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Effects of temperature and anoxia upon the performance of *in situ* perfused trout hearts

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Summary

Rainbow trout (*Oncorhynchus mykiss*) are likely to experience acute changes in both temperature and oxygen availability and, like many other organisms, exhibit behavioural selection of low temperatures during hypoxia that acts to reduce metabolism and alleviate the demands on the heart. To investigate whether low temperature protects cardiac performance during anoxia, we studied the effects of an acute temperature change, from 10°C to either 5°C, 15°C or 18°C, upon the performance of *in situ* perfused trout hearts before, during and after exposure to 20 min of anoxia. Routine cardiac workload mimicked *in vivo* conditions at the given temperatures, and the effects of anoxia were evaluated as maximal cardiac performance before and after 20 min of anoxic perfusion. Functional data were related to maximal activities of glycolytic enzymes and energetic status of the heart at the termination of the experiment.

At high oxygenation, maximum cardiac output and power output increased with temperature (Q_{10} values of 1.8 and 2.1, respectively) as a result of increased heart rate. Hypoxia tolerance was inversely related to temperature. At 5°C, the hearts maintained routine cardiac output throughout the 20 min period of anoxia, and maximal cardiac performance was fully restored following reoxygenation. By contrast, cardiac function failed sooner during anoxia as temperature was increased and maximal performance after reoxygenation was reduced by 25%, 35% and 55% at 10°C, 15°C and 18°C, respectively. Increased functional impairment following anoxic exposure at elevated temperature occurred even though both cardiac glycolytic enzyme activity and the rate of lactate production were increased proportionally with cardiac work. Nonetheless, there was no indication of myocardial necrosis, as biochemical and energetic parameters were generally unaffected by anoxia.

Key words: fish, trout, *Oncorhynchus mykiss*, temperature, cardiovascular, recovery, *in situ* perfusion, hypoxia, anoxia, glycolytic metabolism.

Introduction

Cardiac function is vital for transport of respiratory gases, nutrients and hormones and removal of waste products. The latter three functions remain crucial under conditions of extreme oxygen deficiency, where oxidative phosphorylation is curtailed, so it is not surprising that hearts of anoxia-tolerant species such as the painted turtle (*Chrysemys picta*) and the crucian carp (*Carassius carassius*) remain functional, albeit at a reduced level, when oxygen is not available in the environment (Jackson, 2000, 2002; Nilsson, 2001). These species have a remarkable ability to fully recover cardiac function after a prolonged lack of oxygen (Gesser, 1977; Wasser et al., 1990, 1992; Hicks and Wang, 1998). This response contrasts with mammalian hearts, which generally succumb quickly during severe hypoxia and, in time, exhibit considerable myocardial necrosis (Allen et al., 1985; Heusch and Schulz, 1996). The pronounced anoxia tolerance of turtles and carp is linked to a marked depression of metabolic rate, which is further enhanced at low temperatures, so that anaerobic metabolism can balance energy consumption and reduce the rate of waste production (Jackson, 2000, 2002). Moreover, these species reduce or slow the acidosis that usually develops with anoxia (see Nilsson, 2001; Jackson, 2002).

Rainbow trout (*Oncorhynchus mykiss*) is not particularly hypoxia tolerant, but may encounter substantial variations in oxygen availability in both natural settings and when kept in aquaculture. When exposed to hypoxia, rainbow trout exhibit the typical piscine cardio-respiratory response (e.g. Randall, 1982) and, in common with other animals, reduce their preferred body temperature during hypoxia by seeking lower environmental temperatures (Schurmann et al., 1991; Steiner and Branco, 2002). Hypoxia-induced hypothermia diminishes

metabolism, as well as the critical oxygen level (Schurmann and Steffensen, 1997; Ott et al., 1980), and serves a protective role by alleviating the demands on the cardiovascular system. The importance of suppressing cardiac demands is well illustrated by the finding that the 70% reduction in force production by anoxic myocardial strips from rainbow trout occurs faster, albeit ultimately to the same extent, at 20°C compared with 10°C (Hartmund and Gesser, 1992). While reduced temperature confers benefits to prolong the activity of anoxic cardiac tissue by reducing metabolism and the rate of the ensuing lactic acidosis, decreased temperature also diminishes glycolytic capacity, which may affect the balance between energy production and energy consumption.

Using *in situ* perfused hearts from trout, we investigate the effects of temperature on routine and maximal cardiac performance during normoxia, anoxia and following recovery from anoxia. We tested the hypothesis that the rainbow trout heart benefits from lowered temperatures during anoxia (severe hypoxia) despite an anticipated depression of glycolytic capacity. Measurements were taken before, during and after a 20 min exposure to anoxia in preparations that were acutely exposed to 5, 15 and 18°C, as well as in preparations measured at the acclimation temperature of the fish (10°C). To mimic *in vivo* conditions, the hearts were set to work at increasingly higher loads with increasing temperature, and maximal cardiac performance was measured both before and following recovery from anoxia. These functional variables are related to glycolytic capacity and energetic status of the cardiac muscle, which were determined at the end of the experiment.

Materials and methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum; mass=482±15 g, *N*=33) were obtained from a local fish farm (Sun Valley Trout Farm, Langley, BC, Canada) and held in a large fibreglass tank (2000 litre) continuously supplied with dechlorinated tapwater. The fish were maintained under natural photoperiod at a temperature of 10±1°C and fed commercial trout pellets three times a week.

Surgical procedures

Fish were anaesthetised in an oxygenated buffered solution of tricaine methane sulfonate (MS-222; 0.15 g l^{-1} MS-222 plus 0.15 g l^{-1} sodium bicarbonate) and transferred to an operating table where their gills were irrigated with oxygenated water containing diluted anaesthetic (0.075 g l^{-1} MS-222 plus 0.075 g l^{-1} sodium bicarbonate) at 8–10°C. Fish were then injected with 1.0 ml kg^{-1} of heparinised (100 U ml^{-1}) saline *via* the caudal blood vessels, and the perfused heart preparation was prepared as described by Farrell et al. (1986). Briefly, an input cannula was introduced into the sinus venosus through a hepatic vein, and perfusion with heparinised (10 IU ml^{-1}) saline containing 7.5 nmol l^{-1} adrenaline was immediately commenced (the adrenaline concentration was higher for 18°C hearts; see below). An output cannula was inserted into the ventral aorta at a point confluent with the bulbus arteriosus and tied firmly in place with a single silk thread. Finally, both ducts of Cuvier were occluded with sutures, thereby crushing the cardiac branches of the vagus nerve and eliminating all venous return to the heart except for the perfusate delivered to the heart *via* the input cannula. Thus, the *in situ* preparation isolated the heart in terms of perfusate delivery and collection, as well as autonomic nervous control, while leaving the pericardium intact.

