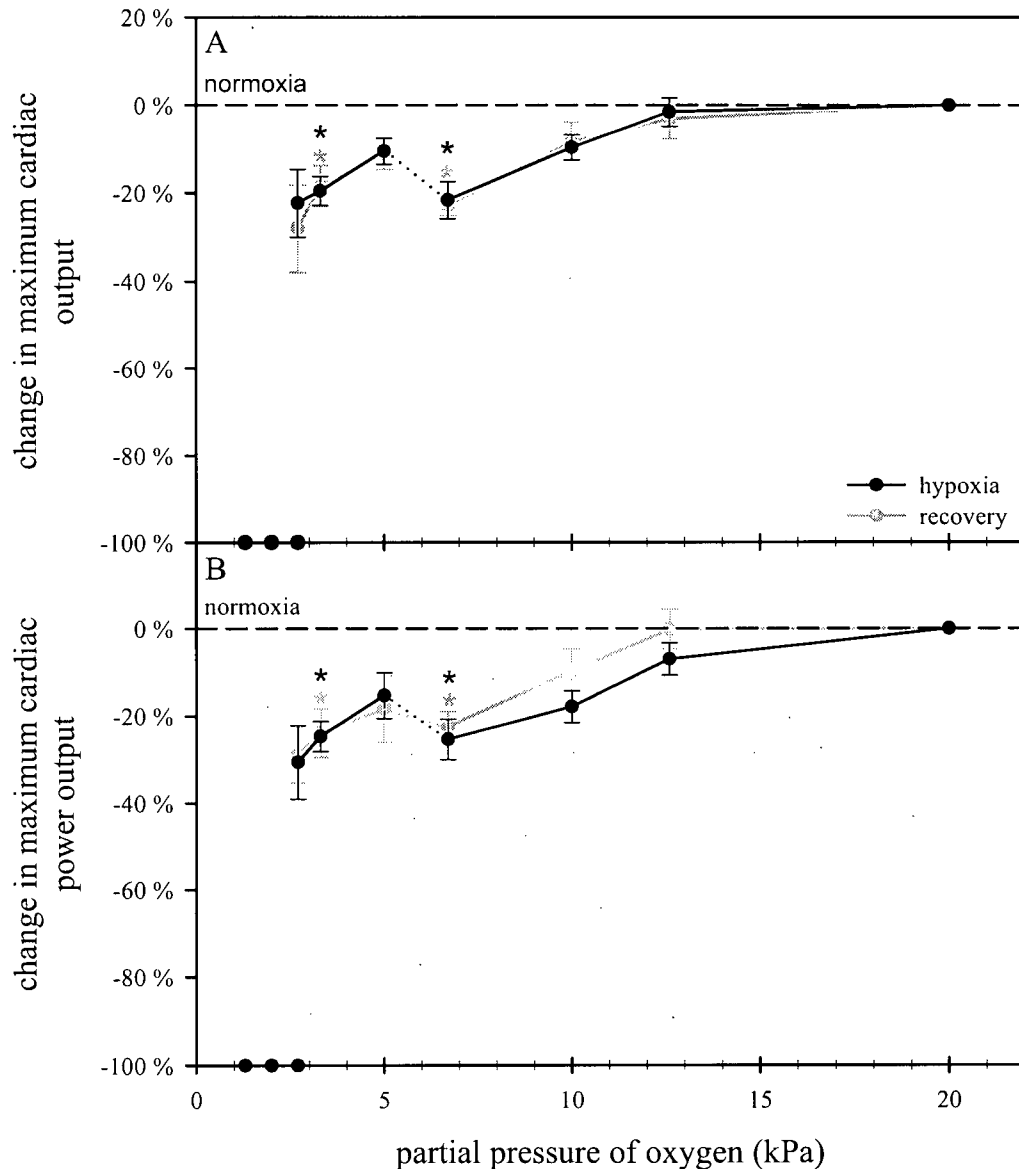


Figure 2.3: The maximum cardiac output (A) and maximum cardiac power output (B) of perfused rainbow trout hearts at 10°C under hypoxic perfusate. The grey line indicates the level of recovery during a subsequent exposure to normoxic conditions. Individual groups of hearts were exposed to specific hypoxia levels as indicated on the x-axis. Results shown are from two different series of experiments, as indicated by the separate (and discontinuous) line segments; however, experimental protocols were identical up until this point. The number of hearts used for each experiment was series III: 12.6 kPa (n = 6), 10 kPa (n = 10), 6.7 kPa (n = 6), and series IV: 5.0 kPa (n = 7), 3.3 kPa (n = 8), 2.7 kPa (n = 5). Values are reported as percent change from an original assessment under normoxic conditions. One-way repeated measures ANOVA and Holm-Sidak multiple comparisons tests were used to compare treatment means and each heart acted as its own control. Values shown are means \pm SEM. *Denotes a significant difference from normoxia ($\alpha = 0.05$).

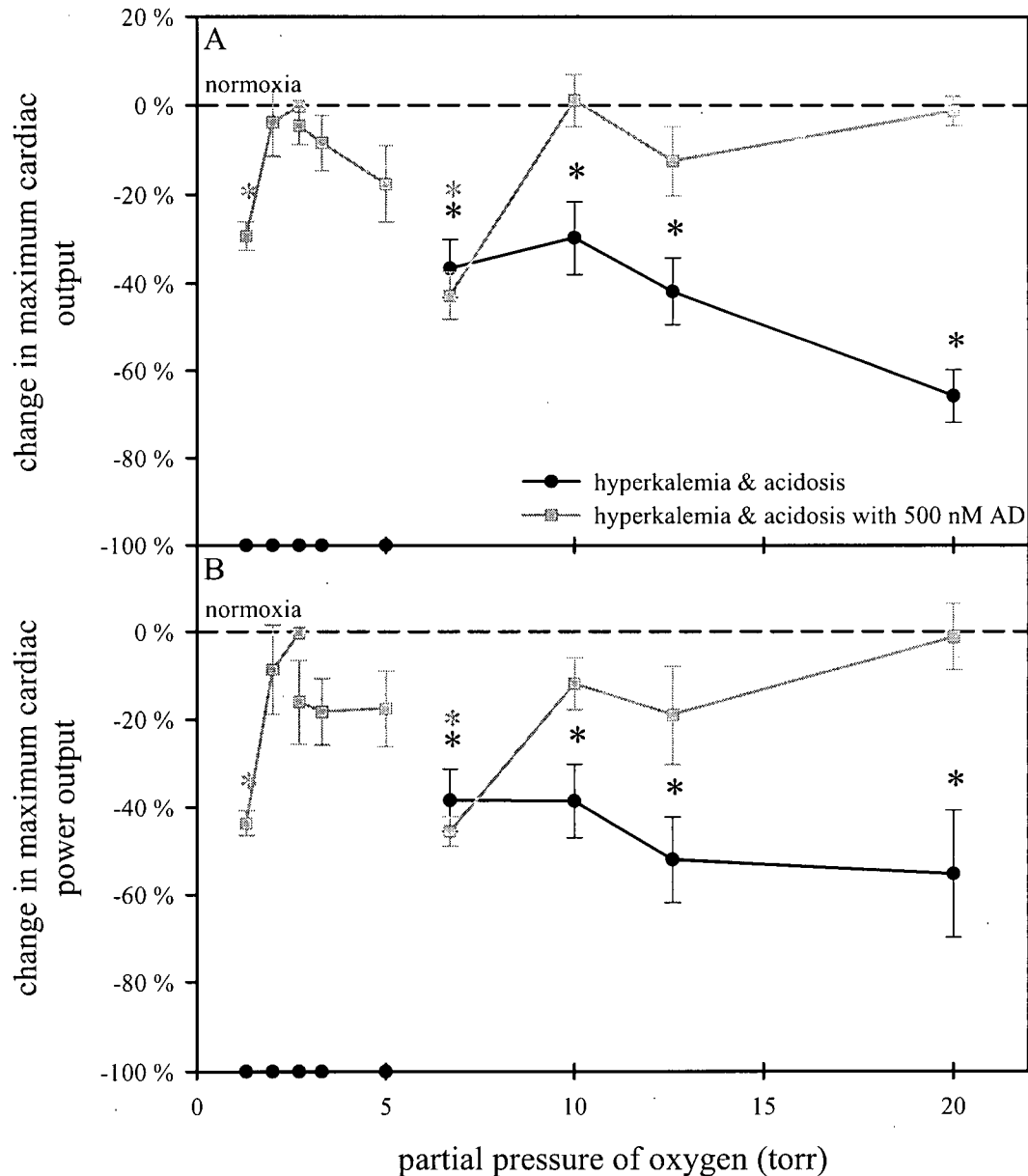


Figure 2.4: The maximum cardiac output (A) and maximum cardiac power output (B) of perfused rainbow trout hearts at 10°C under hypoxic, hyperkalemic (5 mM), acidotic (pH 7.5) perfusate with both tonic (5 nM) and maximal (500 nM) adrenergic stimulation. Individual groups of hearts were exposed to specific hypoxia levels as indicated on the x-axis, and results are shown from several series of experiments, each indicated as a separate line segment. The series were as follows: Series III: 12.6 kPa (n = 6), 10 kPa (n = 10), 6.7 kPa (n = 6), series IV: 5.0 kPa (n = 7), 3.3 kPa (n = 8), 2.7 kPa (n = 5), and series V: 2.7 kPa (n = 6), 2.0 kPa (n = 6), and 1.3 kPa (n = 4). Values are reported as percent change from an original assessment under normoxic conditions. One-way repeated measures ANOVA and Holm-Sidak multiple comparisons tests were used to compare treatment means and each heart acted as its own control. Values shown are means \pm SEM. *Denotes a significant difference from normoxia ($\alpha = 0.05$).

