The Journal of Experimental Biology 207, 1005-1015
Published by The Company of Biologists 2004
doi:10.1242/jeb.00824

All rainbow trout (*Oncorhynchus mykiss*) are not created equal: intra-specific variation in cardiac hypoxia tolerance

Heather A. Faust[1,*], A. Kurt Gamperl[1,†] and Kenneth J. Rodnick[2]

[1]*Department of Biology, Portland State University, PO Box 0751, Portland, OR 97207-0751, USA* and
[2]*Department of Biological Sciences, Idaho State University, Pocatello, ID 82309-8007, USA*

*Present Address: Life Sciences Department, Monterey Peninsula College, 980 Fremont Street, Monterey, CA 93940, USA
†Author for correspondence at present address: Ocean Sciences Center, Memorial University of Newfoundland, St John's, Newfoundland, Canada. A1C 5S7 (e-mail: kgamperl@mun.ca)

Accepted 8 December 2003

Summary

All of our previous work, and that of other investigators, shows that the trout heart only partially recovers following brief exposure to severe hypoxia or anoxia (i.e. it is hypoxia-sensitive). However, in preliminary studies, we found evidence to suggest that rainbow trout reared at a farm in Oregon (USA) have a significant degree of inherent myocardial hypoxia tolerance. To evaluate whether hearts from these trout are indeed hypoxia-tolerant, and thus to determine whether intra-specific variation in rainbow trout myocardial hypoxia tolerance exists, we measured *in situ* cardiac function and monitored myoglobin and lactate dehydrogenase (LDH) release (both indices of myocardial damage) in hearts that were exposed to varying durations (10–30 min) of severe hypoxia (P_{O_2}=5–10 mmHg). There was a strong positive relationship between the duration of severe hypoxia and the degree of post-hypoxic myocardial dysfunction. However, the resulting dysfunction was modest, with hearts exposed to 30 min of severe hypoxia recovering 77% of their initial maximum cardiac output. Furthermore, myoglobin was not detected in the perfusate, and ventricular LDH activity did not vary in response to the duration of severe hypoxia. These data (1) indicate that trout from this farm have extremely hypoxia-tolerant hearts; (2) suggest that considerable intra-specific variation exists in trout myocardial hypoxia tolerance; and (3) provide preliminary evidence that trout hearts are not irreversibly damaged, but are merely 'stunned', following brief periods (10–30 min) of severe hypoxia.

Key words: rainbow trout, *Oncorhynchus mykiss*, intra-specific variation, cardiac hypoxia tolerance, heart, lactate dehydrogenase.

Introduction

A broad scope of myocardial hypoxia tolerance exists among the more than 20 000 species of fish, and this is often a reflection of the species' environmental history and/or activity level. Extremely athletic species such as the tuna have moderately to extremely hypoxia-sensitive hearts (Bushnell et al., 1990). In contrast, fish that survive in low oxygen environments, including the carp (Gesser, 1977) and eel (Bailey et al., 1999), as well as relatively sluggish species such as the hagfish (Axelsson et al., 1990), maintain myocardial performance during hypoxia and/or recover full cardiac function following oxygen deprivation for extended periods. Mechanisms responsible for inter-specific variations in myocardial hypoxia-tolerance have been extensively studied (reviewed in Driedzic and Gesser, 1994). However, intra-specific variation in myocardial hypoxia-tolerance has not been examined in fish, despite evidence that heart metabolism and glycolytic enzymatic activities can vary greatly between fish populations (Podrabsky et al., 2000).

The rainbow trout is generally considered to be a hypoxia-sensitive species (Gesser, 1977; Dunn and Hochachka, 1986; Arthur et al., 1992; Gamperl et al., 2001). However, we have identified an aquaculture facility in Oregon (USA) that produces rainbow trout which appear to have a considerable degree of inherent myocardial hypoxia tolerance. For example, although Gamperl et al. (2001) report that 15 min of severe hypoxia (P_{O_2}<5 mmHg; 1 mmHg=133.3 Pa) with only 5 min of physiological afterload (50 cmH_2O; 1 cmH_2O=98.07 Pa) reduced post-hypoxic cardiac output in trout from a British Columbia (Canada) facility by 38%, this protocol had no effect on post-hypoxic cardiac function in these Oregon-reared trout. In the current study, we determine the degree of myocardial hypoxia-tolerance displayed by these rainbow trout, and investigate whether myoglobin or lactate dehydrogenase release from the myocardium can be used as indices of myocardial damage (necrosis) in the *in situ* perfused trout heart. This latter goal is important for future studies of myocardial hypoxia tolerance and preconditioning in fishes. The loss of cardiac function following severe hypoxia could occur in response to temporary contractile dysfunction (i.e. stunning; Ferrari et al., 1999) or permanent cellular necrosis,

and the quantification of cell death remains the most widely accepted end-point for identifying myocardial preconditioning (Wolff et al., 2000).

Materials and methods

Fish husbandry

Adult female rainbow trout *Oncorhynchus mykiss* Walbaum (437–736 g) were purchased from Clear Creek Rainbow Ranch (Oregon City, Oregon, USA) and transported in insulated tanks to the Aquatic Vertebrate Facility at Portland State University (PSU). Once at PSU, these fish were held in 1000 l indoor tanks for a minimum of 10 days before experimental use. Water temperature was maintained at 10±1°C using a 3/4 Horsepower heat pump (model AHP-6, Aquanetics Systems, Inc., San Diego, CA, USA), photoperiod was 12 h:12 h light:dark, and fish were fed trout pellets *ad libitum* every other day. The tanks were supplied with municipal water that was continuously dechlorinated by slowly dripping sodium thiosulfate (50 g l^{-1}) (Aquatic Eco-Systems, Inc., Apopka, FL, USA) into the tank, and briner's grade (77%) calcium chloride (General Chemical Corporation, Parsippany, NJ, USA) was added to maintain calcium hardness at 80–140 p.p.m. The water in each tank was gradually replaced at a rate of approx. 1300 l each day, and each tank was continuously aerated to ensure that oxygen remained near saturation levels. Biological and mechanical filters were used to remove suspended solids and to maintain ammonia nitrogen levels below 2 p.p.m. Calcium hardness and ammonia nitrogen levels were monitored on a weekly basis using La Motte test kits (Chestertown, MD, USA).

Surgical procedures

All procedures were approved by the Animal Care Committee at PSU, and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Trout were anesthetized in an oxygenated, buffered solution of tricaine methane sulfonate (0.1 g l^{-1} MS-222; 0.1 g l^{-1} sodium bicarbonate) and transferred to an operating table where their gills were irrigated with oxygenated, buffered anesthetic (0.05 g l^{-1} MS-222; 0.05 g l^{-1} sodium bicarbonate) at 4–6°C. Fish were then injected with 1.0 ml of heparinized (100 i.u. ml^{-1}) saline *via* the caudal vessels, and an *in situ* heart preparation was obtained as detailed in Farrell et al. (1986).