Once surgery was completed (~15–20 min after netting), the fish was immersed in a temperature-controlled saline bath regulated at the test temperature (either 5, 10, 15 or 18°C). The input cannula was attached to an adjustable constant-pressure reservoir, and the output cannula was connected to a constant pressure head. For routine normoxic conditions, output pressure (P_{out}) was set to 4.9 kPa to simulate *in vivo* ventral aortic blood pressure at rest, and input pressure to the heart (P_{in}) was adjusted to give a routine cardiac output that was physiological for the particular test temperature (approximately 12, 16, 20 and 24 ml min^{-1} kg^{-1} for 5, 10, 15 and 18°C, respectively; Table 1) (Kiceniuk and Jones, 1977; Thorarensen et al., 1996). As a result, Q_{10} of routine cardiac output and power output varied between 1.6 and 1.8 for the different temperature intervals (average 1.7), which resembles *in vivo* Q_{10} values (~2; Driedzic and Gesser, 1994). In the absence of autonomic input, heart rate was set by the intrinsic pacemaker and the stimulation provided by the tonic level of adrenaline in the perfusate. The 7.5 nmol l^{-1} of adrenaline used for 5, 10 and 15°C hearts is similar to that of plasma in resting rainbow trout (Milligan et al., 1989). However, as in previous studies of perfused rainbow trout hearts studied at temperatures approaching their upper lethal limit (Farrell et al., 1996), our preparations needed considerably higher adrenergic stimulus to maintain stable cardiac output and heart rate at 18°C. Therefore, at 18°C the perfusate contained 50–100 nmol l^{-1} adrenaline. Cardiac output was maintained at a routine level for 20 min to allow heart function to stabilise and to ensure temperature equilibration with the saline bath. Hearts that required a P_{in} of >0.05 kPa to reach routine cardiac flow rate ($\dot{Q}$) were discarded because this indicates either a dysfunctional pericardium or improper input cannula placement in the preparation. However, two out of seven hearts at 18°C were allowed to start with marginally higher input pressures because these preparations appeared normal in all other respects. Adjustments of P_{in} were made continuously to maintain routine $\dot{Q}$.

The perfusate contained 124 mmol l^{-1} NaCl, 3.1 mmol l^{-1} KCl, 0.93 mmol l^{-1} $MgSO_4.7H_2O$, 2.52 mmol l^{-1} $CaCl_2.2H_2O$, 5.6 mmol l^{-1} glucose, 6.4 mmol l^{-1} TES salt and 3.6 mmol l^{-1} TES acid. The TES buffer simulates the buffering capacity of trout plasma and the normal change in blood pH with temperature ($\Delta pK/dT$ of 0.016 pH units $deg.^{-1}$). The perfusate was renewed every 20 min, as adrenaline rapidly degrades. The coronary circulation that normally supplies the outer 30–40% of the

rainbow trout heart (Farrell et al., 1986, 1988a) was not perfused in our preparation. To compensate, the perfusate was gassed with 100% O_2, and Gamperl et al. (2001) argued that this level of oxygenation can supply a sufficient amount of O_2 to the outer myocardial layer because the O_2 tension in the perfusate is more than 10× that routinely found in venous blood in rainbow trout (Farrell and Clutterham, 2003). As a result, maximal performance of the *in situ* perfused rainbow trout heart is comparable with, or even higher than, the maximal performance measured *in vivo* (Farrell et al., 1986, 1991). For the anoxic (severe hypoxic) exposures, the perfusate reservoir was gassed with 100% N_2 for at least 1 h, which resulted in a partial oxygen pressure (P_{O_2}) of <0.5 kPa. Oxygen transfer between the surrounding bath and the heart was minimised by covering the preparation with a loose-fitting plastic lid and bubbling the saline bath with 100% N_2, beginning 5 min before the onset of anoxia.

Experimental protocols

The experimental protocols were designed to examine the effects of temperature on normoxic, anoxic and post-anoxic performance. Maximal and routine performance was assessed, and recovery of cardiac performance was measured after exposure to a standardised 20-min period of anoxia. During anoxia, P_{in} was gradually raised to, but not above, 0.25 kPa in an attempt to maintain routine $\dot{Q}$. If 0.25 kPa was insufficient, flow was allowed to decline. Following anoxia, P_{out} was reduced to 1.0 kPa for 10 min to aid the recovery of cardiac output.

Fish were randomly assigned to one of five experimental treatment groups (N=6 or 7). The fish tested at 5°C were significantly larger than those tested at 18°C, but the ratio between ventricle mass and body mass (0.089±0.002%) was similar for all temperature groups (Table 1). The five experimental treatments were as follows: (A) Oxygenated control tested at 10°C; these hearts were only exposed to oxygenated saline and were intended to reveal any decay in cardiac performance over time; (B–E) anoxic exposure; hearts were tested at 5, 10, 15 or 18°C, using routine cardiac outputs of 12, 16, 20 or 24 ml min^{-1} kg^{-1}, respectively. The various protocols, which all lasted 140 min, are depicted schematically in Fig. 1. After stabilisation for 20 min at the routine cardiac output, maximum cardiac output was assessed under normoxic conditions (see maxtest protocol below). Subsequently, the hearts were allowed to recover at routine cardiac output for 55 min, whereupon they were exposed to 20 min of anoxia, followed by 30 min of normoxic recovery before a second maxtest was performed.

Assessment of cardiac performance and viability

Assessment of cardiac performance was based on repeated measurements of routine and maximum cardiac performance, while viability was based on biochemical measures. Maximal cardiac performance (maxtest) was determined by increasing P_{in} up to 0.45 kPa to maximise stroke volume and reach maximal flow rate ($\dot{Q}_{max}$). Then, P_{out} was progressively

Table 1. *Routine and maximal cardiac performance of* in situ *perfused trout hearts at 5, 10, 15 and 18°C during the oxygenated conditions at the start of the experiments*

Treatment	Temp. (°C)	Fish mass (g)	Ventricle mass (g)	Routine $\dot{Q}$ (ml kg^{-1} min^{-1})	P_{in} at routine $\dot{Q}$ (kPa)	f_H (min^{-1})	V_S (ml kg^{-1})	$\dot{Q}_{max}$ (maxtest$_1$) (ml kg^{-1} min^{-1})	PO_{max} (maxtest$_1$) (mW g^{-1} ventricle)	V_{Smax} (maxtest$_1$) (ml kg^{-1})
Control	10	508±39^{a,b} (7)	0.42±0.03^{a,b} (7)	15.0±0.2 (7)	–0.06±0.02^{a} (7)	55.0±1.3^{a} (7)	0.27±0.01^{a} (7)	43.9±2.4^{a,b} (7)	5.1±0.4^{a,b} (7)	0.89±0.04^{a} (7)
Anoxic challenge	5	561±24^{a} (7)	0.51±0.02^{a} (7)	11.5±0.5 (7)	–0.02±0.02^{a} (7)	39.6±0.5^{b} (7)	0.29±0.01^{a} (7)	34.3±1.2^{b} (7)	3.3±0.3^{b} (7)	0.98±0.03^{a} (7)
Anoxic challenge	10	450±32^{a,b} (6)	0.40±0.03^{a,b} (6)	16.0±0.5 (6)	–0.04±0.02^{a} (6)	57.8±2.8^{a} (6)	0.28±0.02^{a} (6)	52.8±2.5^{a,c} (6)	5.5±0.3^{a} (6)	0.97±0.07^{a} (6)
Anoxic challenge	15	454±13^{a,b} (6)	0.41±0.02^{a,b} (6)	19.7±0.4 (6)	–0.03±0.01^{a} (6)	61.0±2.5^{a} (6)	0.33±0.02^{a} (6)	61.9±2.3^{c} (6)	6.5±0.3^{a} (6)	1.07±0.05^{a} (6)
Anoxic challenge	18	427±20^{b} (7)	0.39±0.03^{b} (7)	22.8±0.6 (7)	0.00±0.02^{a} (7)	81.9±3.9^{c} (7)	0.28±0.2^{a} (7)	75.6±4.8^{d} (7)	8.7±0.8^{c} (7)	1.03±0.06^{a} (7)