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Chapter 3: The hypoxic threshold for maximum cardiac performance during simulated exercise conditions at 18°C.

Introduction

Like many animals, prolonged exercise in rainbow trout (*Oncorhynchus mykiss*) greatly alters the composition of the venous blood, such that it becomes hypoxic (Kiceniuk and Jones, 1977; Steffensen and Farrell, 1998; Farrell and Clutterham, 2003), hyperkalemic (Holeton *et al.*, 1983; Turner *et al.*, 1983; Perry *et al.*, 1987; Thomas *et al.*, 1987; Nielsen and Lykkeboe, 1992; Holk and Lykkeboe, 1998), and acidotic (Kiceniuk and Jones, 1977; Graham *et al.*, 1982; Holeton *et al.*, 1983; Turner *et al.*, 1983). These changes can be even more extreme following exhaustive exercise (Wood *et al.*, 1983; Milligan, 1996). Such changes in venous blood composition can detrimentally affect cardiac performance, and are particularly taxing for the rainbow trout whose heart is nourished predominantly by venous blood (Santer and Greer Walker, 1980; Farrell *et al.*, 1988). Nevertheless, adrenergic stimulation of cardiac tissue can provide effective protection for all but the worst of these conditions.

Adrenergic stimulation has been shown to individually counteract the negative inotropic and chronotropic effects of hypoxia, hyperkalemia and acidosis (Gesser *et al.*, 1982; Gesser and Jorgensen, 1982; Farrell *et al.*, 1983; 1986; Farrell and Milligan, 1986; Gamperl *et al.*, 1994b; Nielsen and Gesser, 2001; Kalinin and Gesser, 2002). In addition, maximum adrenergic stimulation can protect maximum performance of the *in situ* heart at 10°C from the combined challenges of venous hypoxia, hyperkalemia and acidosis at the levels seen during intense activity and recovery *in vivo* (Hanson *et al.*, 2006). In fact, these

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experiments showed that without adrenergic stimulation, the simulated exercise conditions of acidosis and hyperkalemia will debilitate maximum cardiac performance of the rainbow trout heart even when oxygen is not limiting.

While adrenergic stimulation of the heart clearly plays an important protective role, temperature acclimation is known to alter the response of the rainbow trout heart to adrenaline (Ask *et al.*, 1981; Graham and Farrell, 1989; Farrell *et al.*, 1996). This is significant because rainbow trout can experience a wide range of seasonal temperatures (2°C to 19°C). Specifically, an increase in acclimation temperature is associated with a decrease in adrenergic sensitivity (Graham and Farrell, 1989; Keen *et al.*, 1993), with some of this change being attributed to a temperature-dependent change in ventricular cell surface β -AR density. In fish, cardiac adrenergic stimulation is mediated by the β -adrenoceptor (β -AR) signalling pathway (Ask *et al.*, 1981; Temma *et al.*, 1986; Keen *et al.*, 1993; Gamperl *et al.*, 1994c), and thus changes in acclimation temperature have the potential to alter β -AR mediated cardioprotective mechanisms. With adrenergic receptor density decreasing by ~11% for every 1°C increase in acclimation temperature (Keen *et al.*, 1993; Gamperl *et al.*, 1998), it begs the question how does this reduction in adrenergic effectiveness impact the protection afforded to the heart under the adverse venous conditions experienced by rainbow trout post-exercise?

It has been suggested that in fish, high temperature tolerance may be limited by oxygen availability (Farrell, 2002; Portner *et al.*, 2004). Increasing temperature induces a right-shift of the haemoglobin-oxygen equilibrium curve (Eddy, 1971; Vorger, 1986) and this decrease in haemoglobin-oxygen (Hb-O₂) affinity favours O₂ uptake by tissues, impairs O₂ uptake at the gills and reduces arterial O₂ saturation (Heath and Hughes, 1973). This

decrease in arterial oxygen saturation, combined with a temperature-induced increase in whole body oxygen uptake, decreases venous oxygen tension (P_{vo_2}). P_{vo_2} determines the diffusion gradient of oxygen from venous blood to myocardial tissue and thus it determines oxygen supply for ~70% of the rainbow trout myocardium (Santer and Greer Walker, 1980; Farrell *et al.*, 1988). Thus, while myocardial oxygen needs increase with temperature (Farrell *et al.*, 1985; Graham and Farrell, 1990; Mortensen and Gesser, 1999), the diffusion gradient of oxygen from venous blood is decreasing. The balance between myocardial oxygen supply and demand becomes even more precarious during exercise when myocardial oxygen demand can increase as much as four-fold (Farrell and Jones, 1992) and P_{vo_2} is reduced by ~50% through increased O_2 uptake by skeletal muscles (Kiceniuk and Jones, 1977; Farrell and Clutterham, 2003). If oxygen demand during exercise were to exceed oxygen supply then myocardial hypoxia could ensue resulting in reduced cardiac performance. Furthermore, exercise at high temperature can pose additional challenges when compared to exercise at low temperature as increased temperature exacerbates the potential mismatch between myocardial oxygen supply and oxygen demand induced by exercise.

Temperature-induced myocardial hypoxia can result in a more glycolytic heart. Previous studies (Overgaard *et al.*, 2004a) suggest that although glycolytic capacity increases with temperature, the glycolytic potential of the rainbow trout heart is limited and thus myocardial failure during severe hypoxia ($P_{o_2} < 0.5$ kPa) is primarily due to insufficient anaerobic energy production. In addition, glycolysis results in intracellular acidosis which reduces myocardial contractility and debilitates cardiac performance.

The cardiorespiratory adjustments induced by high temperature effect the venous oxygen reserve such that at high temperature the point at which myocardial oxygen supply

balances demand, i.e. the venous oxygen threshold, will be higher than that seen at low temperature. Furthermore, if myocardial oxygen availability is the only determinant of cardiac performance, one would predict that the venous oxygen threshold for cardiac collapse would increase proportionally to the increase in myocardial oxygen consumption. On the other hand, if adrenergic mechanisms are what fails then one would expect the threshold to increase to a greater extent. This study tests the hypothesis that maximum cardiac performance during prolonged exercise at high temperature is limited by oxygen delivery. It is predicted that the hypoxic threshold for cardiac collapse under hyperkalemic and acidotic conditions with maximal adrenergic stimulation will increase with temperature, and thus will be higher at 18°C than previously discovered at 10°C (Chapter 2).

Materials and Methods

Rainbow trout (body mass $476 \text{ g} \pm 19 \text{ g SEM}$; relative ventricular mass $0.080\% \pm 0.002\%$) of both sexes (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish hatchery (Richard Henley Farm, Langley, BC, Canada), held indoors in 1000 L fibreglass tanks and acclimated to a temperature of $18^\circ\text{C} \pm 1^\circ\text{C}$ for a minimum of two weeks prior to experimentation. An *in situ* perfused heart preparation was prepared as described in Farrell *et al.* (1986) and as modified by Farrell *et al.* (1989), in order to perfuse the venous circulation via an input cannula in the sinus venosus and an output cannula in the ventral aorta. The total time to prepare the perfused heart preparation was 15-20 minutes. Following surgery, the fish was transferred to a temperature-controlled, physiological saline bath (124.1 mM NaCl, 2.5 mM KCl, 11.9 mM NaHCO₃, 2.0 mM CaCl₂·2H₂O, 0.2 mM NaH₂PO₄·H₂O, 3.4 mM Na₂HPO₄, 0.9 mM MgSO₄·7H₂O; all chemicals purchased from

Sigma-Aldrich, Oakville, ON, Canada). The input cannula was immediately connected to an adjustable, constant-pressure reservoir, and the output cannula was connected to a separate constant pressure head set at 4.9 kPa to mimic resting *in vivo* ventral aortic blood pressure. The height of the input pressure reservoir was adjusted to set routine cardiac output (Q) at approximately $25.0 \text{ ml min}^{-1} \text{ kg}^{-1}$. Input (P_{in}) and output (P_{out}) pressure were measured through saline-filled side arms (PE50 tubing) connected to disposable pressure transducers (DPT 6100, Smiths Medical, Kirchseeon, Germany). Cardiac outflow was continuously measured through the output line with an in-line electromagnetic flow probe (SWF-4, Zepada Instruments, Seattle, WA, USA). Hearts were equilibrated for 5-10 minutes while receiving control perfusate (see below) before the experiment commenced. The coronary circulation was not perfused in this preparation.