The saline used to perfuse the heart (pH 7.8 at 10°C) contained (in mmol l^{-1}): NaCl (124), KCl (3.1), $MgSO_4.7H_2O$ (0.93), $CaCl_2.2H_2O$ (2.52), glucose (5.0), TES salt (6.4) and TES acid (3.6) (Keen et al., 1993). These chemicals were purchased from Fisher Scientific (Fair Lawn, NJ, USA), with the exception of the TES salt (Sigma Chemical Co., St Louis, MO, USA). Adrenaline bitartrate (15 nmol l^{-1} final concentration; Sigma Chemical Co.) was added to the perfusate every 20 min throughout the experiment to ensure the long-term viability of the perfused trout heart (Graham and Farrell, 1989). The saline was bubbled with 100% O_2 for a minimum of 45 min prior to use. Although the coronary circulation was not perfused, prior research on fish of similar size suggests that this level of oxygenation can supply sufficient O_2 to the outer myocardium such that the maximum performance of the *in situ* heart is comparable (Farrell et al., 1986) and perhaps even higher (Farrell et al., 1991) than that measured *in vivo*. For hypoxic exposures, the perfusate was bubbled with 100% N_2 for a minimum of 2 h prior to the experiments to ensure that P_{O_2} was 5–10 mmHg. Potential oxygen transfer from the experimental bath to the heart was minimized by covering the bath with a loose-fitting plastic lid, and by bubbling 100% N_2 into the bath beginning 5 min prior to the onset of severe hypoxia.

Experiment 1

Before assessing the degree of myocardial hypoxia tolerance displayed by these trout hearts, we wanted to ensure that the hearts were truly exposed to severely hypoxic conditions when perfused with N_2-equilibrated saline in our experimental apparatus.

Two groups (N=7–8) of *in situ* hearts were exposed to 15 min of severe hypoxia, at an output pressure (P_{out}) of 50 cmH_2O (Fig. 1). In addition, one of the groups was also exposed to 1.5 mmol l^{-1} of NaCN. Cardiac output ($\dot{Q}$) and heart rate (f_H) were monitored at 2 min intervals throughout severe hypoxia. In these experiments, the recovery of maximum cardiac output ($\dot{Q}_{MAX}$) was not assessed, due to the potential effects of residual NaCN on *in situ* cardiac performance. In this experiment, the P_{O_2} of the perfusate entering the heart was also measured by collecting perfusate

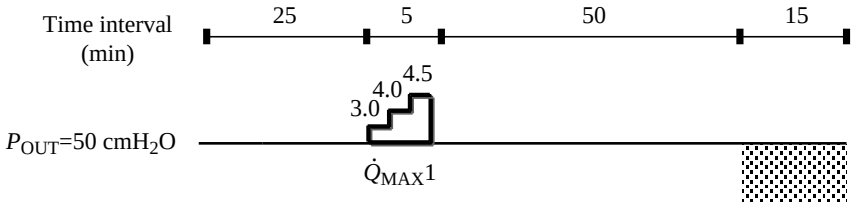

Fig. 1. Schematic diagram of the experimental protocol used to confirm that the *in situ* trout hearts were experiencing severe hypoxia. One group of *in situ* hearts was perfused with severely hypoxic saline (N=8), while a second group of hearts was perfused with severely hypoxic saline containing 1.5 mmol l^{-1} of sodium cyanide (NaCN) (N=7). Time intervals are marked in min above the protocol. The solid line represents the end-diastolic pressure developed by the ventricle. P_{OUT} was set to a physiologically realistic level of 50 cmH_2O. The steps identify the maximum cardiac output tests ($\dot{Q}_{MAX}$), where P_{IN} was raised sequentially from 3 cmH_2O to 4 cmH_2O, and finally to 4.5 cmH_2O. During all periods of oxygenated cardiac perfusion, $\dot{Q}$ was maintained at a physiologically resting level of 16–17 ml min^{-1} kg^{-1}, by adjusting P_{IN} as needed. The shaded rectangle represents the period of severe hypoxia (P_{O_2}=5–10 mmHg). During hypoxia, P_{IN} was not adjusted and $\dot{Q}$ was allowed to fall.

samples (1–2 ml) in gas tight Hamilton syringes and injecting each sample into a water-jacketed E-101 oxygen electrode at 10°C (Cameron Instrument Company, Port Aransas, TX, USA). P_{O_2} (in mmHg) was read from an OM-200 dual channel oxygen meter (Cameron Instrument Company, Port Aranas, TX, USA).

Experiment 2

This experiment assessed the degree of myocardial hypoxia tolerance displayed by trout from Clear Creek Rainbow Ranch. In this experiment, each protocol was separated into 3 main sections: (1) stabilization and $\dot{Q}_{MAX}1$, (2) the experimental period and (3) recovery and $\dot{Q}_{MAX}2$. All cardiovascular variables (input pressure, P_{IN}; output pressure, P_{OUT}; and $\dot{Q}$) were manipulated in an identical manner during the initial and final portions of each protocol. However, the protocols were unique in terms of the duration of severe hypoxia administered during the experimental period.

Stabilization and $\dot{Q}_{MAX}1$

Once the fish was placed into the experimental bath and connected to the perfusion apparatus, P_{IN} was set to achieve a physiologically relevant $\dot{Q}$ (16–17 ml min^{-1} kg^{-1}; Kiceniuk and Jones, 1977), and P_{OUT} was maintained at 10 cmH_2O for 5 min. Thereafter, P_{OUT} was raised to 50 cmH_2O, a level comparable to *in vivo* arterial pressures (Kiceniuk and Jones, 1977). After allowing the heart to stabilize at a P_{OUT} of 50 cmH_2O for 5 min, P_{IN} was gradually increased until $\dot{Q}$ reached 30 ml min^{-1} kg^{-1}. This initial cardiac stretch, which was maintained for 20 s, allowed any air bubbles to be cleared from within the heart and provided an initial assessment of cardiac viability. Hearts were discarded if they required more than a 3 cmH_2O increase in P_{IN} to reach a $\dot{Q}$ of 30 ml min^{-1} kg^{-1}, and were assumed to have either poor cannula placement, cannula obstruction or myocardial damage.

Following the cardiac stretch, all hearts were maintained at a $\dot{Q}$ of 16–17 ml min^{-1} kg^{-1} for 20 min before their initial maximum cardiac output ($\dot{Q}_{MAX}1$) was determined. Maximum cardiac output ($\dot{Q}_{MAX}$) was achieved by increasing P_{IN} in a stepwise fashion from that required to achieve resting cardiac output (~ –1.0 cmH_2O) to 3.0 cmH_2O, to 4.0 cmH_2O, and finally to 4.5 cmH_2O (Fig. 2). Each stepwise increase in P_{IN} was