Dissimilar letters indicate significant difference (P<0.05).
N is indicated by the number in parentheses.

increased to attain maximal power output (PO_{max}). After the maxtest, $\dot{Q}$ and P_{out} were returned to routine levels. Routine cardiac performance was measured continuously throughout the experiment and maximal performance was assessed at the start of the experiment during normoxia before other experimental manipulations ($maxtest_1$) and repeated 30 min after anoxia ($maxtest_2$) (Fig. 1). In this way, each heart served as its own control for statistical analysis.

Perfusate from the outflow was sampled at 2 min intervals during the 20 min anoxic challenge (arrows in Fig. 1) to measure lactate efflux from the heart. Samples were stored at –80°C until analysis with an automated YSI 2300 Stat Plus glucose and L-lactate analyser (YSI Inc., Yellow Springs, OH, USA). Lactate efflux rate (nmol lactate g^{-1} ventricle min^{-1}) was calculated as:

$$\text{Lactate efflux rate} = [\text{perfusate lactate}] \times \dot{Q}/M_v , \quad (1)$$

where perfusate lactate concentration is in mmol l^{-1}, $\dot{Q}$ is in ml min^{-1} and M_v is ventricular mass (g).

Maximal rate of lactate efflux during anoxia was defined as the maximal value measured at stable heart rate (f_H), and lactate measurements were discarded if f_H or $\dot{Q}$ were unstable or approached zero. Thus, due to rapidly failing flow during anoxia at 18°C, no lactate efflux rates are reported at that temperature.

Myocardial enzyme activities and high-energy phosphate concentrations were measured to evaluate myocardial necrosis and to determine whether energy balance had been restored after the various treatments. Ventricular tissue was sampled 5 min after $maxtest_2$, i.e. approximately 40 min after the conclusion of the anoxic period. Tissue was quickly excised and freeze-clamped with aluminium tongs precooled in liquid nitrogen, split into several pieces that were weighed and stored at –80°C. Biochemical analysis was performed on 50–200 mg muscle samples homogenised in 50 volumes of 50% glycerol in 20 mmol l^{-1} sodium phosphate buffer (pH 7.4), 5 mmol l^{-1} β-mercaptoethanol, 0.5 mmol l^{-1} EDTA and 0.02% bovine serum albumin (BSA). Activities of the glycolytic enzymes lactate dehydrogenase and pyruvate kinase were measured at 20°C as described by Chi et al. (1983) and Christensen et al. (1994), respectively, with some modifications. The lactate dehydrogenase reaction solution contained 100 mmol l^{-1} imidazole adjusted to pH 7.4, 1 mmol l^{-1} sodium pyruvate, 0.3 mmol l^{-1} NADH and 0.05% BSA. Pyruvate kinase activity was measured in a reaction solution consisting of 50 mmol l^{-1} triethanolamine, 75 mmol l^{-1} KCl, 8 mmol l^{-1} $MgSO_4$, 0.8 mmol l^{-1} phosphoenolpyruvate, 1 mmol l^{-1} ADP, 0.3 mmol l^{-1} NADH and 60 U ml^{-1} lactate dehydrogenase that was adjusted to pH 7.5. Both enzymatic activities were expressed relative to ventricular protein content, which was measured from similar homogenates (without BSA) using Sigma kit no. 690-A (Sigma Chemicals, Oakville, ON, Canada). In addition to the measurements of enzymatic activity at 20°C, enzymatic activities were also measured at 5, 10 and 15°C in six randomly chosen samples to obtain the temperature sensitivity of these glycolytic enzymes.

Myocardial high-energy phosphates were measured using high-preformance liquid chromatography (HPLC; Bøtker et al., 1994). Briefly, a 30–60 mg piece of ventricle was homogenised in 1.6 ml of 0.42 mol l^{-1} perchloric acid (PCA) in a glass–glass homogeniser. The homogenate was then centrifuged for 10 min at 3400 ***g***, and the supernatant was separated into two 200 µl portions. One portion was used for measurement of creatine compounds (phosphocreatine and creatine) and was neutralised with 100 µl KOH (1 mol l^{-1}), while the other, used for subsequent measurement of adenylates (ATP, ADP and AMP), was neutralised with 100 µl of $KHCO_3$ (2 mol l^{-1}) and Tris (0.1 mol l^{-1}). After neutralisation, both portions were kept on ice for 10 min to

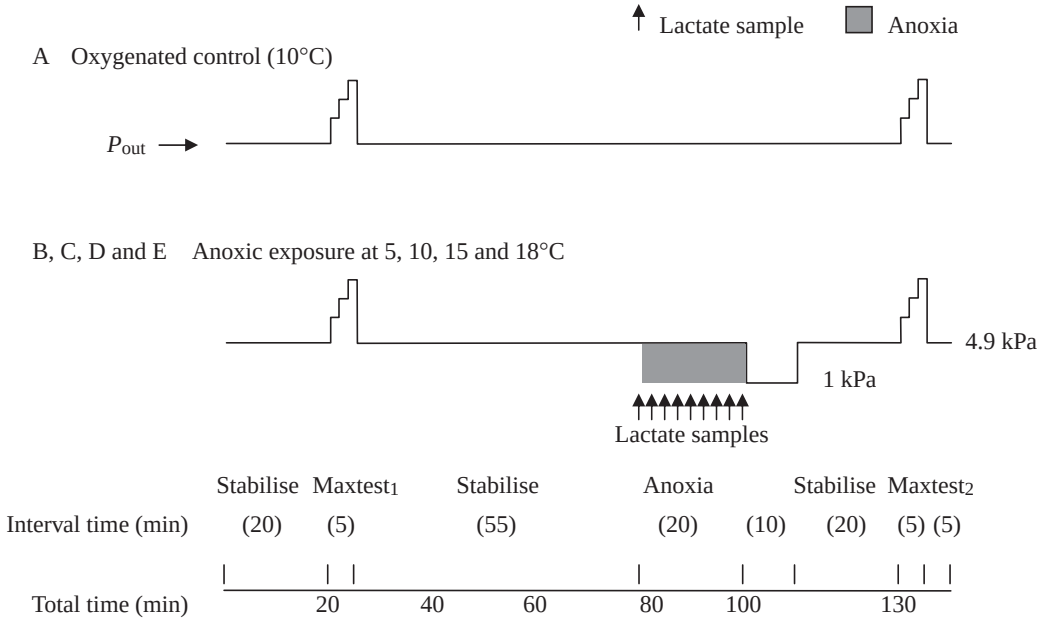

Fig. 1. Graphical presentation of the experimental protocols used to examine cardiac performance in oxygenated and anoxic *in situ* perfused trout hearts. Output pressure (P_{out}) is represented by the solid line, and the two maximal performance tests are shown as the progressive increases in P_{out}. The anoxic exposure is shown with grey bars. During both routine oxygenated and anoxic conditions, P_{in} was adjusted to as much as 0.25 kPa to maintain cardiac flow rate ($\dot{Q}$) at 12, 16, 20 or 24 ml min^{-1} kg^{-1} at 5, 10, 15 and 18°C, respectively. Arrows indicate samples for lactate measurements of the perfusate.

