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

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Summary

As rainbow trout approach exhaustion during prolonged exercise, they maintain maximum cardiac output despite the fact their venous blood, which bathes the heart, becomes hypoxic, acidotic and hyperkalemic. Because these factors are individually recognized to have detrimental inotropic and chronotropic effects on cardiac performance, we hypothesized that adrenergic stimulation is critical in maintaining maximum cardiac performance under these collectively adverse conditions *in vivo*. To test this hypothesis, maximum cardiac performance in the presence and absence of maximal adrenergic stimulation was assessed with *in situ* rainbow trout hearts using relevant hyperkalemic (5.0 mmol l^{-1} K^+), acidotic (pH 7.5) and hypoxic challenges. With tonic adrenergic stimulation (5.0 nmol l^{-1} adrenaline), hearts produced only 44.8±14.6% of their normal maximum cardiac output when exposed under normoxic conditions (20 kPa) to the hyperkalemic, acidotic perfusate, indicating that *in vivo* there was no refuge from cardiac impairment even if venous blood was fully oxygenated. By contrast, maximum adrenergic stimulation (500 nmol l^{-1} adrenaline), fully protected maximum cardiac performance under hyperkalemic and acidotic conditions over a wide range of oxygen availability, from normoxia to 2.0 kPa, a venous oxygen tension close to routine values *in vivo*. Extending the level of hypoxia to 1.3 kPa resulted in a 43.6±2.8% decrease in maximum cardiac output, with hearts failing when tested at 1.0 kPa. Our results suggest that adrenergic stimulation of the trout heart is critical in maintaining maximum performance during prolonged swimming tests, and probably during all forms of exhaustive activity and recovery, when venous blood is hyperkalemic, acidotic and hypoxic.

Key words: acidosis, adrenaline, exercise, heart, hyperkalemia, hypoxia, maximum cardiac performance, *Oncorhynchus mykiss*, $P_{V_{O_2}}$, rainbow trout, teleost.

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 mmol l^{-1} to 3.4–4.7 mmol l^{-1} (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 ($\dot{Q}$) 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 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). A 60% decrease was observed in isometric force of rainbow trout ventricular strips exposed to hypercapnic acidosis (pH 6.9) (Kalinin and Gesser, 2002), whereas a ~10% decrease in maximum cardiac power output (PO_{max}) was observed (Farrell et al., 1986), 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 $\dot{Q}$ 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 μmol l^{-1} adrenaline (AD) (Farrell et al., 1983). Concurrent adrenergic stimulation (100 μmol l^{-1} AD) also maintained cardiac performance in isolated cardiac muscle strips, whereas 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 mmol l^{-1} and 12.5 mmol l^{-1} K^+) reduces the contractive force of isolated heart strips by ~50% (Kalinin and Gesser, 2002) to ~100% (Nielsen and Gesser, 2001). 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 (K^+>5.5 mmol l^{-1}) 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 μmol l^{-1} AD) of isolated cardiac muscle completely eliminated a 50% decrease in contraction force associated with 10 mmol l^{-1} K^+ (Kalinin and Gesser, 2002), and the complete loss of contractile force associated with 12.5 mmol l^{-1} 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 ($P_{V_{O_2}}$). 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 to the remaining myocardium increases during exercise (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 $\dot{Q}_{max}$ and PO_{max} by ~50% and ~80%, respectively (Farrell et al., 1989), which seems a rather high threshold given that $P_{V_{O_2}}$ 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, although 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 mmol l^{-1}), 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±12 g; relative ventricular mass =

0.087±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°C (±1°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 for a minimum of 2 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 i.u. ml^{-1}) *via* the caudal vessels. An *in situ* perfused heart preparation was prepared (Farrell et al., 1986; 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 nmol l^{-1} adrenaline (arenaline bitartrate salt; AD) and 10 i.u. heparin ml^{-1}. 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 min. 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 mmol l^{-1} NaCl, 2.5 mmol l^{-1} KCl, 11.9 mmol l^{-1} $NaHCO_3$, 2.0 mmol l^{-1} $CaCl_2$-$2H_2O$, 0.2 mmol l^{-1} $NaH_2PO_4.H_2O$, 3.4 mmol l^{-1} Na_2HPO_4, 0.9 mmol l^{-1} $MgSO_4.7H_2O$; all chemicals were 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 ($\dot{Q}$) at approximately 17.0 ml min^{-1} 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 the temperature could be maintained at 10°C (Brinkman Instruments Inc., Mississauga, ON, Canada). Hearts were allowed to equilibrate for 5–10 min 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 nmol l^{-1} 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 nmol l^{-1} 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±89 nmol l^{-1} (Butler et al., 1986).