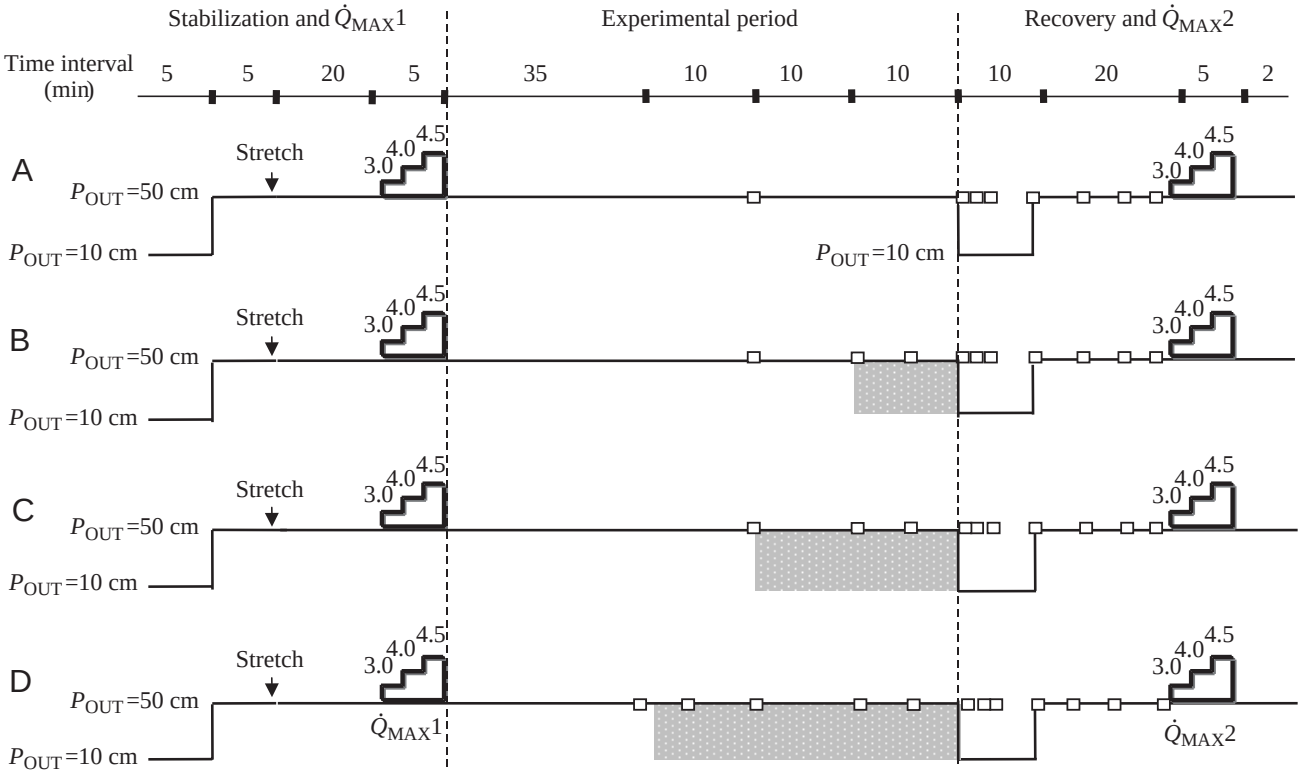

Fig. 2. Experimental protocols used to examine the effect of duration of severe hypoxia (P_{O_2}=5–10 mmHg) on *in situ* trout heart function. Hearts were exposed to one of four treatment protocols: (A) control (oxygenated perfusion) (*N*=7), (B) 10 min (*N*=7), (C) 20 min (*N*=7) or (D) 30 min (*N*=8) of severe hypoxia. In each protocol, the solid line represents the end-diastolic pressure developed by the ventricle, determined by adjusting the height of the output pressure (P_{OUT}) head. P_{OUT} was set to either a physiologically realistic level of 50 cmH_2O, or a sub-physiological level of 10 cmH_2O. The arrows (↓) mark the initial cardiac stretch, where input pressure (P_{IN}) was raised to elicit a cardiac output ($\dot{Q}$) of 30 ml min^{-1} kg^{-1}. The steps identify the maximum cardiac output tests ($\dot{Q}_{MAX}1$ and $\dot{Q}_{MAX}2$), where P_{IN} was raised sequentially from 3 cmH_2O to 4 cmH_2O, and finally to 4.5 cmH_2O. The shaded rectangles indicate periods of induced severe hypoxia (P_{O_2}=5–10 mmHg). During hypoxia, P_{IN} was not adjusted and $\dot{Q}$ was allowed to fall. During all periods of oxygenated cardiac perfusion, $\dot{Q}$ was maintained at a physiologically resting level of 16–17 ml min^{-1} kg^{-1} by adjusting P_{IN} as needed. The small, open squares represent points at which perfusate samples (1 ml) were collected for biochemical analysis.

maintained for approximately 20 s, and resting $\dot{Q}$ was quickly re-established after $\dot{Q}_{MAX}$ was reached. The entire $\dot{Q}_{MAX}$ test took approx. 5 min to complete. After $\dot{Q}_{MAX}1$ had been measured, hearts were randomly assigned to a treatment group.

Experimental period

Hearts were exposed to either (A) a control treatment (oxygenated perfusion) (N=7), or to severe hypoxia for (B) 10 min, (N=7), (C) 20 min (N=7) or (D) 30 min (N=8) (Fig. 2). To ensure that the total length of each treatment was equal, despite the variable durations of hypoxic exposure, the period of resting (oxygenated) cardiac function preceding hypoxia ranged from 35 to 65 min.

Throughout the experimental period, P_{OUT} was set at 50 cmH_2O. During all periods of oxygenated perfusion $\dot{Q}$ was maintained at a resting level of 16–17 ml min^{-1} kg^{-1} by adjusting P_{IN}. However, P_{IN} was not increased to maintain $\dot{Q}$ during severe hypoxia because several preliminary experiments showed that the *in situ* hearts failed to regain contractile function when an attempt was made to maintain pre-hypoxic workloads (data not shown).

Recovery and $\dot{Q}_{MAX}2$

Immediately following the main hypoxic period, the *in situ* heart was perfused with oxygenated saline, and a resting $\dot{Q}$ of 16–17 ml min^{-1} kg^{-1} was quickly restored (within 2–4 min). This was accomplished by setting P_{OUT} at a sub-physiological level (10 cmH_2O) and gradually increasing P_{IN}. Lowering P_{OUT} to 10 cm H_2O facilitated the rapid recovery of cardiac function following hypoxia by reducing the work required by the heart to generate a given flow. Following this 10 min period of reduced after-load, P_{OUT} was restored to 50 cmH_2O and the heart was allowed to recover for 20 min before the final maximum cardiac output test ($\dot{Q}_{MAX}2$) was administered (Fig. 2). This test was performed using the same procedures described for the $\dot{Q}_{MAX}1$ test.

After $\dot{Q}$ had been restored to 16–17 ml min^{-1} kg^{-1} following $\dot{Q}_{MAX}2$, two tests were performed to ensure that the input cannula was securely tied into the sinus venosus, and that the *in situ* heart was isolated from the saline in the experimental bath. First, it was confirmed that $\dot{Q}$ rapidly fell to 0 after the input cannula was clamped off with a pair of haemostats. Second, with the haemostats still clamping the input cannula, and the tubing connected to the output cannula raised to 100 cmH_2O, we ensured that there was no backflow of perfusate. After completing these tests, the ventricle was rapidly excised, blotted to remove residual saline, and weighed.

Perfusate samples (1 ml) for protein and myoglobin analyses were collected immediately prior to the main hypoxic challenge, and at 2, 4, 6, 10, 15, 20 and 30 min following hypoxia (Fig. 2). During the control treatments, perfusate samples were taken at points equivalent to those used in the other treatment groups. The perfusate and ventricular samples were immediately frozen in liquid nitrogen, and stored at –70°C for subsequent biochemical analysis.

Data collection and analysis

Cardiac function was continuously monitored throughout each experiment by measuring $\dot{Q}$, P_{IN} and P_{OUT}. Cardiac output (ml min^{-1}) was measured using a Model T206 small animal blood flow meter in conjunction with a pre-calibrated in-line flow probe (2 N, Transonic Systems Inc., Ithaca, NY, USA). Gould Statham pressure transducers (P23 ID, Oxnard, CA, USA) were used to measure P_{IN} and P_{OUT} (cmH_2O). The pressure transducers were calibrated daily against a static column of water, where zero pressure (0 cmH_2O) was set equal to the saline level in the experimental bath. In addition, the recorded input and output pressures were corrected to account for the resistance in the tubing between the points of pressure measurement and the heart, using predetermined calibrations.