Maximum cardiac performance was assessed sequentially under normoxic (control) and simulated exercise conditions (defined below) in a manner similar to that described by Hanson *et al.* (2006). By initially measuring both maximum cardiac output (Q_{max}) and maximum cardiac power output (PO_{max}) under normoxia, each heart acted as its own control. To determine Q_{max} , P_{in} was gradually increased in increments of $\sim 0.05 \text{ kPa}$ until cardiac output reached a plateau (usually around 0.4 kPa). To assess PO_{max} , P_{in} was left at its maximum and P_{out} was increased in a stepwise fashion in $\sim 0.5 \text{ kPa}$ increments until PO reached a plateau. After PO_{max} was determined, P_{out} and P_{in} were returned to resting levels and the heart was allowed to recover ($\sim 5 \text{ min}$) before being exposed to the next perfusate. Hearts were exposed to each perfusate for a total of 15 minutes; this time period ensured continued viability of the photosensitive adrenaline (which was renewed with each change in perfusate) while remaining physiologically relevant, as P_{vo_2} can take more than 20 minutes

to return to normal following exhaustive exercise (Farrell and Clutterham, 2003). Under some extreme conditions individual hearts succumbed (i.e. cardiac output approached zero) before the end of the 15-min period. In this case, the duration was noted and normoxic conditions restored. Hearts that were unable to complete both cardiac performance tests were considered to have failed under that test condition.

The purpose of these experiments was to determine the hypoxic threshold for maximum cardiac performance under levels of respiratory acidosis (pH 7.5) and hyperkalemia (5.0 mM) similar to those found in the plasma of exercising rainbow trout *in vivo* (Milligan and Wood, 1987; Nielsen and Lykkeboe, 1992). Adrenaline was used to simulate tonic and maximal adrenergic cardiac stimulation. For the control perfusate, 5 nM AD was used to produce a tonic level of adrenergic stimulation consistent with that found in resting rainbow trout *in vivo* (Milligan *et al.*, 1989). In addition, *in vivo* studies in rainbow trout have reported post-exercise [AD] as high as 186-212 nM (Butler *et al.*, 1986; Milligan *et al.*, 1989), and previous *in situ* studies at 18°C (Farrell *et al.*, 1996) suggest that 200 nM AD produces maximal adrenergic stimulation of rainbow trout hearts. Consequently, 500 nM of AD was added to the perfusate to ensure maximal adrenergic stimulation during the simulated exercise conditions.

The experimental protocol consisted of two different test conditions: (1) control (normoxia), and (2) the combined hyperkalemic, acidotic and hypoxic exposure. In order to determine the hypoxic threshold a series of experiments was done to test hearts at various levels of hypoxia. Regardless of the hypoxia level being tested, hearts were exposed to the following sequence of perfusates: (1) control with 5 nM AD, (2) hypoxia, 5.0 mM K⁺ and pH 7.5 with 500 nM AD, and (3) control with 5 nM AD.

Control (normoxia): All preparations started under this condition. Freshwater trout saline (124.1 mM NaCl, 2.5 mM KCl, 0.9 mM MgSO₄·7H₂O, 2.5 mM CaCl₂·2H₂O, D-glucose 5.6 mM, 11.9 mM NaHCO₃) was gassed with 0.5% CO₂ and balance air to achieve a pH of 7.9 and a P_{O_2} of 20 kPa.

Hyperkalemia, acidosis and hypoxia: The control perfusate was made hyperkalemic by adding additional KCl to increase the [K⁺] to 5.0 mM. To achieve a pH of 7.5, the concentration of NaHCO₃ was decreased to 10.1 mM and the solution was equilibrated with a gas mixture containing 1.0% CO₂. In addition, the perfusate was made acidotic and hypoxic by equilibrating it with 1.0% CO₂ and an amount of oxygen corresponding to the desired level of hypoxia (balance nitrogen). Each heart preparation was tested at one hypoxic level and the levels of hypoxia in kPa were 10 (n = 4 fish), 8.3 (n = 5 fish), 6.7 (n = 8 fish), 5.6 (n = 5 fish), 5.1 (n = 3 fish), 4.0 (n = 5 fish). Premixed, calibrated gases (Praxair, Vancouver, BC, Canada) and Wostoff gas pumps (M303/a-F, Bochum, Germany) were used to generate gas mixtures.

All experimental data was collected using data acquisition software (Labview version 5.1, National Instruments, Austin, TX, USA), which allowed for real-time measurements of f_H , P_{in} , P_{out} , Q , and PO . Statistical differences within test groups were determined by one-way repeated measures analysis of variance (ANOVA). When warranted, the Holm-Sidak procedure was used for *post hoc* multiple comparisons. SigmaStat (3.0, SPSS Inc., San Rafael, CA, USA) was used for all statistical analysis ($\alpha = 0.05$).

Results

The combined hyperkalemic, acidotic exposure with maximum adrenergic stimulation did not significantly alter maximum cardiac output (Q_{max}) at any oxygen tension ≥ 5.6 kPa as compared with control (Fig. 3.1). One of five preparations failed at 8.3 kPa, two of five preparations failed at 5.6 kPa, all four preparations tested at 5.1 kPa failed and all five preparations tested at 4.0 kPa failed (data not shown).

Q_{max} was fully restored under subsequent control conditions >8.3 kPa but remained significantly ($38.6\% \pm 9.2\%$) lower than control at 8.3 kPa (Fig. 3.1). Similarly, under the combined hyperkalemic and acidotic conditions with maximum adrenergic stimulation, PO_{max} was significantly ($43.3\% \pm 7.9\%$) lower than control at 6.7 kPa, and recovery was incomplete at both 6.7 and 8.3 kPa, as indicated by significant decreases in PO_{max} under control conditions (Fig. 3.2).

Cardiac failure was primarily due to significant decreases in f_H . Heart rate was significantly reduced with the combined hyperkalemic and acidotic perfusate at 6.7 kPa (Fig. 3.3) and under the subsequent control conditions at both 6.7 kPa and 5.1 kPa. In contrast, there were no significant changes in cardiac stroke volume (V_s) at any of the hypoxia levels tested (Fig. 3.4).

Discussion

Previous studies have shown that the *in situ* perfused heart preparation utilized in this study can perform at levels approximating maximum *in vivo* cardiac performance and remain stable for several hours (Farrell *et al.*, 1986; Farrell, 2002; Overgaard *et al.*, 2004b). The values obtained in this study for maximum cardiac output (54.0 ± 4.7 ml min⁻¹ kg⁻¹) and

maximum cardiac power output (5.9 ± 0.06 mW g ventricle⁻¹) under control conditions are comparable, albeit slightly lower, than previous *in situ* studies at 18°C where Q_{max} ranges from 63-78 ml min⁻¹ kg⁻¹ and PO_{max} ranges from 8.8-9.3 mW kg ventricle⁻¹ (Keen and Farrell, 1994; Farrell *et al.*, 1996). Also, it should be noted that the success rate for preparations at 18°C (87%) is lower compared with previous work conducted at 10°C by the same investigators (100%). Consequently, the reported cardiac performance levels may reflect an overestimate due to the removal of poor performers, a problem noted earlier (Farrell *et al.*, 1996).

The goal of this experiment was to determine the hypoxic threshold for maximum cardiac performance under the conditions experienced during and after exhaustive exercise. The hypoxic threshold for cardiac collapse was found to be 5.1-5.6 kPa, while the hypoxic threshold for maximum cardiac performance was 5.1-5.6 kPa for Q_{max} and 6.7 kPa for PO_{max} . At 10°C the hypoxic threshold for maximum cardiac performance is reported as 2.0 kPa for both Q_{max} and PO_{max} (Hanson *et al.*, 2006), which is very similar to previously reported P_{vo_2} values *in vivo* (Kiceniuk and Jones, 1977; Farrell and Clutterham, 2003) and lower than the half maximal haemoglobin-oxygen saturation (P_{50}) value of 3.1 (Perry and Reid, 1992). As predicted, the hypoxic threshold for maximum cardiac performance increases with acclimation temperature. Specifically, the 8°C change in acclimation temperature between these two studies is associated with a 3.6-4.7 kPa increase in the hypoxic threshold. Moreover, at high temperature there is the additional concern of poor recovery following the subsequent return to control conditions, which is not seen at 10°C.

Due to the oxygen buffering capacity of haemoglobin, it is to be expected that the hypoxic threshold for a saline-perfused heart would be higher than the P_{vo_2} threshold *in vivo*.

The sigmoidal shape of the haemoglobin-oxygen equilibrium curve and its high oxygen capacitance would maintain a relatively stable PO_2 over a wide range of oxygen extraction given the myocardial oxygen demand (Farrell, 1986). In contrast, due to the linear nature of oxygen solubility in saline and its lower oxygen capacitance, cardiac oxygen removal from saline will decrease PO_2 more so than oxygen extraction from blood leading to a reduced diffusion gradient. Consequently, the oxygen buffering capacity of haemoglobin will facilitate lower P_{VO_2} thresholds *in vivo*. Despite these considerations, the difference in hypoxic thresholds for 10°C and 18°C appears unusually large. At 18°C exercising rainbow trout quit swimming with a P_{VO_2} of 3.2-3.9 kPa (Farrell and Clutterham, 2003), a value less than the P_{50} value of 4.7 kPa at this temperature (Eddy, 1971). The difference is therefore about 2.4 kPa for Q_{max} and 2.8 kPa for PO_{max} , when the O_2 demand is somewhat higher. Such differences are unlikely to be a result of myocardial O_2 extraction from saline reducing PO_2 .