ensure precipitation of perchlorate. The neutralised portions were then centrifuged for 5 min (3400 ***g***) and the supernatant stored at –80°C until further analysis. Creatine compounds and adenylate compounds were separated using HPLC (Waters, Milford, MA, USA) with a 10 cm crompack C18 microsphere-column of 3 μm particle size (Varian, Palo Alto, CA, USA). Creatine compounds were measured at a wavelength of 210 nm using a mobile phase of aqueous buffer containing 0.02 mol l^{-1} KH_2PO_4 and 2.3 mmol l^{-1} tetrabutyl ammonium hydrogen sulphate (TBAHS) run at 1.5 ml min^{-1}. Adenosine nucleotides were measured at a wavelength of 254 nm using a mobile phase of 25% methanol and 75% aqueous buffer containing 0.06 mol l^{-1} KH_2PO_4 and 0.011 mol l^{-1} TBAHS run at 1 ml min^{-1}. Cardiac energy status was expressed as adenylate energy charge: ([ATP]+ 0.5[ADP])/([ATP]+[ADP]+[AMP]) and phosphorylation potential: $[PCr]/[Cr]^2$ (Meyer, 1988).

Muscle lactate was measured spectrophotometrically as described by Lowry and Passonneau (1972) from the same homogenates as those used for adenylate measurements. Briefly, lactate was measured from the change in absorbance following addition of lactate dehydrogenase (15 U ml^{-1}) in a reaction buffer containing 50 mmol l^{-1} glutamic acid, 50 mmol l^{-1} 2-amino-2metyl-propanolol, 1.5 mmol l^{-1} NAD and glutamate-pyruvate transaminase (3 U ml^{-1}). Muscle glycogen was measured according to Bergmeyer (1983). After homogenisation in 0.6 mol l^{-1} PCA, glycogen in the homogenate was transformed to glucose by incubation for 3 h at 35°C in an acetate buffer (0.1 mol l^{-1}, pH 4.8) containing amyloglucosidase. Subsequently, glucose was measured spectrophotometrically in a 0.3 mol l^{-1} triethanolamine buffer containing 4 mmol l^{-1} $MgSO_4$, 0.4 mmol l^{-1} ATP and 0.4 mmol l^{-1} NADP before and after addition of hexokinase and glucose-6-phosphate dehydrogenase. Muscle glycogen content is, therefore, presented as a glucose concentration and calculated as: [glucose] in homogenate – [glucose] in homogenate supernatant (no glycogen transformation).

Instrumentation set-up and data analysis

An in-line electromagnetic flow probe (Zepeda Instruments, Seattle, WA, USA) was used to record ventral aortic flow ($\dot{Q}$), and disposable pressure transducers (model DPT-6100; PVB Medizintechnik, Hauptstraße, Germany) were used to measure inflow and outflow pressures. Pressure transducers were calibrated daily against a static water column and referenced to the surface of the saline bath regularly. Pressure transducer signals were amplified with a Senselab amplifier (Somedic Sales AB, Hörby, Sweden), while the flow signal was amplified using a DC amplifier (Gould, Cleveland, OH, USA). All signals were recorded at 5 Hz on an in-house programmed computer-assisted data acquisition system (National Instruments, Austin, TX, USA), and block averages were calculated every 5 s. The measured values for P_{in} and P_{out} were adjusted for cannulae resistances so that the reported pressures represented those in the sinus venosus and in the bulbus arteriosus, respectively. f_H was measured by counting pressure pulses over 10 s periods. Ventricular power output (*PO*; mW g^{-1} ventricle) was calculated as:

$$PO = \dot{Q} \times (P_{out} - P_{in}) \times 0.0167/M_v , \quad (2)$$

where $\dot{Q}$ is in ml min^{-1}, P_{in} and P_{out} are in kPa, and 0.0167 is the conversion factor to mW.

Stroke volume (V_S) was calculated as:

$$V_S = (\dot{Q}/M_b)/f_H , \quad (3)$$

where $\dot{Q}$ is in ml min^{-1}, f_H is in min^{-1} and M_b is body mass (kg).

Statistics

All statistical analyses were performed using SigmaStat for Windows 2.03 (SPSS Inc., Chicago, IL, USA). One-way analyses of variance (ANOVAs) were used to compare parameters between treatment groups, including: (1) body and ventricular mass; (2) P_{in} required to attain routine cardiac output; (3) routine values of V_S and f_H; (4) maximal cardiac performance during $maxtest_1$; (5) relative recovery of function during $maxtest_2$; (6) biochemical status of the muscle samples obtained at the end of the experiments (including pyruvate and lactate dehydrogenase activity at 20°C, protein, adenylate, creatine, glycogen and lactate content, adenylate energy charge and $[PCr]/[Cr]^2$). A nonparametric test (Mann–Whitney) was used when data were not normally distributed. Repeated-measures ANOVAs were performed for comparisons of: (1) maximum cardiac performance within a treatment group at the initial and second test and (2) P_{in}, *PO*, $\dot{Q}$, V_S, and f_H during the 20 min anoxic challenge relative to the control (pre-anoxic) conditions. Unless otherwise stated, statistical differences were identified using a Bonferroni *post-hoc* test, and $P<0.05$ was used as the level of statistical significance. All data are reported as means ± S.E.M.

Results

Initial cardiac performance under oxygenated conditions

Differences in the initial routine cardiac output among temperature groups were due solely to heart rate (mean Q_{10} ~1.7), as routine cardiac output was attained with a similar filling pressure and stroke volume for all test temperatures (Table 1). Similarly, maximal stroke volume was not significantly affected by temperature (Table 1), so maximal cardiac and power output increased with Q_{10} values of 1.8 and 2.1, respectively, under oxygenated conditions.

Input pressure was increased gradually during the experiment to maintain routine cardiac output (Fig. 2). After anoxia, it was often necessary to increase P_{in}, and the increase in P_{in} was particularly pronounced at 18°C (Fig. 2A).

Cardiac performance during anoxia

Normoxic cardiac performance increased progressively with elevated temperature, while cardiac anoxia tolerance was inversely related to temperature (Figs 2, 3). Hearts maintained cardiac output throughout anoxia at 5°C (Fig. 2E), but anoxia

tolerance decreased with increased temperature, as indicated by the pronounced and rapid decline of cardiac output in spite of the increased input pressure (Fig. 2). Routine cardiac output was significantly reduced after 15 min and 20 min of anoxia at 15°C and 10°C, respectively, and significantly depressed after only 5 min of anoxia at 18°C. Nevertheless, cardiac output was similar at the end of anoxia (approximately 5 ml min^{-1} kg^{-1}) in all groups at 10°C or warmer.