Control (normoxic) condition

All preparations started under this condition. Freshwater trout saline (124.1 mmol l^{-1} NaCl, 2.5 mmol l^{-1} KCl, 0.9 mmol l^{-1} $MgSO_4.7H_2O$, 2.5 mmol l^{-1} $CaCl_2.2H_2O$, D-glucose 5.6 mmol l^{-1}, 11.9 mmol l^{-1} $NaHCO_3$) 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 that additional KCl was added to increase the $[K^+]$ to either 5.0 mmol l^{-1} or 7.5 mmol l^{-1}.

Acidosis

To achieve a pH of 7.5, the concentration of $NaHCO_3$ in the control perfusate was decreased to 10.1 mmol l^{-1} 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 $[K^+]$ to either 5.0 mmol l^{-1} or 7.5 mmol l^{-1}.

Hyperkalemia, acidosis and hypoxia

A hyperkalemic (5.0 nmol l^{-1}), 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 ($\dot{Q}_{max}$) and maximum cardiac power output (PO_{max}) under normoxic, control conditions each heart acted as its own control. To determine $\dot{Q}_{max}$, 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 min; this time period ensured continued viability of the photosensitive AD (which was renewed with each change in perfusate) while remaining physiologically relevant, as $P\mathrm{v}_{O_2}$ can take more than 20 min to return to normal following exhaustive exercise (Farrell and Clutterham, 2003). Under some extreme conditions individual hearts did not perform for 15 min, 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 mmol l^{-1} (Thomas et al., 1987) but previous studies done on isolated cardiac muscle strips have tested higher concentrations of 5.0–12.5 mmol l^{-1}. The order of the test conditions was: (1) control, (2) 5 mmol l^{-1} K^+ (3) 7.5 mmol l^{-1} K^+ (4) control and (5) 7.5 mmol l^{-1} K^+ with 500 nmol l^{-1} AD.

Series II (acidosis and hyperkalemia)

The purpose of series II was to quantify under normoxic conditions (a) the 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 mmol l^{-1} K^+, pH 7.5, (3) control, (4) 5.0 mmol l^{-1} K^+ and pH 7.5 with 500 nmol l^{-1} AD, and (5) 7.5 mmol l^{-1} K^+ and pH 7.5 with 500 nmol l^{-1} AD. In addition, three preliminary preparations were tested using 7.5 mmol l^{-1} K^+ and 5 nmol l^{-1} 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 mmol l^{-1} 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 mmol l^{-1}) 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 mmol l^{-1} K^+ and pH 7.5 with 5 nmol l^{-1} AD and (5) hypoxia, 5.0 mmol l^{-1} K^+ and pH 7.5 with 500 nmol l^{-1} 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 nmol l^{-1} 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 $P\mathrm{v}_{O_2}$ 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 mmol l^{-1} K^+ and pH 7.5 with 500 nmol l^{-1} 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 that 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 mmol l^{-1} K^+ and pH 7.5 with 500 nmol l^{-1} 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, $[K^+]$, $P\mathrm{v}_{O_2}$ 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}, $\dot{Q}$ 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 P=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-dependent manner when compared to control (Fig. 1). The 30% decrease in $\dot{Q}_{max}$ and PO_{max} with 5.0 mmol l^{-1} K^+ was caused by a 25% decease in f_H and a 10% decrease in maximum stroke volume (V_S). Similarly, the 60% reduction in $\dot{Q}_{max}$ and PO_{max} with 7.5 mmol l^{-1} K^+ was caused by a 45% decrease in f_H and a 25% decrease in maximum V_S.