If changes in saline PO_2 due to myocardial oxygen extraction were the sole determinant of maximum cardiac performance, the venous oxygen threshold should increase with temperature in proportion to myocardial oxygen needs. Based on the data of Graham and Farrell (1989), myocardial oxygen needs increase by < 1 kPa for an 8°C temperature increase. However, over a temperature increase of the same magnitude the venous oxygen threshold increases 3.6 kPa, from 2 kPa at 10°C (Hanson *et al.*, 2006) to 5.6 kPa at 18°C (this study). If myocardial oxygen extraction was indeed the culprit, the expected venous oxygen threshold would be ~3 kPa and not 5.6 kPa. Consequently, it can be concluded that limited myocardial oxygen supply from saline is not the sole determinant of the high hypoxic threshold at 18°C. Accordingly, a temperature-induced loss of adrenergic effectiveness

known to occur at 18°C (Graham and Farrell, 1989; Keen *et al.*, 1993) seems like a significant contributing factor to making the rainbow trout myocardium more hypoxia sensitive at 18°C compared with 10°C under the hyperkalemic and acidotic conditions typically encountered during intense activity and recovery.

Another possibility to account for the higher hypoxic threshold at 18°C could be an increased reliance on the coronary circulation at higher temperature, which would make sense if P_{vo_2} is being compromised *in vivo*. The role and plasticity of coronary circulation is poorly understood. Nevertheless, previous studies on rainbow trout have shown that an increase in acclimation temperatures is associated with an increase in both the proportion of compact myocardium (Farrell *et al.*, 1988; Simonot, 2006) and the density of coronary vasculature (Egginton and Cordiner, 1997). The coronary circulation provides arterial blood to 30% of the ventricle (Santer and Greer Walker, 1980; Tota, 1983; Davie and Farrell, 1991). Coronary blood flow increases during exercise (Gamperl *et al.*, 1994b; 1994a) up to 2 fold (Gamperl *et al.*, 1995); likely to reflect the increased oxygen needs of the compact myocardium. The importance of increased coronary circulation during exercise is highlighted by the 37% decrease in cardiac performance seen in coronary-ligated rainbow trout during a hypoxic swimming challenge (Steffensen and Farrell, 1998). Agnisola *et al.* (2003) demonstrated that coronary perfusion significantly improved the performance of isolated saline perfused rainbow trout hearts at 10°C, such that their performance was not significantly different from that seen under control conditions; however, their performance was still 44% lower than the PO_{max} generated under similar conditions by perfused hearts *in situ* with only cardiac circulation (Hanson *et al.*, 2006). Blood perfusion of the coronary circulation can help improve cardiac performance by increasing myocardial oxygen

consumption (Agnisola *et al.*, 2003), which may help support lower thresholds *in vivo*, however work in other species (eels) suggests that blood perfusion helps PO_{max} more so than Q_{max} (Davie *et al.*, 1992). Clearly, the lack of a coronary circulation in the perfused heart preparation will overestimate the hypoxic thresholds for maximum cardiac performance, but to what degree the dependence on the coronary circulation increases at high temperature will have to await *in vivo* experiments.

In conclusion, during exercise at high temperature, the negative effects of hypoxia, hyperkalemia and acidosis that would be alleviated through adrenergic stimulation are probably compromised by a decrease in adrenergic effectiveness (Graham and Farrell, 1989; Keen *et al.*, 1993). This study showed a temperature-induced loss of adrenergic protection and as a result, the venous oxygen threshold required to maintain maximum cardiac performance at 18°C was relatively high under the conditions associated with intense activity and recovery.

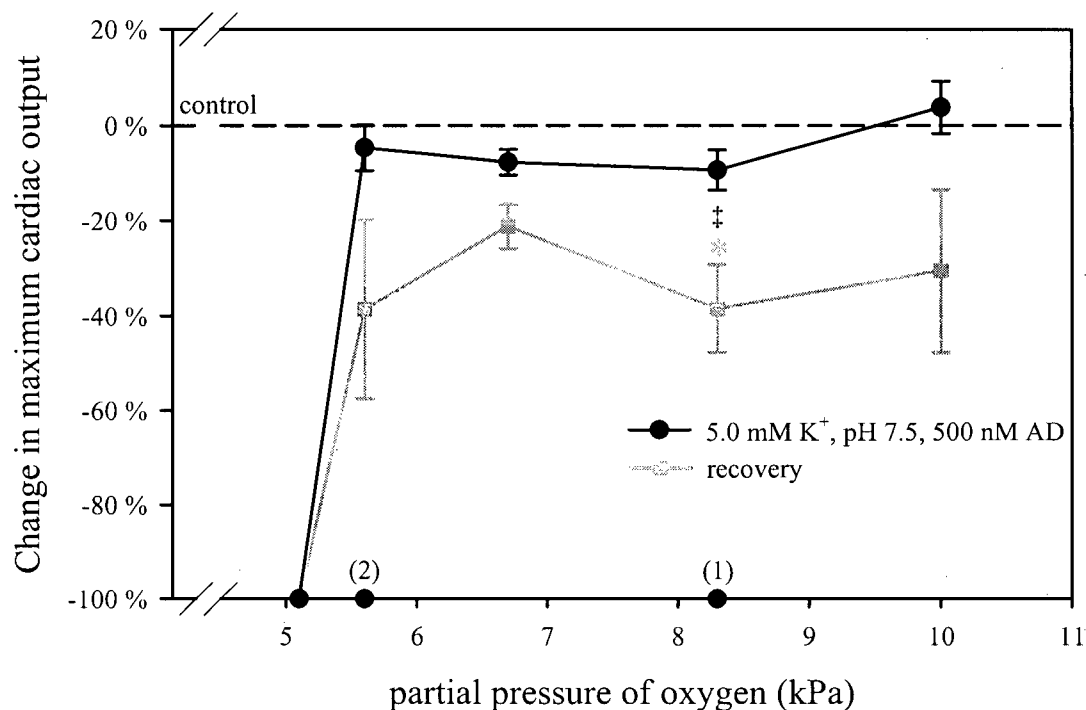


Figure 3.1: The maximum cardiac output of perfused rainbow trout hearts at 18°C under hypoxic, hyperkalemic (5 mM), acidotic (pH 7.5) perfusate with maximal (500 nM) adrenergic stimulation. The grey line indicates the level of recovery during a subsequent exposure to control conditions. Individual groups of hearts were exposed to specific hypoxia levels as indicated on the x-axis. The number of hearts used at each level of hypoxia was 10 kPa (n = 4 fish), 8.3 kPa (n = 5 fish), 6.7 kPa (n = 8 fish), 5.6 kPa (n = 5 fish), 5.1 kPa (n = 3 fish), 4.0 kPa (n = 5 fish). Values are reported as percent change from an original assessment under control conditions. One-way repeated measures ANOVA and Holm-Sidak multiple comparisons tests were used to compare treatment means and each heart acted as its own control. Values shown are means \pm SEM. *Denotes a significant difference from control while ‡ denotes a significant difference between the hypoxic, hyperkalemic, acidotic exposure and recovery ($\alpha = 0.05$).