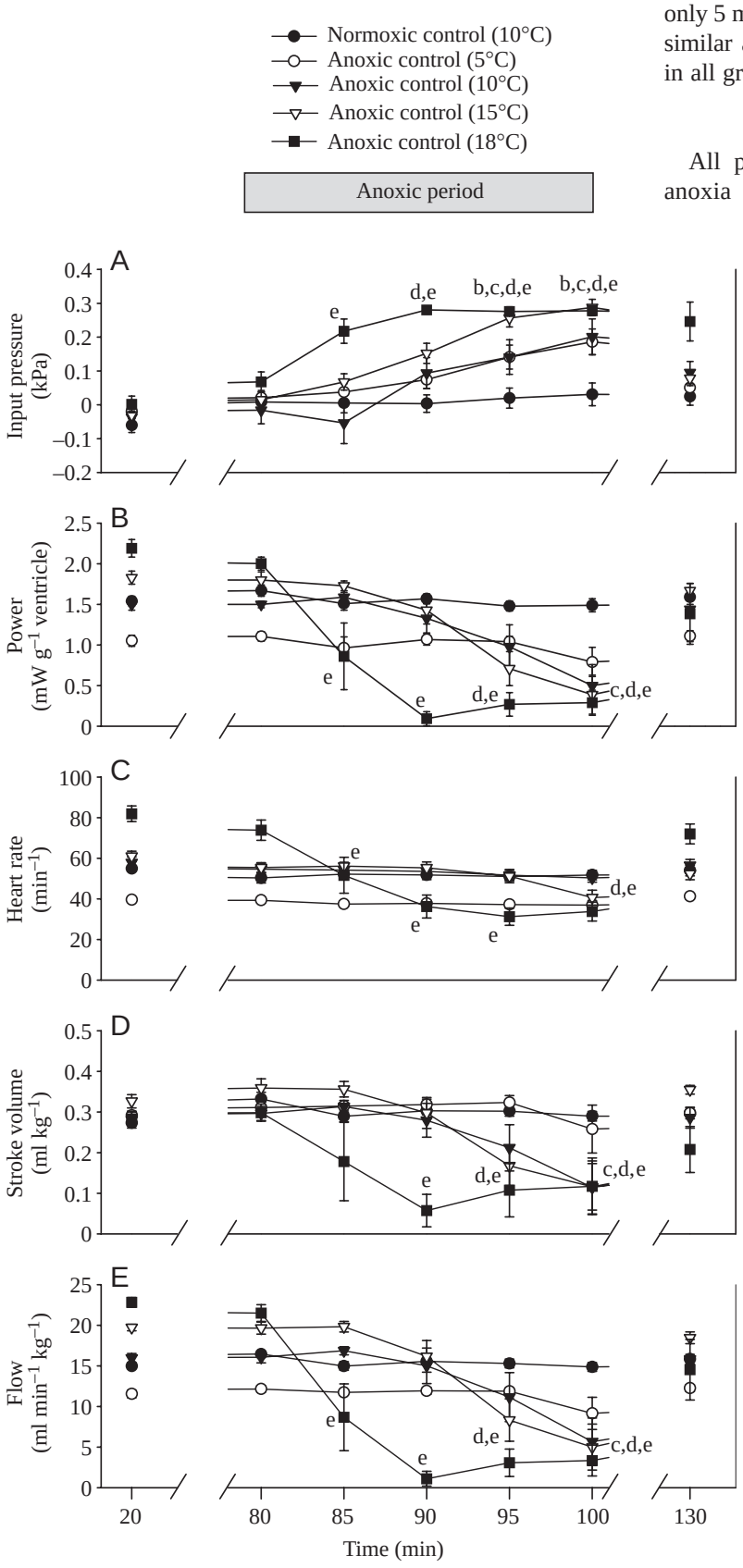

Fig. 2. Routine cardiac performance before, during and after the anoxic period as a function of time. Representative measurements were taken; (1) before $maxtest_1$; (2) 0, 5, 10, 15 and 20 min into the anoxic test; and 3) before $maxtest_2$. (A) Input pressure was adjusted in an attempt to maintain flow at 12, 16, 20 or 24 ml min^{-1} kg^{-1} for 5, 10, 15 and 18°C hearts (see text for further explanation). (B) Power output. (C) Heart rate. (D) Stroke volume. (E) Cardiac output. Letters indicate significant changes within each group during anoxia relative to values at the onset of anoxia: [b]differences for hypoxic hearts at 5°C, [c]hypoxic hearts at 10°C, [d]hypoxic hearts at 15°C and [e]hypoxic hearts at 18°C.

Cardiac performance after anoxic recovery

All preparations were allowed 30 min to recover from anoxia before $maxtest_2$. Maximal performance of the oxygenated control at 10°C was unchanged over time (Fig. 3). While maximal performance was not affected by anoxia at 5°C, the ability of the *in situ* perfused heart to recover from anoxic exposure was inversely related to temperature and the reduction of maximal performance was 55% at 18°C (Fig. 3). The reduction in maximal cardiac performance following recovery from anoxia that was observed at temperatures above 5°C was mainly caused by a reduction in stroke volume, while post-anoxic heart rate was only marginally affected (Fig. 3C,D). Curiously, the absolute levels for maximum cardiac output and power output of the recovered hearts did not vary significantly with temperature and were ~40 ml min^{-1} kg^{-1} and ~3.8 mW g^{-1} ventricle, respectively, at all temperatures (Fig. 3A,B). Heart rate was unaffected except at 15°C (Fig. 3D).

The effect of acute temperature changes on glycolytic metabolism

When measured at 20°C, maximal enzymatic activities of lactate dehydrogenase (LDH) and pyruvate kinase (PK) were similar in all experimental groups (Table 2), indicating that all hearts possessed similar capacities for glycolytic energy production. However, maximal enzymatic activity was temperature dependent (Fig. 4) with Q_{10} values of 1.9 and 2.3 for LDH and PK, respectively, between 5°C and 20°C. The Q_{10} values were considerably higher for both enzymes between 5°C and 10°C compared with between

10°C and 20°C (Q_{10}=2.5 *vs* 1.6 for LDH and 5.0 *vs* 1.6 for PK).

The temperature dependency of the glycolytic capacity was reflected in a temperature dependency of lactate efflux from the anoxic perfused hearts (Fig. 5). We estimate the highest rates of lactate production to be 2.0, 2.7 and 4.1 μmol min^{-1} g^{-1} ventricle at 5, 10 and 15°C, respectively (Fig. 5), which yielded a Q_{10} of 2.1, which is comparable with the Q_{10} of the glycolytic enzymes. (We were unable to quantify lactate production reliably at 18°C as cardiac output failed to stabilize almost immediately after the onset of anoxia.)