Signals from the flow meter and the pressure transducers were amplified and filtered using a Model MP100A-CE data acquisition system (BIOPAC Systems Inc., Santa Barbara, CA, USA). The acquired signals were then analyzed and stored using Acqknowledge Software (BIOPAC Systems). Although data were continuously collected, cardiovascular function was only analyzed at specific intervals during each experiment. The resting P_{IN} required to maintain a $\dot{Q}$ of 16–17 ml min^{-1} kg^{-1} was measured prior to the $\dot{Q}_{MAX}1$ and $\dot{Q}_{MAX}2$ tests. A rise in resting P_{IN} over the course of the experiments was used as an index of diminished resting cardiac function. Because $\dot{Q}_{MAX}$ tests were administered at the beginning and at the end of each experiment (Figs 2 and 3), reductions in $\dot{Q}_{MAX}$ and maximum stoke volume (V_S) were used as measures of reduced maximum heart function. Heart rate (f_H) and $\dot{Q}$ were also measured at regular intervals (every 2–5 min) throughout the hypoxic period to provide an index of cardiac function during hypoxia.

Heart rate was calculated by measuring the number of systolic peaks during a 20–30 s interval and stroke volume V_S was calculated as $\dot{Q}/f_H$.

Biochemical assessment of myocardial damage

To determine when myoglobin concentrations in the perfusate would probably be at their maximum, the total concentration of protein in the perfusate was quantified between 2 and 30 min post-hypoxia using the Bradford dye-binding procedure (Bradford, 1976), with bovine serum albumin (BSA) as the standard. No protein was detected in the perfusate leaving *in situ* hearts exposed to the control treatment or to 10 min of severe hypoxia. However, protein was detected in two-thirds of the hearts exposed to 20 and 30 min of severe hypoxia at 2, 4 or 6 min of recovery (range 0.3–10 μg ml^{-1}).

Myoglobin

Based on the results of the protein assay, the amount of myoglobin released from the heart was assessed in perfusate samples collected 4 min following hypoxia. Samples (20 μl) were solubilized in Laemmli sample buffer containing 2% SDS and dithiothreitol (final concentration 1 mmol l^{-1}) at 70°C for 10 min. In addition, a frozen tissue standard of trout cardiac muscle (approx. 50 mg) was homogenized in 19 volumes of

filtered (0.22 μm), ice-cold extraction buffer (20 mmol l^{-1} Hepes, 250 mmol l^{-1} sucrose, 1 mmol l^{-1} EDTA, pH 7.5). This homogenate was then centrifuged (600 ***g***) for 10 min at 4°C, and the supernatant was used for myoglobin measurements.

All samples were electrophoresed (BioRad Mini Protean II, Hercules, CA, USA) at 140 mV for 2 h with a 17.5% Tricine–SDS polyacrylamide resolving gel. Immediately after electrophoresis, proteins were transferred to PVDF membranes (0.45 μm; Immobilon-P, Millipore Corp., Bedford, MA, USA) using a Mini Transblot apparatus (BioRad) set at 150 mA for 50 min. These membranes were then soaked overnight at 4°C in phosphate-buffered saline (PBS) (pH 7.4) containing 5% nonfat dry milk (Carnation, Los Angeles, CA, USA). Immunoblotting was performed with a polyclonal rabbit anti-myoglogin antibody (Sigma #M8648; diluted 1:1000 in PBS containing 0.1% BSA and 0.02% sodium azide) for 60 min at 25°C. Membranes were then washed in PBS containing 1% Triton X-100, and incubated for 60 min at 25°C with goat anti-rabbit HRP-conjugated IgG (BioRad) that was diluted 1:15 000 in PBS containing 0.1% BSA without sodium azide. Enhanced chemiluminescence (Amersham Life Sciences, Buckinghamshire, UK) was used to visualize bands using the Fluor-S Multi-imaging system (BioRad). The relative amount of protein in each band was quantified using scanning densitometry and Quantity One software (BioRad).

Lactate dehydrogenase

Excessive dilution caused by high perfusate flow rates (16–17 ml min^{-1} kg^{-1}) and the absence of perfusate recirculation may prevent the detection of metabolic enzymes in the perfusate leaving *in situ* hearts (Gamperl et al., 2001). Therefore, LDH activity remaining in the ventricle was quantified following each of the treatments.

In addition, LDH activity was quantified in ventricles from a group of baseline (non-experimental) trout (N=6) that were sampled directly from the holding tanks. Baseline trout were euthanized using cerebral percussion, then the hearts were quickly (<30 s) excised and allowed to beat in ice-cold saline for 1 min to clear any residual blood from within the heart. Ventricular LDH activities in the control and baseline hearts were compared to determine whether any myocardial cell death occurred as a result of surgery and/or the duration of the experimental protocol. Further, the LDH activity in the baseline ventricles was compared to literature values in order to evaluate whether elevated ventricular LDH activities could explain the enhanced hypoxia tolerance observed in this population of rainbow trout.

Samples of frozen ventricle (50–100 mg) were homogenized in 9 volumes of ice-cold extraction buffer (50 mmol l^{-1} Hepes, 1 mmol l^{-1} EDTA, and 2 mmol l^{-1} dithiothreitol, pH 7.6 at 10°C) using a motorized Duall-21 ground-glass homogenizer. The reaction medium containing whole homogenate, 50 mmol l^{-1} Hepes, 1 mmol l^{-1} KCN, 0.17 mmol l^{-1} NADH and 1 mmol l^{-1} pyruvate (omitted from controls), was maintained at pH 7.6 and 10°C. LDH activity was analyzed using a Perkin-Elmer Lambda 6 UV/VIS spectrophotometer (Norwalk, CT, USA) equipped with a thermostatically controlled recirculating water bath and water-jacketed cuvette holder. The reaction was followed at 340 nm for 5 min. The analytical grade biochemicals used in this analysis were purchased from Sigma Chemical Co.

Statistics

All statistical analyses were performed using StatView Software (SAS Institute Inc., Cary, NC, USA). One-way analyses of variance (ANOVAs), followed by Fisher's protected least significant difference (PLSD) *post-hoc* tests, were used to compare parameters between the treatment groups, including: (1) body and ventricular mass, (2) resting cardiac function ($\dot{Q}$, V_S and f_H) prior to $\dot{Q}_{MAX}1$, (3) maximum cardiac function ($\dot{Q}$, V_S and f_H) at $\dot{Q}_{MAX}1$, (4) the percent change in maximum cardiac performance ($\dot{Q}_{MAX}2$ *versus* $\dot{Q}_{MAX}1$), (5) the percent change in resting P_{IN} prior to $\dot{Q}_{MAX}1$ *versus* $\dot{Q}_{MAX}2$ and (6) average ventricular LDH activities. Repeated-measures ANOVAs were performed for all comparisons of (1) maximum myocardial performance ($\dot{Q}_{MAX}1$ *versus* $\dot{Q}_{MAX}2$) within each treatment group, (2) resting P_{IN} (prior to $\dot{Q}_{MAX}2$ *versus* prior to $\dot{Q}_{MAX}1$) within each treatment group and (3) the loss of cardiac function ($\dot{Q}$ and f_H) during 30 min of severe hypoxia between the treatment groups. The level of statistical significance used in each analysis was P<0.05. All percentage data were arc-sine transformed prior to running any statistical tests.

Results

Experiment 1

The P_{O_2} of the perfusate entering the heart during the hypoxic periods was always less than 10 mmHg. Cardiac output fell at a similar rate when the *in situ* hearts were exposed to 15 min of severe hypoxia or 15 min of severe hypoxia with NaCN. In addition, cardiac output at the end of the hypoxic period was not significantly different between the two groups (24% and 28% of pre-hypoxic values) (Fig. 3). These data strongly suggest that *in situ* hearts in both groups were experiencing severe myocardial hypoxia, if not anoxia.