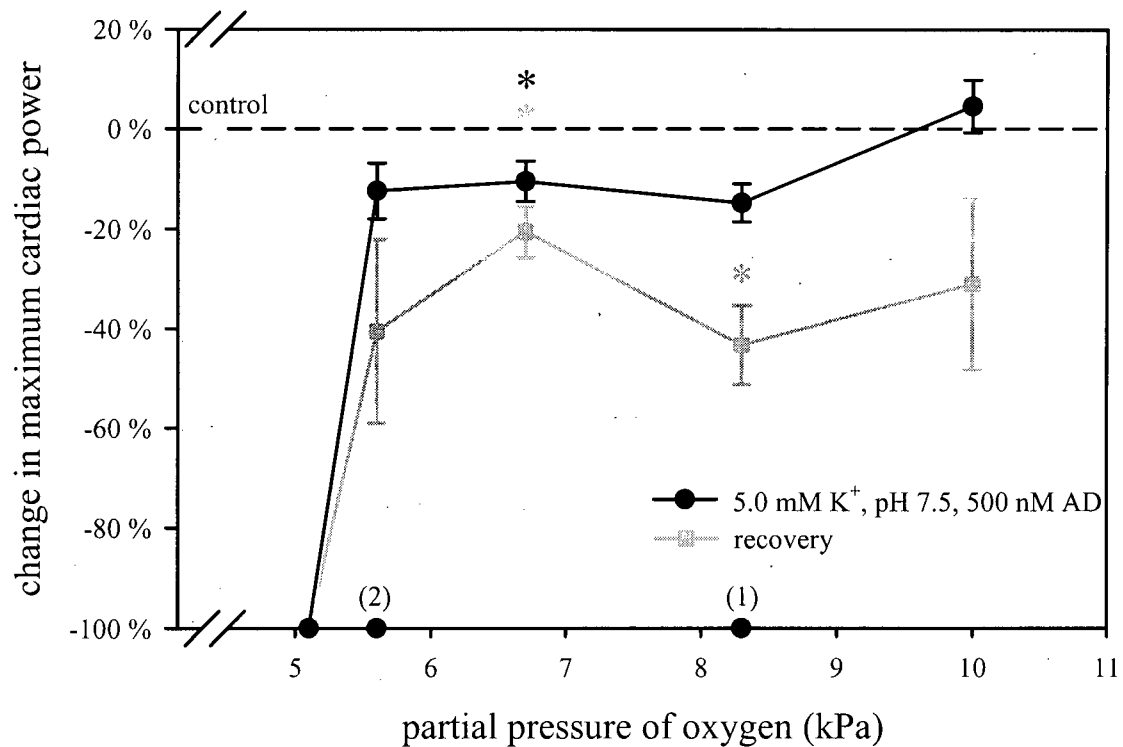


Figure 3.2: The maximum cardiac power output of perfused rainbow trout hearts at 18°C under hypoxic, hyperkalemic (5 mM), acidotic (pH 7.5) perfusate with maximal (500 nM) adrenergic stimulation. The grey line indicates the level of recovery during a subsequent exposure to control conditions. Individual groups of hearts were exposed to specific hypoxia levels as indicated on the x-axis. The number of hearts used at each level of hypoxia was 10 kPa (n = 4 fish), 8.3 kPa (n = 5 fish), 6.7 kPa (n = 8 fish), 5.6 kPa (n = 5 fish), 5.1 kPa (n = 3 fish), 4.0 kPa (n = 5 fish). Values are reported as percent change from an original assessment under control conditions. One-way repeated measures ANOVA and Holm-Sidak multiple comparisons tests were used to compare treatment means and each heart acted as its own control. Values shown are means \pm SEM. *Denotes a significant difference from control while † denotes a significant difference between the hypoxic, hyperkalemic, acidotic exposure and recovery ($\alpha = 0.05$).

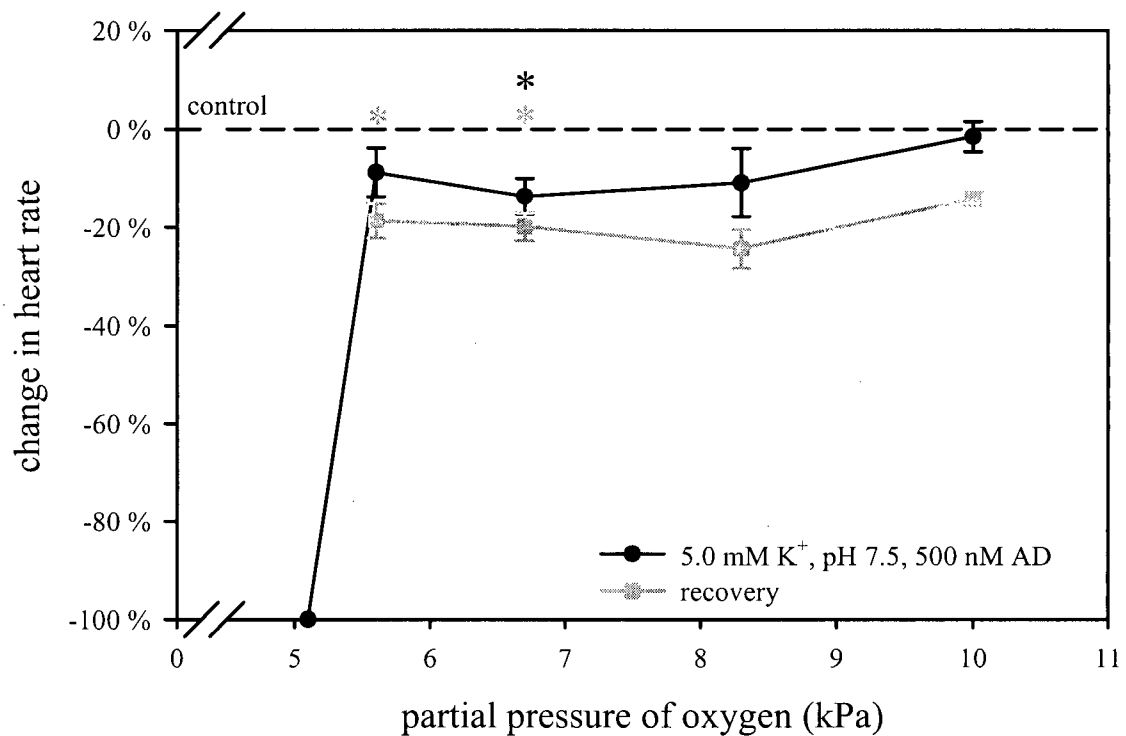


Figure 3.3: Heart rate at maximum cardiac output of perfused rainbow trout hearts at 18°C under hypoxic, hyperkalemic (5 mM), acidotic (pH 7.5) perfusate with maximal (500 nM) adrenergic stimulation. The grey line indicates the level of recovery during a subsequent exposure to control conditions. Individual groups of hearts were exposed to specific hypoxia levels as indicated on the x-axis. The number of hearts used at each level of hypoxia was 10 kPa (n = 4 fish), 8.3 kPa (n = 5 fish), 6.7 kPa (n = 8 fish), 5.6 kPa (n = 5 fish), 5.1 kPa (n = 3 fish), 4.0 kPa (n = 5 fish). Values are reported as percent change from an original assessment under control conditions. One-way repeated measures ANOVA and Holm-Sidak multiple comparisons tests were used to compare treatment means and each heart acted as its own control. Values shown are means \pm SEM. *Denotes a significant difference from control ($\alpha = 0.05$).

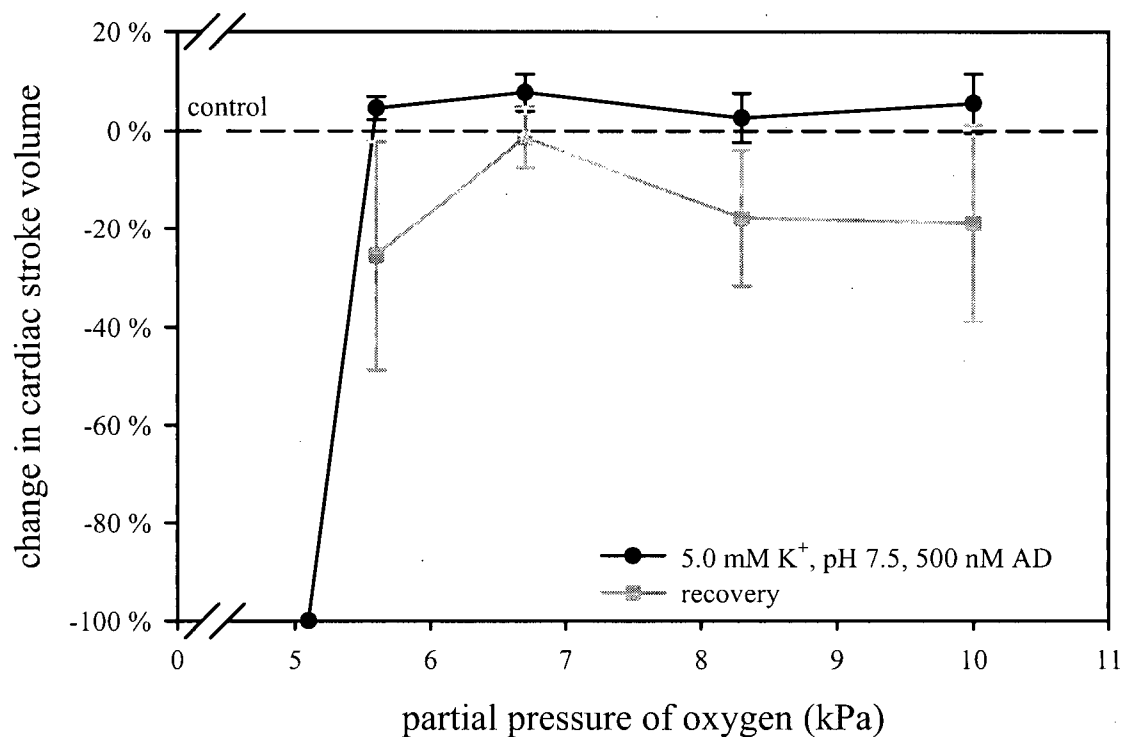


Figure 3.4: Cardiac stroke volume during maximum cardiac output of perfused rainbow trout hearts at 18°C under hypoxic, hyperkalemic (5 mM), acidotic (pH 7.5) perfusate with maximal (500 nM) adrenergic stimulation. The grey line indicates the level of recovery during a subsequent exposure to control conditions. Individual groups of hearts were exposed to specific hypoxia levels as indicated on the x-axis. The number of hearts used at each level of hypoxia was 10 kPa (n = 4 fish), 8.3 kPa (n = 5 fish), 6.7 kPa (n = 8 fish), 5.6 kPa (n = 5 fish), 5.1 kPa (n = 3 fish), 4.0 kPa (n = 5 fish). Values are reported as percent change from an original assessment under control conditions. One-way repeated measures ANOVA showed no significant differences between treatment means. Values shown are means \pm SEM.