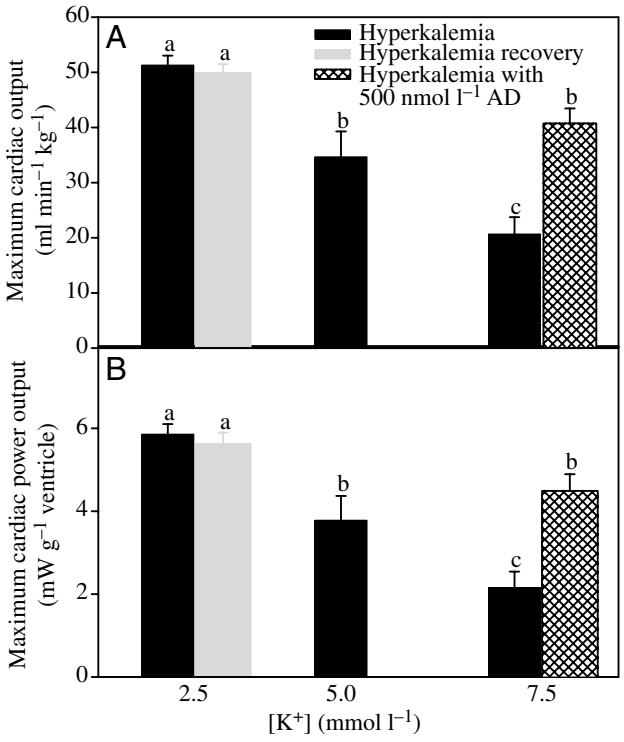

Fig. 1. The effects of hyperkalemia on maximum cardiac output (A) and maximum cardiac power output (B) of perfused rainbow trout hearts at 10°C, pH 7.9. Values are reported as mean ± s.e.m. Individual hearts (N=9) were exposed to the following sequence of perfusates: (1) control (normoxia), (2) 5 mmol l^{-1} K^+, (3) 7.5 mmol l^{-1} K^+, (4) control (recovery) and (5) 7.5 mmol l^{-1} K^+ with 500 nmol l^{-1} adrenaline (AD). Repeated measures one-way ANOVA and a Holm-Sidak multiple comparisons test were used to compare treatment means. Different letters denote significant differences at P=0.05.

A noticeable arrhythmia also developed near the end of the 7.5 mmol l^{-1} K^+ exposure. Despite these effects of hyperkalemia, maximum cardiac performance was fully restored when hearts were returned to control conditions (Fig. 1). Maximal adrenergic stimulation significantly improved maximum cardiac performance of hyperkalemic (7.5 mmol l^{-1} K^+) hearts, with increases in both $\dot{Q}_{max}$ and PO_{max} to within 20% (P<0.05) of their original performance under control conditions (Fig. 1).

Hyperkalemia combined with acidosis

The results for 5 mmol l^{-1} hyperkalemia alone, and in combination with acidosis (pH 7.5), are presented in Fig. 2.