Experiment 2

Ventricular mass (0.52±0.02 g) and relative ventricular mass (0.09±0.003%) were not significantly different between the various treatments. Therefore, $\dot{Q}$ and V_S are reported on a mass-specific basis (ml min^{-1} kg^{-1} body mass).

Initial cardiac function under oxygenated conditions

Prior to $\dot{Q}_{MAX}1$, resting cardiovascular function was not significantly different between treatments. When the data for all groups was combined, a P_{IN} of –1.0±0.4 cmH_2O was required to maintain a resting $\dot{Q}$ of 17.0±0.2 ml min^{-1} kg^{-1}, and resting f_H and V_S values were 69.3±3.7 beats min^{-1} and 0.26±0.02 ml kg^{-1}, respectively. Furthermore, there were no significant differences in $\dot{Q}$ (54.4±3.1 ml min^{-1} kg^{-1}), V_S (0.95±0.05 ml kg^{-1}) or f_H (58.4±3.2 beats min^{-1}) at $\dot{Q}_{MAX}1$.

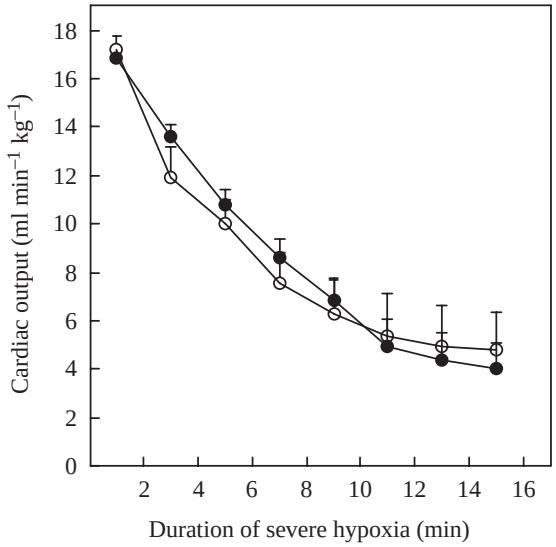

Fig. 3. Cardiac output ($\dot{Q}$) during 15 min of severe hypoxia (filled circles; N=8), and during 15 min of severe hypoxia with 1.5 mmol l^{-1} of sodium cyanide (NaCN) added to the hypoxic perfusate (open circles; N=7). $\dot{Q}$ was averaged over 2 min intervals during severe hypoxia. There was no significant difference in $\dot{Q}$ between the treatment groups, at any point during severe hypoxia.

Cardiac function during severe hypoxia

Cardiac performance was compromised to varying degrees during severe hypoxia. Some *in situ* hearts generated positive flow throughout the hypoxic challenge, while $\dot{Q}$ fell to zero in others. In addition, many *in situ* hearts developed an irregular f_H during severe hypoxia, and/or during initial recovery from the hypoxic challenge. Overall cardiovascular function decreased at a similar rate in hearts exposed to 10, 20 or 30 min of severe hypoxia (Fig. 4). However, in all treatments, $\dot{Q}$ (Fig. 4A) decreased more rapidly than f_H (Fig. 4B). Cardiac output decreased by 66% (falling from 16.8±0.4 ml min^{-1} kg^{-1} to 5.7±1.2 ml min^{-1} kg^{-1}) during the first 10 min of severe hypoxia, and ultimately reached 2.4±0.9 ml min^{-1} kg^{-1} (approx. 15% of the initial value) after 30 min of severe hypoxia (Fig. 4). In contrast, f_H slowed by approx. 20% every 10 min, and reached 30.5±7.1 beats min^{-1} (approx. 40% of the initial value) by 30 min of severe hypoxia (Fig. 4B). Because $\dot{Q}$ fell more dramatically and more rapidly than f_H, it is clear that V_S was also reduced (by approx. 64%) during the 30 min hypoxic challenge (data not shown).

Cardiac function following reperfusion

A significantly greater P_{IN} (as compared with pre-hypoxic values) was required to maintain a resting $\dot{Q}$ of 16–17 ml min^{-1} kg^{-1} following 10 min (0.8±0.1 cmH_2O) and 30 min (1.1±0.3 cmH_2O) of severe hypoxia (Fig. 5). Maximum f_H also increased following the control (by 7.0±1.8 beats min^{-1}) and 20 min (by 5.5±1.9 beats min^{-1}) of severe hypoxia treatments (Fig. 6). However, these increases in resting P_{IN} and maximum f_H were not significantly different when all groups were compared (Figs 5B and 6B, respectively). Therefore, the effect of the duration of severe hypoxia on cardiac function was evaluated by comparing changes in maximum V_S and $\dot{Q}_{MAX}$.

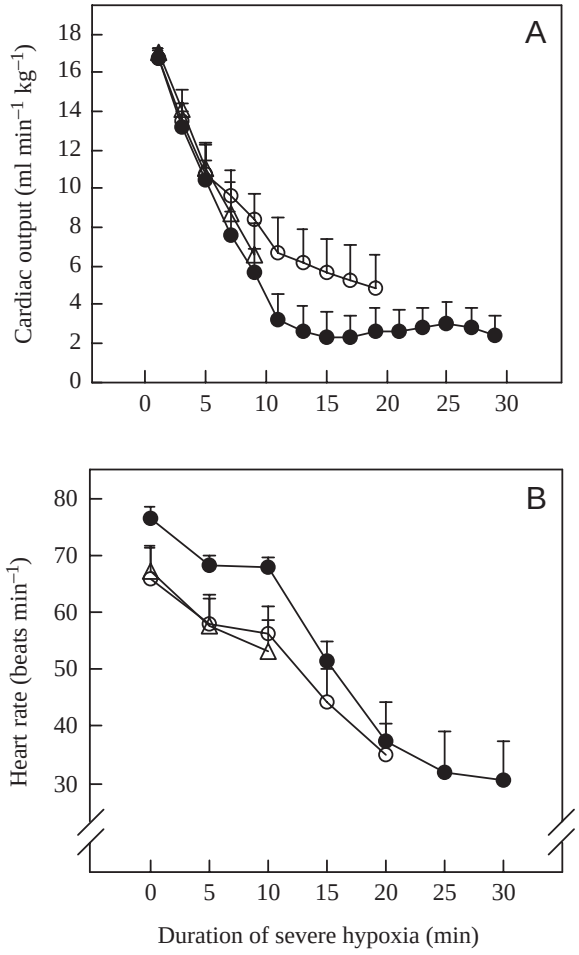

Fig. 4. Decreases in (A) cardiac output $\dot{Q}$ and (B) heart rate f_H during 10 min (triangles; N=7), 20 min (open circles; N=7) and 30 min (filled circles; N=8) of severe hypoxia. Repeated-measures ANOVA showed that there were no significant differences between the treatments. Data for control fish are not shown because these hearts were not exposed to severe hypoxia, and $\dot{Q}$ was maintained at 16–17 ml min^{-1} kg^{-1}.

Maximum V_S and $\dot{Q}_{MAX}$ were not affected by the control protocol. However, a strong negative relationship existed between the duration of severe hypoxia and the recovery of maximum cardiac function (Fig. 6B). Maximum $\dot{Q}$ decreased by 4.2±2.2%, 15.3±5.6% and 23.0±5.0% after hearts were exposed to 10, 20 or 30 min of severe hypoxia, respectively. Further, although the reduction in V_S following 10 min of severe hypoxia was not significantly greater as compared with the control treatment, significant decreases in V_S were recorded after both 20 min (23.2±5.5%) and 30 min (27.4±6.5%) of severe hypoxia.