Biochemical and energetic state of the myocardium following recovery

There were only very limited differences in myocardial state after recovery from anoxia. Enzymatic capacities of PK and LDH, glycogen content and total creatine were similar between temperature groups (Table 2), indicating a lack of cellular rupture and absence of energetic depletion of the myocardium. In general, energetic status was similar between groups, although adenylate charge was significantly lower at 18°C compared with 5°C, coupled with significantly lower total adenylates at 18°C. Hearts at 18°C were characterised by a higher level of muscle lactate compared with those at 5°C and 15°C, which may indicate that myocardial homeostasis had not fully recovered at 18°C.

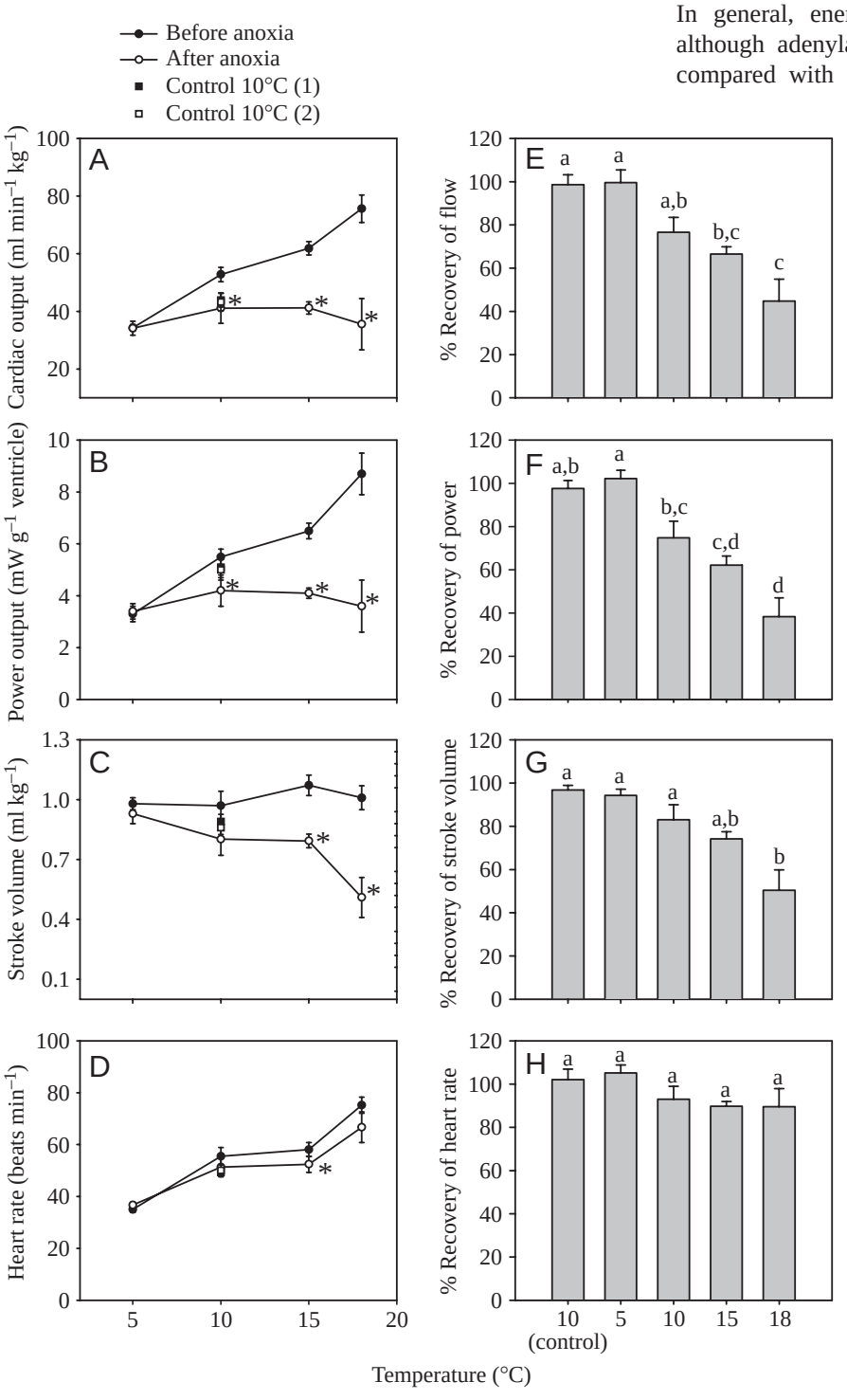

Fig. 3. Maximal performance of *in situ* trout hearts measured before and after recovery from 20 min of anoxia. (A–D) Line plots of cardiovascular variables before (filled symbols) and after (open symbols) recovery from 20 min anoxia. (A) Cardiac output, (B) power output, (C) stroke volume, (D) heart rate. (E–H) Recovery of cardiac output, power output, stroke volume and heart rate after anoxia, expressed as the second maxtest relative to the first maxtest. Significant differences (P<0.05) between the initial and second maxtest are indicated with an asterisk, while dissimilar letters indicate significant differences between the relative recovery of cardiac performance. Control 10°C (1) and (2) indicate measurements taken at the onset and conclusion of the experiment, respectively.

Discussion

Routine and maximum cardiac performance under oxygenated conditions

Because pre-anoxic measurements of maximal cardiac performance were made at all experimental temperatures, we can assess the effect of acute temperature changes on performance. Most previous studies on rainbow trout have measured the fish at their respective acclimation temperature (reviewed by Farrell et al., 1996; Farrell, 2002).

Maximal flow and power output reported in our study are generally similar to those in previous *in situ* and *in vivo* studies. While our values for maximal cardiac performance at 10°C and 15°C are

Table 2. *Biochemical and energetic status of trout hearts following the 30 min post-anoxic recovery period*

Treatment	Temp. (°C)	Total adenylates (mmol l^{-1})	Total creatine (mmol l^{-1})	Protein content (% of wet mass)	Muscle glucogen (µmol glucose g^{-1} ventricle)	Muscle lactate (µmol g^{-1} ventricle)	PK activity (µmol g^{-1} protein min^{-1})	LDH activity (µmol g^{-1} protein min^{-1})	Adenylate charge	PCr/Cr^2
Control	10	4.98±0.10^{a,b} (5)	14.08±0.72^{a} (5)	0.17±0.01^{a} (6)	19.9±4.1^{a} (5)	2.4±0.4^{a,b} (5)	561.4±43.6^{a} 6)	1891±235^{a} (6)	0.86±0.01^{a,b} (4)	0.18±0.06^{a} (4)
Anoxic challenge	5	4.99±0.08^{b} (7)	15.24±0.42^{a} (7)	0.14±0.01^{a} (7)	24.5±5.1^{a} (7)	1.6±0.2^{a} (7)	666.1±41.5^{a} (7)	2132±242^{a} (7)	0.88±0.02^{a} (7)	0.28±0.05^{a} (7)
Anoxic challenge	10	4.16±0.23^{a} (5)	14.42±0.95^{a} (6)	0.17±0.01^{a} (6)	10.4±3.4^{a} (6)	2.8±0.5^{a,b}(6)	567.3±53.9^{a} (6)	2136±85^{a} (6)	0.84±0.02^{a,b} (3)	0.27±0.06^{a} (3)
Anoxic challenge	15	4.54±0.08^{a,b} (6)	14.09±0.35^{a} (6)	0.17±0.01^{a} (6)	14.5±4.5^{a} (6)	1.5±0.4^{a} (6)	570.0±31.8^{a} (6)	1979±106^{a} (6)	0.84±0.01^{a,b} (6)	0.21±0.04^{a} (6)
Anoxic challenge	18	3.28±0.26^{c} (7)	13.15±1.01^{a} (7)	0.15±0.01^{a} (7)	16.5±4.0^{a} (7)	3.9±0.5^{b} (7)	609.1±39.6^{a} (7)	2267±164^{a} (7)	0.80±0.01^{b} (5)	0.15±0.02^{a} (7)