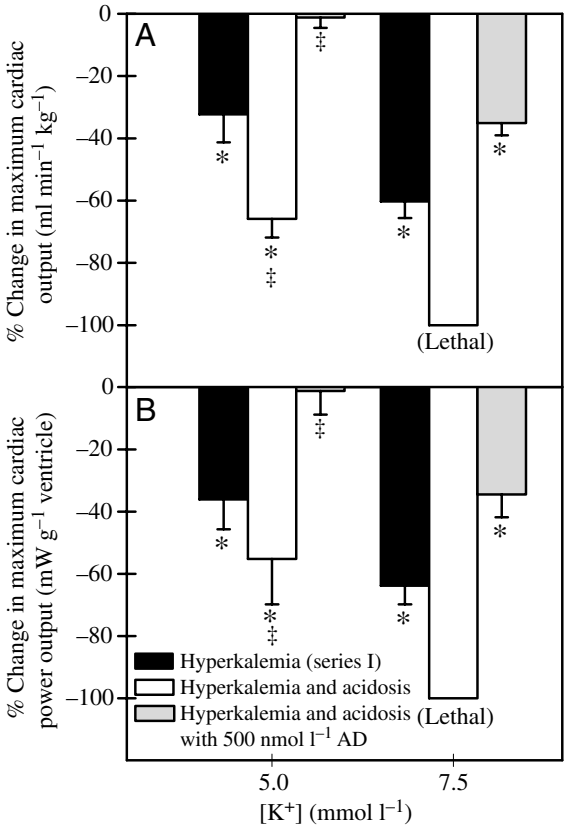

Fig. 2. The effects of hyperkalemia and acidosis (pH 7.5) on maximum cardiac output (A) and maximum cardiac power output (B) of perfused rainbow trout hearts at 10°C. Values are reported as mean ± s.e.m. Individual hearts (N=8) were exposed to the following sequence of perfusates: (1) control (normoxia), (2) 5.0 mmol l^{-1} K^+, pH 7.5, (3) control (recovery), (4) 5.0 mmol l^{-1} K^+ and pH 7.5 with 500 nmol l^{-1} adrenaline (AD), and (5) 7.5 mmol l^{-1} K^+ and pH 7.5 with 500 nmol l^{-1} AD. Values from series I are presented for comparison purposes (see Fig. 1). One-way ANOVA and a Holm–Sidak multiple comparisons test were used to compare treatment means. *Significant difference from control; ‡a significant difference from pH 7.9 at that particular level of hyperkalemia, P=0.05. Three preliminary preparations were exposed to 7.5 mmol l^{-1} K^+ and 5 nmol l^{-1} 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.

Hyperkalemia and acidosis significantly decreased (P<0.05) both $\dot{Q}_{max}$ (–65.9±6.0%) and PO_{max} (–55.2±14.6%) from control, and to a greater (20–30%) degree when compared to 5.0 mmol l^{-1} K^+ alone. The additional 20–30% decrease in $\dot{Q}_{max}$ was mainly due to a further decrease (–62.0±6.6%, P<0.05) in f_H as maximum V_S was still only depressed by 10% (9.73±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 mmol l^{-1} K^+ resulted in rapid, irrecoverable cardiac collapse (associated with severe cardiac arrhythmia). Maximal adrenergic stimulation completely prevented the debilitating effect of 5 mmol l^{-1} K^+ and acidosis, allowing the hearts to perform at control levels of $\dot{Q}_{max}$ and PO_{max} (Fig. 2). Moreover, concurrent maximum adrenergic stimulation allowed hearts to perform under the previously lethal conditions of 7.5 mmol l^{-1} K^+ and acidosis, but with both $\dot{Q}_{max}$ and PO_{max} ~35% lower than control (P<0.05; Fig. 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 Fig. 3. Hypoxia at 12.6 kPa and 10 kPa had no significant effect on $\dot{Q}_{max}$ and PO_{max} either during hypoxia or with subsequent normoxic exposure (Fig. 3). However, hypoxic levels between 6.7 and 3.3 kPa not only significantly decreased $\dot{Q}_{max}$ and PO_{max} by 10–25% (Fig. 3), maximum performance did not show any immediate recovery during subsequent normoxia from the level seen under hypoxia. At 2.7 kPa, three out of eight 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 $\dot{Q}_{max}$ 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 $\dot{Q}_{max}$ and PO_{max} by 38% to 66% (P<0.05) when $P_{v_{O_2}}$ ~6.7 kPa (Fig. 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 $\dot{Q}_{max}$ and PO_{max} with 5 mmol l^{-1} K^+ and acidosis at 12.6 kPa and 10 kPa. Although the protective effect of AD was apparently lost at 6.7 kPa (Fig. 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 $\dot{Q}_{max}$ nor PO_{max} were significantly different from control (Fig. 4). At hypoxia levels of 1.3 kPa, however, maximal adrenergic