Biochemical markers of myocardial damage

Myoglobin was not detected in the perfusate 4 min

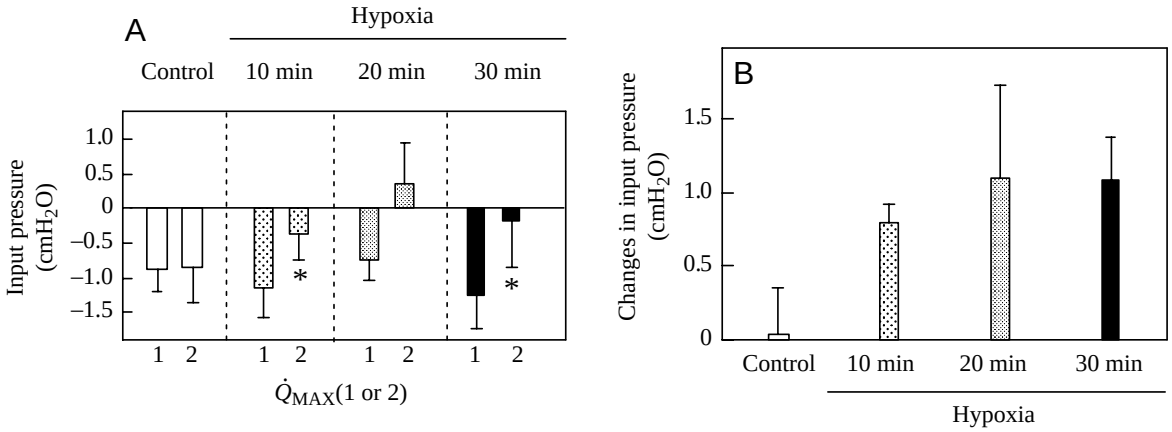

Fig. 5. The effect of increasing the duration of severe hypoxia on the input pressure P_{IN} required to maintain a resting cardiac output $\dot{Q}$ of 16–17 ml min^{-1} kg^{-1}. **(**A**)** P_{IN} recorded prior to $\dot{Q}_{max}1$ and prior to $\dot{Q}_{max}2$; **(**B**)** the change in resting P_{IN} between $\dot{Q}_{max}1$ and $\dot{Q}_{max}2$ (N=6–7 in each group). *Significant difference (P<0.05) identified using repeated-measures ANOVA. One-way ANOVA did not reveal any significant differences in the change in P_{IN} between treatments.

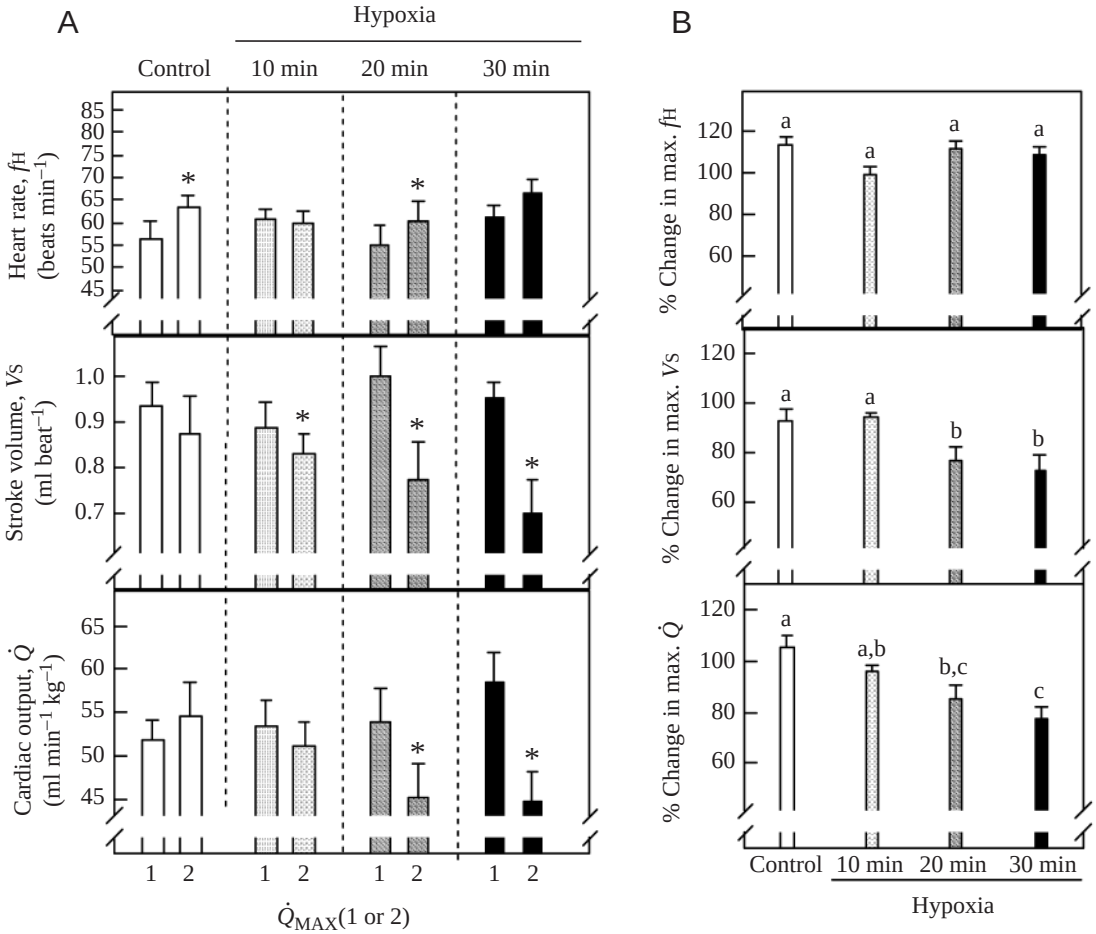

Fig. 6. The effect of increasing the duration of severe hypoxia on maximum cardiac performance of *in situ* rainbow trout hearts. Hearts were exposed to 0 min (control), or 10 min, 20 min and 30 min of severe hypoxia (N=7–8 in each group). (A) Comparison of maximum cardiac performance between $\dot{Q}_{max}1$ and $\dot{Q}_{max}2$; *significant difference (P<0.05) as determined by repeated-measures ANOVA. (B) The percent change in maximum cardiac performance at $\dot{Q}_{max}2$ relative to $\dot{Q}_{max}1$. Dissimilar letters indicate a significant difference (P<0.05) between treatments, as determined using one-way ANOVA.

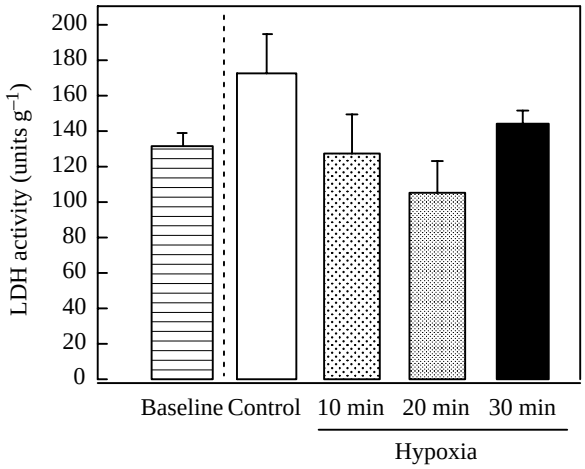

Fig. 7. Activity of lactate dehydrogenase (LDH) in baseline (non-experimental) ventricles, and in ventricles exposed to each of the treatments in Experiment 2. Ventricular LDH values are means + S.E.M., where 1 unit = conversion of 1 μmol substrate to product per min. There were no significant differences in average LDH activities between any of these groups (P<0.05), as determined using one-way ANOVA (N=4–7 in each group).

following severe hypoxia, even though the trout heart standard was clearly labeled by the polyclonal rabbit anti-myoglobin antibody (data not shown). For baseline (non-experimental) fish, ventricular LDH activity was 131.1±7.6 units g^{-1} wet mass (1 unit of LDH activity = conversion of 1 μmol substrate to product per min), a value not significantly different from that measured in the control group (172.3±21.9 units g^{-1} wet mass) (Fig. 7). Furthermore, ventricular LDH activity did not vary significantly in response to the duration of hypoxic exposure (Fig. 7). These results suggest that myocardial necrosis did not occur in response to severe hypoxia.