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Chapter 4: The effect of temperature acclimation on myocardial β -adrenoceptor density and ligand binding affinity in African Catfish (*Claris gariepinus*)

Introduction

The β -adrenoceptor (β -AR) signalling pathway is known to mediate important cardiac inotropic and chronotropic actions of adrenaline and noradrenaline in fish (Ask *et al.*, 1981; Temma *et al.*, 1986; Gamperl *et al.*, 1994). The main β -AR subtype mediating these cardiac actions in rainbow trout (*Oncorhynchus mykiss*) is a β_2 subtype (Ask *et al.*, 1980, 1981; Keen *et al.*, 1993; Gamperl *et al.*, 1994), although other sub-types have been identified in other fish species (see Nilsson, 1983; Farrell and Jones, 1992). Temperature acclimation can alter the response of the rainbow trout heart to adrenaline (Ask *et al.*, 1981; Farrell *et al.*, 1996) and some of this change has been attributed to a temperature-dependent change in cell surface β -AR density (B_{\max}) (Keen *et al.*, 1993; Shiels *et al.*, 2002). For example, the increase in adrenergic sensitivity with cold acclimation in rainbow trout hearts is associated with an increase in β_2 -AR density (Keen *et al.*, 1993; Gamperl *et al.*, 1998) and this adrenergic stimulation is extremely important in preserving the L-type Ca^{2+} current in isolated trout myocytes exposed to cold temperature (Shiels *et al.*, 2000, 2003). The objective of this study was to determine whether a tropical species, African catfish (*Clarias gariepinus*), exhibits a similar intraspecific plasticity in β -AR density in response to acclimation temperature as

rainbow trout. It was expected that β -AR density would be inversely related to acclimation temperature.

Materials and Methods

Fish

African catfish (*Claris gariepinus*) were originally obtained from a live fish supplier in Singapore and held at National University of Singapore for >6 months (with temperature exhibiting the normal diurnal fluctuation between 23-28°C). For the temperature acclimation experiment, fish were transferred into small fiberglass aquaria (2 per temperature group) that were then gradually adjusted to the three experimental temperatures (15°C, 22°C, 32°C) over a period of 2-6 days (i.e. approximately 1°C change per day). Fish in all three acclimation groups were then held at experimental temperature for 28 days before sampling. The fish were quickly killed by a blow to the head, the ventricle was excised, rinsed in a 0.8% NaCl solution and frozen in liquid nitrogen. Ventricles were frozen at -80°C before being shipped on dry ice to Simon Fraser University, where they were stored at -80°C for no more than 2 months prior to analysis. Body mass ranged from 296 to 707 g and relative ventricular mass from 0.05% to 0.11%

Earlier studies of cardiac β -AR in fish have included rainbow trout, therefore they were used as a reference species to ensure consistency of experimental techniques. Rainbow trout (*Oncorhynchus mykiss*) were purchased from Sun Valley Trout Farms (Langley, BC, Canada) and held in an outdoor tank (14°C) for at least 3 weeks prior to sampling. The fish were killed by a quick blow to the head after which the cardiac ventricle was quickly excised, rinsed in saline (composition in mM: NaCl, 124.1; KCl, 3.1; MgSO₄, 0.90; CaCl₂, 2.5; TES

free acid, 7.0; TES Na salt, 3.3; pH 7.85 at 15°C), gently massaged to remove blood and frozen in liquid nitrogen. Tissues were stored at -80°C for no longer than three months prior to analysis. Body mass ranged from 338 to 476 g and relative ventricular mass from 0.08% to 0.13%

Quantification of β_2 -adrenoceptors

Cell surface β_2 -adrenoceptor density (B_{\max}) and binding affinity (K_d) were determined for ventricular punches, using the tritiated ligand technique of Watson-Wright *et al.* (1989), as modified for fish hearts by Gamperl *et al.* (1994). Frozen ventricles were first rinsed in saline (see above) several times to remove any remaining blood. Ventricles were then partially refrozen at -80°C before being sliced (350 μm thickness) with a McIlwain tissue chopper (Brinkman, Rexdale, ON, Canada). Tissue slices were placed in ice-chilled saline while ventricular tissue punches (2 mm diameter by 350 μm thickness) were taken. For rainbow trout, the punches were taken almost exclusively from the compact myocardium, while for African catfish the compact myocardium was not as well defined and punches were taken from the outer regions of the ventricular wall. Gamperl *et al.* (1998) showed that there was no significant difference between K_d for the compact and spongy myocardium of rainbow trout, but that B_{\max} was 14% higher in spongy myocardium. Single punches were placed in separate wells of a 24-well tissue culture plate (Flow Laboratories, McLean, VA), with each well containing 500 μL of saline and various concentrations (0.05-3.5 nM) of the hydrophilic β_2 -adrenoceptor ligand [^3H] CGP-12177 (CGP specific activity 55 and 52 Ci/mol; Amersham Life Science). To determine non-specific binding, separate tissue punches were incubated at each concentration with the competitive β_2 -adrenoceptor

antagonist timolol (10 μ M). All tissue punches were incubated at 0°C for 2 h, which was sufficient for equilibrium binding to occur (Gamperl *et al.*, 1994). The culture plates were agitated frequently during incubation to prevent the formation of a depletion layer. Following incubation, punches were washed twice in ice-chilled saline, placed into 8 ml scintillation vials containing 2 ml of Ecolite scintillation fluid (ICN Canada Inc., Mississauga, Ont., Canada), incubated for 18 h and counted with a liquid scintillation counter (LS 6500, Beckman Instruments, USA). Specific binding was calculated by subtracting radioactivity (disintegrations per minute) of the punches incubated with CGP and timolol (4 punches) from the activity of punches incubated with CGP alone (6-8 punches).

A single ligand-binding curve consisted of 6-8 replicates for each ligand concentration. Ventricle size determined the degree of replication for the binding curves. Rainbow trout were large enough to obtain a sufficient number of tissue punches to construct a single binding curve from each ventricle, therefore, the binding curve was replicated six times using six individuals (i.e., $N = 6$; 6 fish). For the African catfish, however, the smaller ventricular size required the pooling of tissue punches from several individuals to construct a single binding curve. The number of replicate binding curves (N) was 3 and the number of fish used was 5 for all three acclimation temperatures (ie, $N = 3$; 5 fish). Quench tests performed with scintillation fluid and heart tissue revealed no significant quenching.

Data analysis

Binding parameters were determined using a Scatchard plot as described by Zivin and Waud (1982) (not shown). The linearity of the Scatchard plots ($r^2 = 0.80-0.88$) indicates the presence of a single adrenoceptor population. Protein content of representative punches was

determined using a Bradford protein assay, using bovine serum albumin (Sigma Chemicals, St. Louis, Missouri) as the standard, so that B_{\max} could be expressed as fmol mg protein⁻¹. All statistical analysis was performed after specific binding was expressed as fmol mg protein⁻¹ using SigmaStat 2.03 (1997). A one-way ANOVA was used to compare B_{\max} and K_d values between the three groups of African catfish, with the Holm-Sidak multiple comparisons procedure being used to determine between group significance. A separate one-way ANOVA was used to compare B_{\max} and K_d values of rainbow trout with those of African catfish. The Holm-Sidak multiple comparisons procedure was used to test whether the B_{\max} and K_d of each subgroup of African Catfish differed significantly from rainbow trout.

Results

The r^2 value for individual CGP-binding curves was always ≥ 0.90 (range = 0.90-0.96). Values are presented as mean \pm SEM (Fig. 4.1). No statistically significant differences were found among β_2 -adrenoceptor density (B_{\max}) values for African catfish acclimated at 15°C, 22°C and 32°C. Nevertheless, ligand binding affinity (K_d) was statistically significantly higher ($p = 0.002$) for the 32°C acclimation group ($K_d = 0.88$) compared with both the 15°C ($K_d = 0.48$) and 22°C acclimation groups ($K_d = 0.46$) (Fig. 4.2). There were no statistically significant differences in the protein content of punches from the three acclimation groups. Protein content among the temperature acclimation groups ranged from 123 ± 12 (32°C) to 145 ± 13 $\mu\text{g mg tissue}^{-1}$ (22°C) and was 154 ± 13 $\mu\text{g mg tissue}^{-1}$ for rainbow trout. For rainbow trout, B_{\max} (26.4 fmol mg protein⁻¹) was

significantly higher ($p < 0.05$) and K_d (0.19 nM) significantly lower ($p < 0.001$) than the values for African catfish at all three acclimation temperatures.

Discussion

B_{max} and K_d values for the β_2 -AR in cardiac tissue of rainbow trout were used as a reference to compare with earlier studies. Our results for rainbow trout at 14°C ($B_{max} = 26.4$ fmol mg protein⁻¹ and $K_d = 0.19$ nM) compare favourably with those of Gamperl *et al.* (1998), who reported a B_{max} of 25 fmol mg protein⁻¹ and a K_d of 0.16 nM for rainbow trout at 14°C. Similarly, Olsson *et al.* (2000) reported a B_{max} of 23 fmol mg protein⁻¹ and a K_d of 0.13 nM for rainbow trout at 12°C. For rainbow trout acclimated to a colder (8°C) temperature, Gamperl *et al.* (1994) reported an almost 2-fold increase in B_{max} to 40 fmol mg protein⁻¹ and an unchanged K_d of 0.16 nM. In view of this concordance, we are confident in the methods used to characterize myocardial β_2 -AR density and binding affinity.