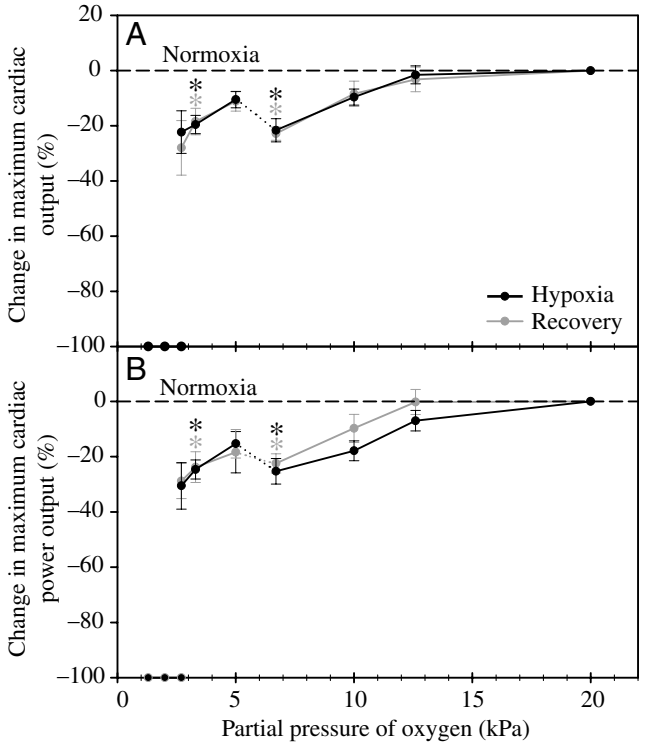

Fig. 3. The maximum cardiac output (A) and maximum cardiac power output (B) of perfused rainbow trout hearts at 10°C under hypoxic perfusate. The grey line indicates the level of recovery during a subsequent exposure to normoxic conditions. Individual groups of hearts were exposed to specific hypoxia 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 in series III: 12.6 kPa (N=6), 10 kPa (N=10), 6.7 kPa (N=6), and in series IV: 5.0 kPa (N=7), 3.3 kPa (N=8), 2.7 kPa (N=5). Values are reported as percentage 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 ± s.e.m. *Significant difference from normoxia (P=0.05).

stimulation only partially protected cardiac performance, as $\dot{Q}_{max}$ and PO_{max} were reduced by 29.4±3.3% and 43.6±2.8% respectively (Fig. 4). Moreover, following the hypoxic, hyperkalemic, acidotic exposure, with 500 nmol l^{-1} AD, hearts exposed to 2.7, 2.0, and 1.3 kPa did not recover 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

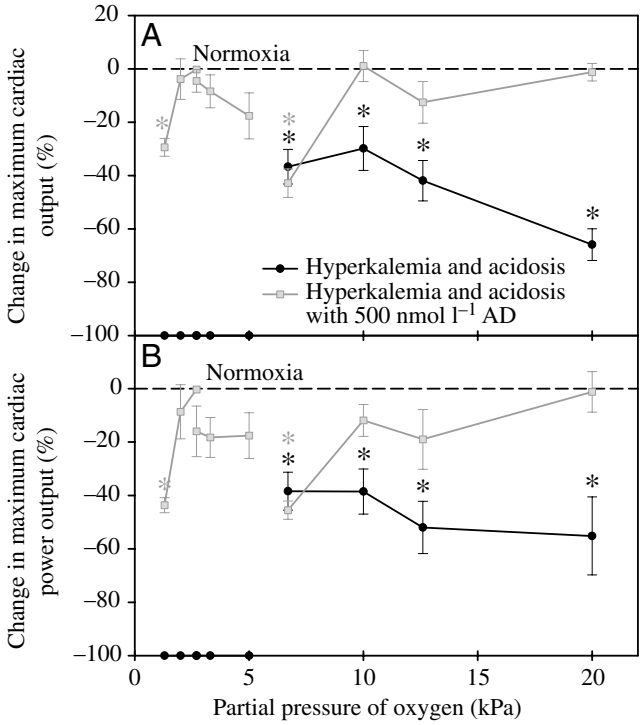

Fig. 4. The change in maximum cardiac output (A) and maximum cardiac power output (B) of perfused rainbow trout hearts at 10°C under hypoxic, hyperkalemic (5 mmol l^{-1}), acidotic (pH 7.5) perfusate with both tonic (5 nmol l^{-1}) and maximal (500 nmol l^{-1}) adrenergic stimulation. Individual groups 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 ± s.e.m. *Significant difference from normoxia (*P*=0.05).