Discussion

In this study, we provide the first evidence that substantial myocardial hypoxia tolerance exists in certain strains of rainbow trout. This is a novel finding, which suggests that intra-specific variation in fish myocardial hypoxia tolerance exists, and that environmental history may significantly influence trout cardiac physiology. Furthermore, this work suggests that myocardial necrosis (cell death) does not occur following exposure to brief (<30 min) periods of oxygen deprivation at 10°C, and that myocardial dysfunction following short periods of severe hypoxia at this temperature is related to ‘stunning’ alone.

Trout cardiac function and hypoxia tolerance

The *in situ* hearts in this study experienced an 85% drop in resting $\dot{Q}$, a 60% fall in f_H, and a 65% decrease in V_S (data not shown) during 30 min of severe hypoxia (P_{O_2}=5–10 mmHg) (Fig. 4). This magnitude of functional loss is very similar to that reported in previous studies of hypoxic cardiac function using *in situ* (Gamperl et al., 2001) or *in vitro* (Gesser, 1977) trout heart preparations. These similarities, in hypoxic myocardial function, when combined with the data from Experiment 1, strongly suggest that the *in situ* hearts in this study were indeed exposed to severe hypoxia and showed a typical functional response to oxygen deprivation.

Although 30 min of severe hypoxia significantly reduced maximum cardiac function in Experiment 2, several pieces of evidence indicate that these *in situ* trout hearts were extremely hypoxia-tolerant, as compared with other rainbow trout. Gamperl et al. (2001) found that *in situ* rainbow trout hearts at Simon Fraser University experienced a significant decline in $\dot{Q}_{MAX}$ (23%) following only 15 min of severe hypoxia with P_{OUT} at 10 cmH_2O. However, the identical protocol to that used by Gamperl et al. (2001) did not significantly affect $\dot{Q}_{MAX}$ in the present study, and a comparable loss of function (23% loss of $\dot{Q}_{MAX}$) could only be achieved by doubling the duration of hypoxia (from 15 to 30 min), and by increasing the workload of the hearts during the hypoxic challenge fivefold (i.e. by making the hearts pump against a physiological P_{OUT} of 50 cmH_2O) (Fig. 2). Furthermore, the degree of functional recovery in these *in situ* trout hearts (approx. 77%) following 30 min of severe hypoxia was much closer to that measured in ventricular strips from the carp (approx. 90%) than from the rainbow trout (approx. 40%; Gesser, 1977) (Fig. 8). The carp is a species capable of surviving up to 4.5 months of environmental hypoxia (Piironen and Holopainen, 1986). Although inter-specific differences in myocardial hypoxia tolerance are expected (Driedzic and Gesser, 1994), these data strongly suggest that significant intra-specific variation in myocardial hypoxia tolerance exists in fishes.

Possible factors leading to enhanced hypoxia tolerance

Water quality

In our previous study (Gamperl et al., 2001), trout of similar age/size were obtained from a rearing facility supplied with groundwater at a constant temperature of 8°C (West Creek Spring Trout Farm, Aldergrove, BC, Canada). In contrast, the hatchery that provided the trout used in this study is supplied with a limited amount of stream water that is subject to seasonal variations in temperature (8–20°C) and oxygen content (minimum 5 mg O_2 l^{-1}). In addition, the holding pens are arranged in series (fry, juveniles, sub-adults, then adults), and water quality deteriorates as maturing fish are moved away from the farm’s water source. It is probable, therefore, that selection occurred at this facility, with only hearty and/or hypoxia-tolerant individuals surviving to adulthood. This selective pressure would have been greatest during the summer when stream temperatures were highest (≥20°C), because high water temperatures lead to (1) increased resting metabolic rates (Q_{10}=1.5–2.0; Brett, 1971; Dickson and Kramer, 1971) and (2) a reduction in the oxygen carrying capacity of water (1.5% for every °C increase in temperature).

Branchial copepod infestations

The trout used in these experiments were active and well

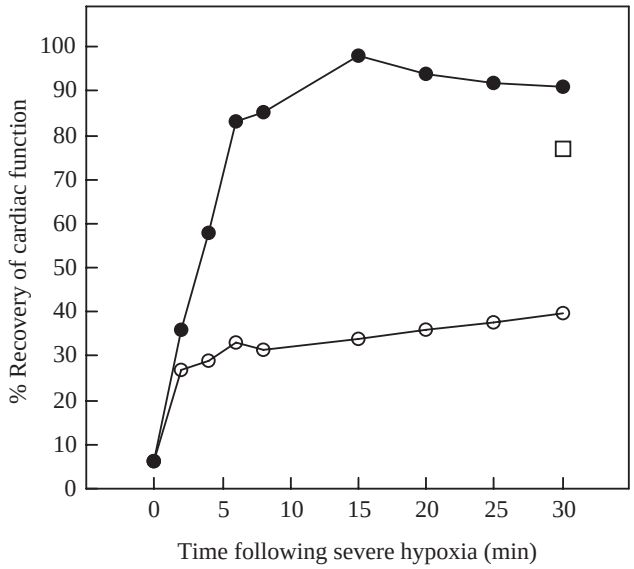

Fig. 8. Recovery of cardiac function following 30 min of severe hypoxia, measured as the percent recovery of maximum force by ventricular strips [Gesser, 1977; open circles, rainbow trout (N=5) and filled circles, carp (N=5)], or as the percent recovery of $\dot{Q}_{MAX}$ in the present study (open square, N=8). The workload during the hypoxic period was similar between studies. The ventricular strips used by Gesser (1977) were developing maximum force, but at a contraction rate (0.2 Hz, 12 contractions min^{-1}) much lower than measured *in vivo*, whereas the power output of the hearts used in the study was approx. one sixth of maximum.

fed. However, branchial copepod infestations (probably *Ergasilis* sp.) were widespread in this population. It is possible that these parasitic infestations reduced the amount of oxygen available to the myocardium and other tissues, and thus magnified the influence of environmental hypoxia (water quality) on fish survival and/or cardiac physiology. Specifically, gill damage caused by these parasites would reduce the gill surface area available for gas exchange, and decrease oxygen uptake from the water. It is also possible that bleeding associated with these infestations led to a reduced haematocrit. A low haematocrit would impair oxygen transport within the blood, and require the heart to work harder in an attempt to maintain oxygen delivery to the tissues during periods of aquatic hypoxia. Such an increase in myocardial energy expenditure might have further exacerbated the effects of reduced blood oxygen content on the cardiac muscle and promoted greater hypoxia tolerance.

Potential adaptations mediating hypoxia tolerance

The average cardiac LDH activity measured in this study (136.0±15.2 units g^{-1} wet mass (Fig. 7) is only slightly higher than that reported for other rainbow trout populations (110 units g^{-1} wet mass) if LDH activity is adjusted to a common temperature (10°C, assuming Q_{10}=2; Driedzic and Gesser, 1994). Although measurements of maximal enzyme activity may not reflect 'physiological flux' through a pathway or even a single step, our measurements of cardiac LDH activity suggest that the enhanced myocardial hypoxia tolerance of these *in situ* hearts is not due to an improved ability to generate ATP through anaerobic glycolysis.