Dissimilar letters indicate significant difference (P<0.05).
N is indicated by the number in parentheses.
Enzymatic activities were measured at 20°C.

slightly lower than those in previous studies (Kiceniuk and Jones, 1977; Farrell et al., 1991, 1996), performance at 18°C closely resembles that reported by Farrell et al. (1996). Conversely, our values at 5°C are considerably higher than those reported at 4°C *in vivo* by Taylor et al. (1996). However, direct comparison is difficult because the level of adrenergic stimulation differs among studies. Here, we used a tonic level of adrenaline (7.5 nmol l^{-1}) at 5, 10 and 15°C to simulate resting *in vivo* plasma concentrations (Milligan et al., 1989). The importance of tonic adrenaline and its variable contribution with temperature was illustrated at 18°C, where adrenaline concentration had to be increased to 50–100 nmol l^{-1} to avoid cardiac arrhythmias. Similar problems have been encountered previously at temperatures above 18°C with rainbow trout hearts (Farrell et al., 1996).

Here, heart rate increased with a Q_{10} of 1.7 from 5°C to 18°C, as *in vivo* (Taylor et al., 1996). In acclimated rainbow trout, the positive chronotropic effect of temperature is reduced at high temperatures, and Farrell et al. (1996) reported a Q_{10} of 1.3 for heart rate between 15°C and 22°C for *in situ* perfused hearts. We observed a much higher Q_{10} (1.9) for heart rate between 15°C and 18°C, but this may have been influenced by the 10-fold higher adrenaline concentration applied to the hearts at 18°C as well as the result of an acute temperature change *versus* temperature acclimation. Recently, using a similar perfused heart set-up, Blank et al. (2002) found that the Q_{10} for heart rate in yellowfin tuna (*Thunnus albacares*) was considerably higher than in rainbow trout (approximately 3 at 10–25°C), but stroke volume was halved with each 10°C

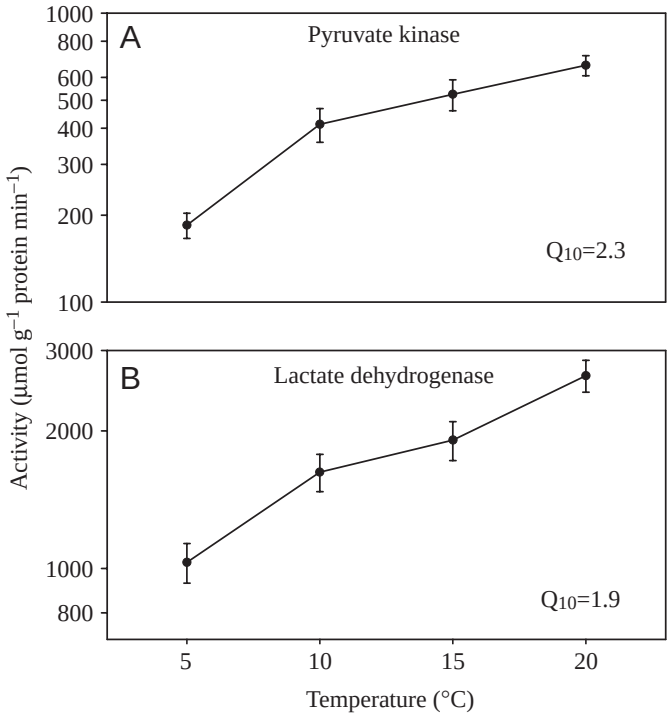

Fig. 4. Activity of the glycolytic enzymes pyruvate kinase (A) and lactate dehydrogenase (B) from rainbow trout measured at 5, 10, 15 and 20°C.

increase in temperature. The reduced routine stroke volume in tuna may reflect either a negative inotropic effect or a reduced cardiac filling time during diastole at the very high heart rates of this species.

Maximum stroke volume measured during the initial maxtest was not affected by temperature (Table 1) and this contrasts with results for acclimated rainbow trout, where maximum stroke volume was reduced at acclimation temperatures above 15°C (Farrell et al., 1996). It is possible that the higher adrenergic stimulation at 18°C explains part of this difference, which would be further exacerbated by the low sensitivity to catecholamines in rainbow trout acclimated to high temperature (Keen et al., 1993). The unchanged maximum stroke volume is, however, in accord with the unchanged twitch force of trout ventricular strips following acute temperature changes (Hartmund and Gesser, 1992; Hove-Madsen, 1992), although negative inotropy with increased temperature was observed at low temperatures in rainbow trout (Hove-Madsen, 1992).

Cardiac performance during anoxia

The major finding of the present study was that anoxia tolerance and recovery of the *in situ* perfused hearts performing at physiologically realistic workloads were inversely related to temperature. Hearts studied at 5°C maintained routine cardiac output throughout 20 min of anoxia and fully recovered maximal performance upon reoxygenation. By contrast, hearts failed within the first 10 min of anoxia and suffered a 55% loss of maximal performance after recovery at 18°C. The loss of function during anoxia at elevated temperature occurred in spite of an increased lactate efflux and glycolytic enzyme activities with elevated temperature. Despite the significant reduction in maximal cardiac performance above 5°C, there was no indication of myocardial necrosis, as biochemical and energetic parameters were generally unaffected, although some minor changes may have occurred at 18°C (Table 2). These results support our hypothesis that low temperature provides benefits towards myocardial hypoxia tolerance. Here, we show that, when devoid of a myocardial oxygen supply, routine cardiac performance can be maintained for a brief period at 5°C and shortly afterwards perform at a maximum level when oxygenation is restored. These results indicate that rainbow trout may have a physiological advantage in exhibiting behavioural hypoxic hypothermia when exposed to hypoxia (Schurmann et al., 1991).