Intraspecific plasticity in ventricular β_2 -AR density in temperate rainbow trout is inversely associated with acclimation temperature (Keen *et al.*, 1993; Gamperl *et al.*, 1998). These two independent studies found that B_{max} could decrease by as much as 11% per 1°C increase in temperature. Our results for the tropical African catfish provide a sharp contrast, with B_{max} ranging from 14.3 to 17.8 fmol mg protein⁻¹ over a 17°C temperature range, which suggests that β -AR plasticity may be species specific. Perhaps stenothermal tropical species do not possess the intraspecific β -AR plasticity exhibited by temperate species such as the rainbow trout, which experience a wide range of seasonal environmental temperatures, and in which β -AR plasticity is critical for maintaining cardiac contractility (Graham and Farrell, 1989; Shiels *et al.*, 2003). Accordingly, acclimation mechanisms, such as cold-induced

increases in β -AR density (Keen *et al.*, 1993; Gamperl *et al.*, 1998), may be superfluous in tropical fish. Further studies are needed to ascertain the exact environmental and physiological triggers for changes in β -AR density in order to determine why temperature-mediated regulation has to date only been observed in a temperate species. Nevertheless, the increase in K_d for African catfish with increasing acclimation temperature is consistent with a decrease in adrenergic sensitivity, as observed in rainbow trout acclimated to elevated water temperature (Keen *et al.*, 1993; Farrell *et al.*, 1996). Thus, it is possible that trout downregulate cardiac adrenergic sensitivity at extremely warm acclimation temperatures by internalizing β -AR, whereas tropical African catfish change binding affinity.

Olsson *et al.* (2000) suggested that across a number of fish species myocardial β -AR density was directly related to acclimation temperature. The present results do not conform with this idea because B_{\max} for African catfish was significantly lower than that measured here and elsewhere for rainbow trout. As a result, we cannot support the suggestion that interspecific differences in B_{\max} are related to temperature differences among species. Indeed, preliminary work in our laboratory shows a wide range for B_{\max} among tropical fish species, which spans that reported for rainbow trout [10.5 ± 0.9 to 47.5 ± 2.9 fmol mg protein⁻¹ for snakehead (*Channa micropeltes*), African catfish (*Clarias gariepinus*), four-eyed sleeper (*Bostrichthys sinensis*), marble goby (*Oxyeleotris marmorata*), black eeltail catfish (*Plotosus canius*), Bengal sergeant (*Abudefdule bengalensis*), and blue spotted fantail ray (*Taeniura lymma*)].

While the lack of an effect of temperature acclimation on B_{\max} in African catfish was clear-cut, there is some concern that the difference between the assay temperature and the range of acclimation temperatures may result in artifacts in the measured K_d values. All of

the assays were conducted and incubated at 0°C to minimize tissue degradation, which is a standard practice for these types of ligand binding assays. Although an assay temperature closer to the acclimation temperature may have improved the assessment of K_d , it would have resulted in variable tissue degradation and subjected receptors to cellular modifications such as desensitization, internalization and degradation, that would have adversely affected B_{max} .

Similarly, it is unlikely that our inability to separate compact and spongy myocardium was a confounding factor in the low B_{max} observed for African Catfish (as compared to rainbow trout and tropical fish of previous studies, Olsson *et al.*, 2000) because only a 14% difference in B_{max} has been observed for these two tissue types (Gamperl *et al.* 1998). In addition, spongy cardiac tissue is known to have a slightly higher B_{max} than compact tissue, and so it is possible that we have overestimated B_{max} for the species comparison. Nonetheless, this degree of variation would not account for the differences we observed between rainbow trout and African catfish.

When fish are sampled, they are briefly exposed to hypoxia. Hypoxia influences B_{max} in mammalian cardiac tissues, with significant (29%-62%) hypoxic downregulation occurring in rat and chick ventricular tissue (Voelkel *et al.*, 1981; Blake *et al.*, 1982; Marsh and Sweeney, 1989; Rocha-Singh *et al.*, 1991). However, this type of β -AR down-regulation did not occur in rainbow trout ventricle tissue after a prolonged 6 h hypoxic exposure (Gamperl *et al.*, 1998). In contrast, β -AR up-regulation occurred in trout red blood cells following a 30 minute hypoxic exposure (Reid *et al.*, 1992). Moreover, adrenergic responsiveness was increased by a concomitant increase in plasma catecholamine levels under hypoxic, but not normoxic conditions (Reid *et al.*, 1992). In view of this, and the fact

that resting adrenergic tonus to the fish heart certainly shows considerable interspecific variability (Axelsson *et al.*, 1987; 1992; Keen *et al.*, 1995), a possibility worth exploring is whether the level of adrenergic stimulation is an important determinant of cardiac β -AR density.

In summary, we suggest that β -AR plasticity in response to temperature acclimation is dissimilar for tropical African catfish and temperate rainbow trout.

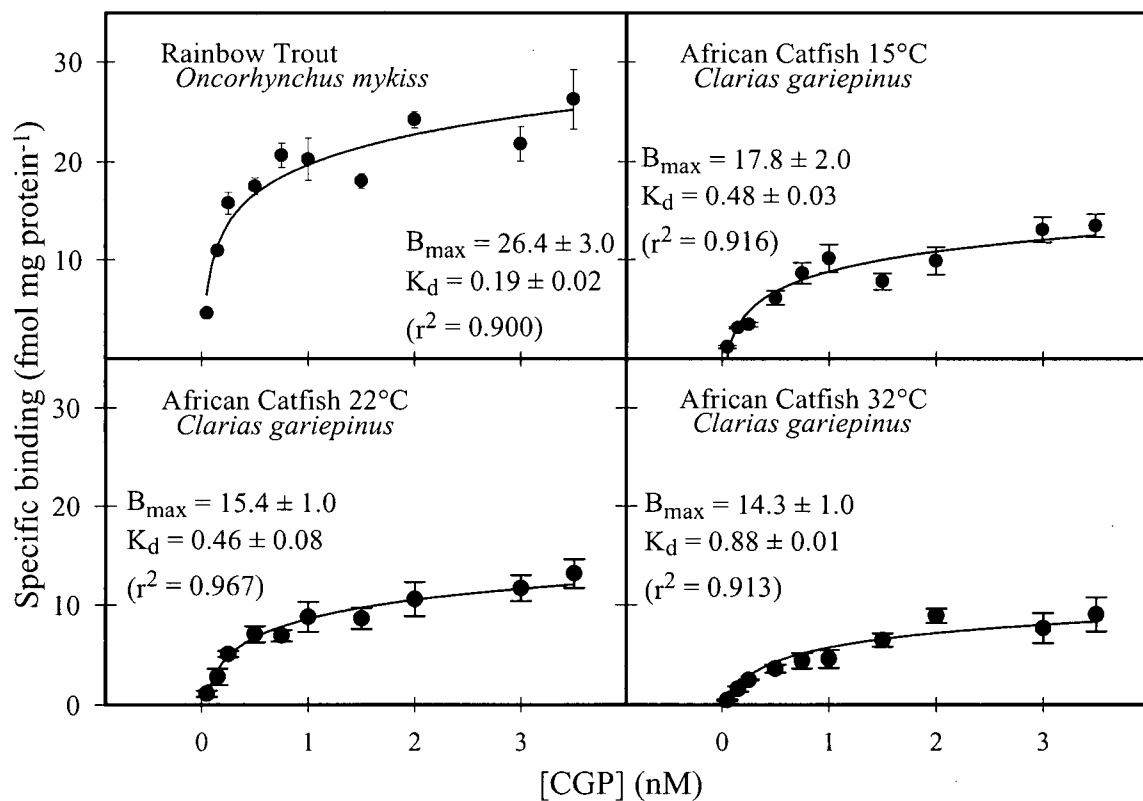


Figure 4.1: Specific binding of [³H]CGP-12177 to ventricular β-adrenoceptors. Due to the small size of some African catfish ventricles they sometimes had to be pooled for a single binding curve. The number of fish used and the number of binding assays performed is as follows: rainbow trout (N=6 binding assays, 6 fish), African catfish 15°C (N=3 binding assays; 5 fish), African catfish 22°C (N=3; 5 fish), and African catfish 32°C (N=3; 5 fish). The β-adrenoceptor density (B_{\max} , fmol mg protein⁻¹), [³H]CGP-12177 dissociation constant (K_d , nM) and r^2 values for each graph are indicated. Values are mean ± SEM.