(Farrell et al., 1986; Farrell, 2002; Overgaard et al., 2004b). Correspondingly, our values for maximum cardiac performance under normoxic conditions ($\dot{Q}_{max}$=51.3± 1.7 ml min^{-1} kg^{-1}; PO_{max}=5.9±0.2 mW g^{-1} ventricle) 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 ml kg^{-1} min^{-1}, whereas maximum power output values ranged from 5.1 to 6.9 mW g^{-1} ventricle (Kiceniuk and Jones, 1977; Farrell et al., 1991; Faust et al., 2004; Gamperl et al., 2004; Overgaard et al., 2004b). Potential variations among studies, as a result of variation in fish stocks, fish size and level of adrenergic stimulation are not accounted for in these comparisons. As our longest experiments lasted for only 100 min, 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 $P_{V_{O_2}}$ 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 showning that isolated perfused hearts with tonic adrenergic stimulation performed routine physiological workloads to 3.3 kPa, but this level of hypoxia decreased $\dot{Q}_{max}$ 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. Rainbow trout exposed to a graded hypoxia challenge released adrenaline when arterial blood oxygen tension (arterial P_{O_2}) fell below 3.4 kPa (Perry and Reid, 1992). Here, we found that hearts exposed to hypoxia of 3.3 kPa with only tonic adrenergic stimulation lasted less than 5 min 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* studies 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) constitutes 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 $P\mathrm{v}_{\mathrm{O}_2}$ values found in trout of around 3–4 kPa. When $P\mathrm{v}_{\mathrm{O}_2}$ 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) by up to twofold (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 (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. (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 min^{-1} since the information was not provided).

An additional factor that sustains lower $P\mathrm{v}_{\mathrm{O}_2}$ 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 $P\mathrm{v}_{\mathrm{O}_2}$, 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 used a fibreoptic micro-optode to measure the $P\mathrm{v}_{\mathrm{O}_2}$ *in vivo* during maximal exercise in rainbow trout (Farrell and Clutterham, 2003) and discovered that at even the most severe exercise intensity $P\mathrm{v}_{\mathrm{O}_2}$ 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 swam coronary-ligated rainbow trout in hypoxic water (5.2 kPa) (Steffensen and Farrell, 1998) and this resulted in a $P\mathrm{v}_{\mathrm{O}_2}$ threshold of 1.3 kPa (10 Torr). From their data, we can estimate that PO_{max} was 4.1 mW g^{-1} ventricle for coronary-ligated fish at this $P\mathrm{v}_{\mathrm{O}_2}$ threshold. [Ventral aortic blood pressure (P_{va}) was ~50 cm H_2O (~4.9 kPa).] We assume that $\dot{Q}_{\mathrm{max}}$ 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. (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 $P\mathrm{v}_{\mathrm{O}_2}$ 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 re-emphasise 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^{-1} ventricle could be maintained for 20 min during anoxia at 10°C (Overgaard et al., 2004a). Although 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 $P\mathrm{v}_{\mathrm{O}_2}$ 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. found (Gamperl et al., 2001) that rainbow trout hearts were stunned when exposed to

extreme hypoxia (P_{O_2}<5 mmHg) at sub-physiological workloads, with $\dot{Q}_{max}$ 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 $\dot{Q}_{max}$ than hearts with no previous exposure to hypoxia.

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 $P_{V_{O_2}}$ levels found in maximally exercising rainbow trout.

Abbreviations

AD	adrenaline
f_H	heart rate
P_{in}	input pressure
PO	cardiac power output
PO_{max}	maximum cardiac power output
P_{O_2}	partial pressure of oxygen
P_{out}	output pressure
P_{VA}	ventral aortic blood pressure
$P_{V_{O_2}}$	venous partial pressure of oxygen
$\dot{Q}$	cardiac output
$\dot{Q}_{max}$	maximum cardiac output
V_S	stroke volume
β-AR	β-adrenoceptor

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