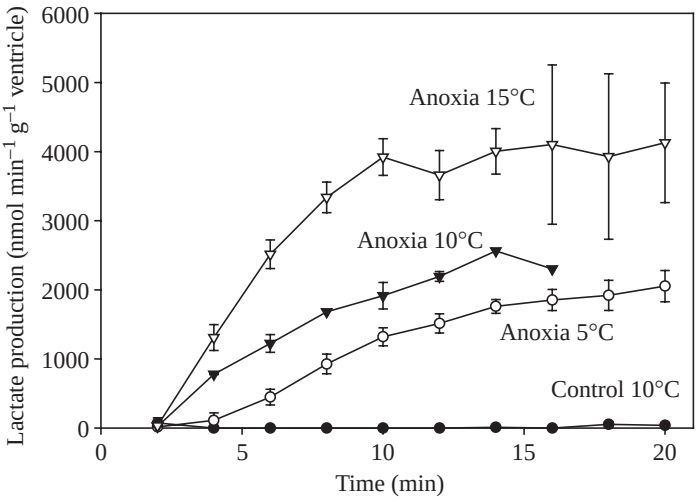

Fig. 5. Lactate efflux from *in situ* trout hearts during oxygenated control (10°C) and at 5, 10 or 15°C during 20 min of anoxia. Measurements were only included if cardiac output was stable.

The inability of the rainbow trout heart to maintain performance during anoxia is most likely caused by insufficient anaerobic energy production. Consistent with our study, Arthur et al. (1992) reported that glycolytic capacity of perfused trout hearts at 16°C could supply only half of the energetic requirement of oxygenated hearts working at routine conditions. Efficiency of rainbow trout hearts is unaffected by severe hypoxia (Arthur et al., 1992; J. Overgaard and H. Gesser, manuscript submitted). Consequently, anoxia substantially reduces phosphocreatine concentration in perfused hearts and cardiac strips (Arthur et al., 1992; Hartmund and Gesser, 1996), and cardiac failure appears to be caused by increased levels of intracellular inorganic phosphates and reduced intracellular pH (Allen et al., 1985; Godt and Nosek, 1989; Arthur et al., 1992). This is supported by the observation that maximum power output and intracellular pH of perfused rainbow trout heart decrease when the extracellular perfusate is made acidic (Farrell et al., 1986, 1988b). Elevated adrenaline concentration can, however, restore performance of an acidotic heart without intracellular pH being restored (Farrell and Milligan, 1986).

Maximal lactate efflux had a Q_{10} of 2.1 between 5°C and 15°C and, similarly, overall Q_{10} values of PK and LDH were 1.9 and 2.3, respectively. Glycolytic capacity, therefore, increases as least as much as routine cardiac performance (Q_{10}=1.7). The increased sensitivity to anoxia at high temperature, therefore, cannot be ascribed to a reduction in glycolytic capacity relative to energy requirements. Instead, it seems that the increased sensitivity is caused by a higher rate of accumulation of waste products with increased temperature, so the intracellular milieu, particularly intracellular phosphate and pH, is disturbed sooner.

Post-anoxic recovery

While several studies have examined the effects of severe hypoxia and anoxia on cardiac function in fish, few studies have previously described post-anoxic recovery of whole working hearts (however, see Gamperl et al., 2001; J. Overgaard, J. A. W. Stecyk, H. Gesser, K. Gamperl, T. Wang and A. P. Farrell, manuscript submitted). Our results show a marked effect of temperature on post-anoxic recovery, where hearts at 18°C only recover 45% of initial performance while hearts were unaffected by 20 min of anoxia at 5°C. The reductions in post-anoxic performance at temperatures above 5°C can mainly be attributed to reductions in the contractile performance of the hearts, as it is stroke volume rather than heart rate that is reduced

following recovery from anoxia. However, the decreased contractile performance of the hearts after anoxia at high temperature cannot be directly explained from impaired myocardial cellular status. Thus, we found no indication of either cellular necrosis or rupture, as both enzyme activity and concentrations of creatine products were similar in all experimental groups (Table 2). Similarly, with the exception of a significant difference in adenylate charge between 18°C and 5°C, there were no significant differences in energetic state between groups, and the values of adenylate charge and phosphorylation potential were similar to those of normoxic trout hearts (Arthur et al., 1992; J. Overgaard and H. Gesser, manuscript submitted). Moreover, the reduction in maximal cardiac performance is unlikely to be caused by energy depletion, as glycogen levels were recovered to a level close to that previously reported for normoxic rainbow trout (Gesser, 2002). The only notable differences were higher levels of muscle lactate and lower total adenylates at 18°C. Lactate levels after anoxia at 18°C were, nevertheless, only moderately higher and unlikely to represent a significant intracellular acidosis. A decrease in total adenylates at 18°C was also reported for anoxic myocardial strips with an inhibited glycolysis (Hartmund and Gesser, 1996), and it is possible that some of the adenylates are lost as adenosine during anoxia. While increased muscle lactate levels could be due to increased glycolytic metabolism during oxygenated perfusion, they may also represent residual lactate from the preceding anoxia. It is possible that lactate accumulated to higher levels at 18°C because the hearts failed early into the anoxic period and had rather low cardiac output during the remaining part of anoxia. The lower recovery of routine performance would also attenuate lactate removal after anoxia.

Given the general lack of cell death and disruption of myocardial energetic and enzymatic status in the failing hearts, we propose that decreased post-anoxic performance is due to myocardial stunning rather than necrosis. Myocardial stunning is defined as the mechanical dysfunction that persists after reoxygenation (reperfusion) despite the absence of irreversible damage (Bolli and Marban, 1999). The severity of myocardial stunning in mammals is associated with both the duration of flow deprivation and temperature, and the decreased contractility is thought to stem from a reduction in Ca^{2+} responsiveness caused by damage of the contractile apparatus of oxygen radicals and/or Ca^{2+} overload (Bolli and Marban, 1999). We suggest increased levels of oxygen radicals to be the primary candidate of stunning in trout, as Ca^{2+} overload only induces modest reductions in twitch force in ectothermic vertebrates compared with mammals (Poupa et al., 1985).

Conclusion

In the present study, we found that maximal and routine cardiac performance increase between 5°C and 18°C with a Q_{10} of 1.7–2.1 due to increased heart rate, while maximal and routine stroke volume were unaffected by acute temperature changes. Anaerobic capacity of the heart, as assessed through measurements of lactate efflux and enzymatic capacity, increased at a similar, but slightly higher, rate to that of routine cardiac output. Even so, 20 min of anoxia resulted in cardiac failure at 10, 15 and 18°C, with the rate of development of cardiac failure and the extent of residual impairment upon re-oxygenation both being temperature dependent. By contrast, hearts at 5°C were not significantly affected by anoxia. Thus, the consequences of anoxia are much more severe at high temperature because the hearts are working at higher rates. We suggest that increased levels of inorganic phosphates and protons cause cardiac failure during anoxia and that the accumulation of waste products is exacerbated once flow can no longer be maintained. Thus, even though glycolytic capacity decreases more than cardiac work with an acute decrease in temperature, the rate at which myocardial homeostasis is disturbed is slower due to the low metabolism. While energetic state was normalised after recovery from anoxia, post-anoxic cardiac performance was indirectly related to the test temperature. We propose that the mechanism underlying post-anoxic failure is myocardial stunning and that the degree of myocardial stunning is influenced by the degree of cardiac failure during the preceding anoxic period. Given these results, we conclude that fish exposed to anoxia have a clear physiological advantage in exhibiting behavioural hypoxic hypothermia, as this should aid them to endure and recover from hypoxic insults.

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