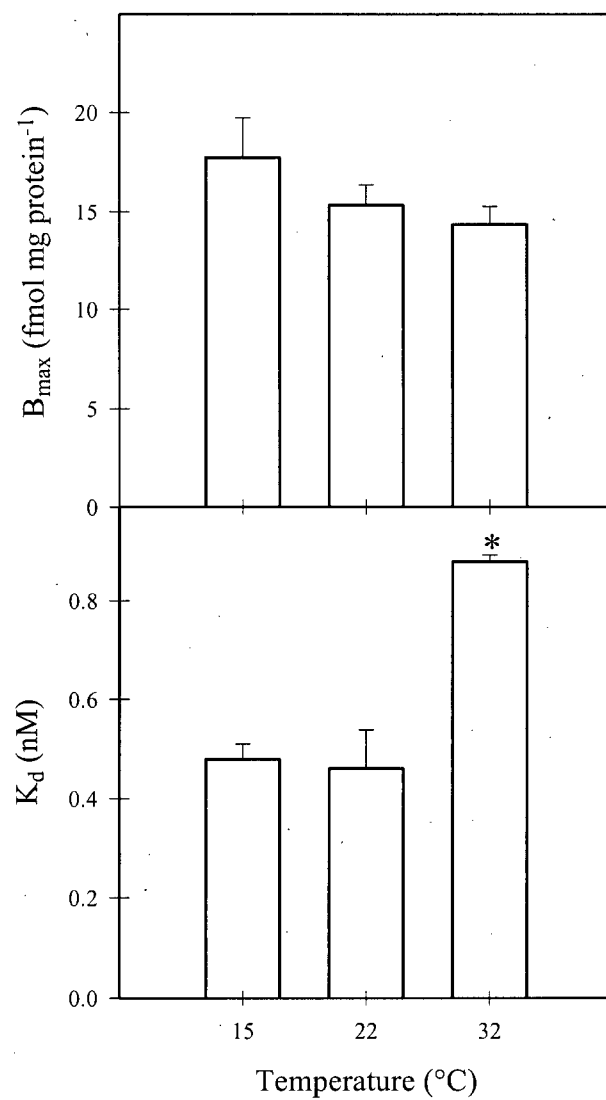


Figure 4.2: [³H]CGP-12177 dissociation constant (K_d) and β -adrenoceptor density (B_{\max}) in African catfish acclimated to 15°C (N=3, 5 fish), 22°C (N=3, 5 fish), and 32°C (N=3, 5 fish). Values are means \pm SEM. * denotes significant differences at $P < 0.05$ (one-way ANOVA)

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Chapter 5: Major findings and conclusions

This thesis examined the role that adrenergic stimulation plays in protecting cardiac performance during exercise-induced changes in venous blood composition, namely hypoxia, hyperkalemia and acidosis. The objectives were five-fold: (1) to quantify the venous hypoxic threshold for maximum cardiac performance using *in situ* perfused hearts, (2) to quantify the inotropic and chronotropic effects of hyperkalemia on *in situ* perfused hearts, (3) to simulate the effects of post-exercise levels of hyperkalemia and acidosis in combination with hypoxia *in situ*, and determine the hypoxic threshold for maximum cardiac performance with and without concurrent adrenergic stimulation, (4) to determine the effect of elevated temperature (18°C) on the *in situ* hypoxic threshold for cardiac collapse associated with a hyperkalemic, acidotic exposure, and (5) to determine whether β -adrenoreceptor density (B_{max}) is affected by warm temperature in the tropical African catfish as it is in the temperate rainbow trout.

Objective 1

I found that the venous hypoxic threshold for maximum cardiac performance in saline perfused rainbow trout hearts was 2.7 kPa. This threshold is similar to previously reported venous oxygen tension (P_{vo_2}) values of 2.0-2.1 kPa observed during maximal exercise *in vivo* (Kiceniuk and Jones, 1977; Farrell and Clutterham, 2003), but higher than reported venous oxygen levels ≤ 1.3 kPa in rainbow trout during environmental hypoxia (Thomas *et al.*, 1994; Steffensen and Farrell, 1998). This difference likely occurs because without exercise under just aquatic hypoxia the heart may not be working as hard and therefore will have a lower cardiac oxygen demand.

Objective 2

My thesis contains the first study to quantify the chronotropic effects of hyperkalemia on fish hearts. I found that with only tonic adrenergic stimulation a physiologically relevant level of hyperkalemia (5.0 mM) reduced cardiac performance by 30%, with the majority of this decrease being due to negative chronotropy. As previous studies have focussed their attention on isolated, paced cardiac tissue, the 25% decrease in heart rate I observed is something previously unreported. In addition, I found that both the negative chronotropic and inotropic effects of 5.0 mM hyperkalemia could be completely eliminated by maximum adrenergic stimulation.

Objective 3

I discovered that if *in situ* perfused rainbow trout hearts were exposed to the hyperkalemic, acidotic conditions experienced in venous blood during intense exercise, they could not reach the maximum level of performance seen during intense exercise and recovery *in vivo*, even if they received fully oxygenated saline. However, maximum adrenergic stimulation of the *in situ* hearts counteracted the detrimental effects of hyperkalemia and acidosis such that hearts could perform maximally at P_{vo_2} levels as low as 2.0 kPa, a hypoxic threshold similar to the P_{vo_2} values reported for maximally exercising fish *in vivo*. This suggests that adrenergic stimulation is obligatory when these adverse conditions are present during intense exercise and recovery.

Objective 4

As acclimation temperature increases above the optimum, maximum cardiac performance is compromised. In rainbow trout this occurs at approximately 18°C (Farrell *et al.*, 1996). In accordance with this observation I found that the hypoxic threshold for maximum cardiac performance shifted dramatically when I tested *in situ* perfused rainbow trout hearts under simulated exercise conditions at 18°C (as compared to my previous experiments at 10°C). The hypoxic threshold increased from 2.0 kPa at 10°C to 5.6 kPa at 18°C. Unlike the situation at 10°C, the *in situ* hypoxic threshold at 18°C is much higher than the *in vivo* threshold of 3.2-3.9 kPa (Farrell and Clutterham 2003). Potential reasons for this discrepancy are discussed below.

Objective 5

Unlike the situation in the temperate rainbow trout, I failed to find any evidence of ventricular B_{max} modulation with acclimation temperature in the tropical African catfish. The B_{max} for African catfish was not significantly different over a 17°C difference in acclimation temperature, whereas in rainbow trout B_{max} varies by as much as 11% for every 1°C change in acclimation temperature (Keen *et al.*, 1993; Gamperl *et al.*, 1998), creating the potential for a three-fold change over this temperature range. My results suggest that the control of ventricular β -AR activated mechanisms in response to temperature acclimation does not appear to be a universal mechanism among fish.

Perspectives

By comparing the results of chapters 2 and 3, a discrepancy in the hypoxic thresholds becomes apparent. At 10°C the *in situ* hypoxic threshold was very similar to *in vivo* values (Kiceniuk and Jones, 1977; Farrell and Clutterham, 2003) and lower than the half maximal haemoglobin-oxygen saturation (P_{50}) value of 3.1 (Perry and Reid, 1992). In contrast, the *in situ* threshold of 5.6 kPa at 18°C was much higher than the *in vivo* threshold of 3.2-3.9 kPa previously observed in exercising rainbow trout (Farrell and Clutterham, 2003), and also higher than the calculated P_{50} value of 4.7 kPa at this temperature (as determined from the data of Eddy, 1971). There are two potential reasons for this discrepancy: (i) the use of saline as a perfusate as opposed to blood or haemoglobin solution and (ii) the loss of critical adrenergic protective mechanisms at 18°C due to the temperature-induced decrease in β -AR density.

Due to the increase in myocardial oxygen extraction at 18°C, the linear nature of the saline-oxygen dissociation curve, and the low oxygen capacitance of saline, the reduction in P_{O_2} as saline passes through the heart may be greater at 18°C than at 10°C. If this prediction were true, saline perfused hearts at 18°C would require a higher venous oxygen threshold in order to maintain an adequate oxygen diffusion gradient to the myocardium. Due to the higher oxygen capacitance of blood and the sigmoidal nature of the haemoglobin-oxygen dissociation curve, this situation would not arise *in vivo*.

To determine if saline perfusion contributed to the above discrepancy in hypoxic thresholds, I needed to find out if temperature affects the reduction in P_{O_2} as saline passes through the heart. Using myocardial oxygen extraction data from Graham and Farrell (1990) and saline-oxygen solubility coefficient (α) values from Graham (1987), I calculated the

change in PO_2 that occurred as saline passed through the heart during several sets of experiments (representing 32 fish) at both 10°C and 18°C. I found that although the change in saline PO_2 was significantly higher at 18°C, the difference was only 0.7 kPa between the two acclimation temperatures. Consequently, it seems unlikely that the corresponding reduction in the oxygen diffusion gradient would be sufficient to explain the 1.7-2.4 kPa discrepancy between the *in vivo* and *in situ* venous oxygen thresholds at 18°C. As saline-oxygen extraction dynamics cannot explain this discrepancy between *in situ* and *in vivo* hypoxic thresholds, it appears that adrenergically activated protective mechanisms may be the key to sustaining lower venous oxygen thresholds *in vivo*.

Nevertheless, the reduction in saline PO_2 due to myocardial oxygen extraction (2.97 ± 0.05 kPa at 10°C and $3.67 \pm .01$ kPa at 18°C) means that *in vivo* venous oxygen thresholds could be several kPa lower than those seen *in situ*. Future studies utilizing either haemoglobin solution or blood perfusion would be needed to determine a more exact threshold. In addition, by examining the difference between the hypoxic thresholds obtained with haemoglobin solution perfusion and blood perfusion, future studies could also discern the importance of erythrocyte β -AR activated compensation mechanisms.

In conclusion, the experiments contained in this thesis demonstrated the vital role that adrenergic stimulation plays in stimulating and protecting maximum cardiac performance during intense activity and recovery. While these effects are acutely temperature sensitive in rainbow trout, this may not be the case for other species.

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