Although cardiac myoglobin was not measured in this study, a greater concentration of myoglobin would increase the efficiency of oxygen use at low P_{O_2} levels (Bailey et al., 1990). Ultimately, myoglobin would help to fuel aerobic metabolism, improve energy availability, and minimize the negative effects associated with the accumulation of anaerobic by-products. There is evidence linking myocardial myoglobin levels and hypoxia-tolerance in both the fish and mammalian literature. Inter-specific differences in fish myoglobin concentration play an important role in the maintenance of cardiac function during hypoxia (Driedzic et al., 1982; Legate et al., 1998), and rats experience a 15% increase in cardiac myoglobin concentration following 2 to 10 weeks of hypoxic adaptation (P_{O_2}=73–90 mmHg; Anthony et al., 1959). However, myoglobin levels were not altered following 4–6 weeks of hypoxic exposure (water P_{O_2}=30–35 mmHg) in the eelpout (*Zoarces viviparous*; Driedzic et al., 1985). This latter study suggests that cardiac myoglobin concentrations do not increase in the fish heart following chronic hypoxic exposure, and that increased levels of myoglobin in the myocardium were not responsible for the enhanced hypoxia tolerance of the rainbow trout hearts used in this study.

A number of sources suggest that an enhanced ability to use exogenous glucose improves post-hypoxic recovery of maximum myocardial function. First, hypoxia stimulates myocytes to take up glucose by recruiting glucose transporters to the cell membrane and/or improving the function of existing glucose transporters (Cartee et al., 1991; Rodnick et al., 1997). Second, exogenous glucose enhances cardiac function during oxygen deprivation in mammals (Apstein et al., 1983; Runnman et al., 1990), in hypoxia-tolerant eels (Driedzic et al., 1978; Bailey et al., 2000) and in the hypoxia-adapted eelpout (*Zoarces viviparous*; Driedzic et al., 1985). The functional protection afforded by exogenous glucose may be mediated by an increased ability to fuel anaerobic glycolysis, as observed by Gamperl et al. (2001). The advantages of elevating anaerobic glycolysis may include (1) an increased production of ATP and (2) the maintenance of vital membrane functions (i.e. ionic balance, membrane potential), particularly if the specific enzymes required for glucose breakdown exist in close proximity to the cell membrane (Runnman et al., 1990). In addition, exogenous glucose may further protect the *in situ* heart by acting as a free radical scavenger during re-oxygenation (Hess et al., 1983). Although glucose uptake was not measured, it is possible that the *in situ* hearts used in this study had an enhanced rate of glucose uptake from the hypoxic perfusate compared to our previous studies (Arthur et al., 1992; Gamperl et al., 2001), and that this enhanced the post-hypoxic recovery of myocardial function. Alternatively, fish used in the current study might have a higher concentration of endogenous glucose (glycogen) that promotes glycolytic activity and post-hypoxic recovery.

Biochemical indices of myocardial damage

The release of cardiac myoglobin and changes in ventricular LDH activity were measured to determine if these biochemical markers could be used as direct indices of *in situ* cardiac cell death. These biochemical markers were selected because both myoglobin (Janier et al., 1994; Kawabata et al., 1998; Stokke et al., 1998) and LDH (McKean and Mendenhall, 1996; Diederichs, 1997) are released from the mammalian heart in response to acute cardiac injury. In addition, the release of cardiac metabolic enzymes such as LDH and creatine kinase has been successfully measured in (1) coronary venous blood (McKean and Landon, 1982; McKean and Mendenhall, 1996), (2) small volumes of perfusate holding *in vitro* heart preparations (Ghosh et al., 2000) and (3) the systemic circulation (Diederichs, 1997).

Although protein was released from the hearts following severe hypoxia, myoglobin was not detected in the perfusate leaving these *in situ* hearts, and ventricular LDH activity did not vary with the duration of severe hypoxia (Fig. 7). It is possible that these direct biochemical indicators of myocardial damage were not detected because either (1) dilution of myoglobin within the large volumes of perfusate pumped by *in situ* heart resulted in concentrations that were below the detection limit of the assay, or (2) 30 min of oxygenated reperfusion was insufficient to remove enough LDH from irreversibly damaged myocytes so that a significant reduction in tissue concentration could be detected. However, it is unlikely that the inadequate time for washout explains the lack of necrosis suggested by our results for perfusate myoglobin and myocardial LDH. McKean and Mendenhall (1996) showed that large amounts of LDH are released from the mammalian heart during the first 30 min of reperfusion. Overgaard et al. (2004) showed that the energetic status (total adenylates, adenylate charge, lactate, glycogen and PCr/Cr^2) of trout hearts exposed to 20 min of severe hypoxia was not significantly different from control hearts after 30 min of oxygenated reperfusion. Finally, anoxic and normoxic non-working myocardial pieces (4–6 mg) from rainbow trout showed no difference in MTT staining until at least 4 h of incubation (J. Overgaard and J. A. W. Stecyk, unpublished). Thus, we hypothesize that *in situ* trout hearts are not irreversibly damaged when exposed to brief (<30 min) periods of severe hypoxia at 10°C, and that myocardial dysfunction during recovery is solely a result of ‘stunning’.

A.K.G. was supported by an American Heart Association Grant (#9960208Z) and by research funds provided by Portland State University. K.J.R. was supported by the MSTI/MSMRI Research Institute of the St Luke’s Regional Medical Center. We thank Debbie Huysoon and Richard Ah You for assistance with fish care, and Dr Stan Hillman for his valuable input.

References

Anthony, A., Ackermann, E. and Strother, G. K. (1959). Effects of altitude acclimation on rat myoglobin. Changes in myoglobin content of skeletal and cardiac muscles. *Am. J. Physiol.* **196**, 512-516.

Apstein, C. S., Gravino, F. N. and Haudenschild, C. C. (1983). Determinants of a protective effect of glucose and insulin on the ischemic myocardium. *Circ. Res.* **52**, 515-526.

Arthur, P. G., Keen, J. E., Hochachka, P. W. and Farrell, A. P. (1992). Metabolic state of the *in situ* perfused trout heart during severe hypoxia. *Am. J. Physiol.* **263**, R798-R804.

Axelsson, M., Farrell, A. P. and Nilsson, S. (1990). Effects of hypoxia and drugs on the cardiovascular dynamics of the Atlantic hagfish *Myxine glutinosa*. *J. Exp. Biol.* **151**, 297-316.

Bailey, J. R., Sephton, D. H. and Driedzic, W. R. (1990). Oxygen uptake by isolated perfused fish hearts with differing myoglobin concentrations under hypoxic conditions. *J. Mol. Cell. Cardiol.* **22**, 1125-1134.

Bailey, J. R., Val, A. L., Almeida-Val, V. M. F. and Driedzic, W. R. (1999). Anoxic cardiac performance in Amazonian and north-temperate-zone teleosts. *Can. J. Zool.* **77**, 683-689.

Bailey, J. R., Rodnick, K. J., MacDougall, R., Clowe, S. and Driedzic, W. R. (2000). Anoxic performance of the American eel (*Anguilla rostrata* L.) heart requires extracellular glucose. *J. Exp. Zool.* **286**, 699-706.

Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Brett, J. R. (1971). Energetic responses of salmon to temperature. A study of some thermal relations in the physiology and freshwater ecology of sockeye salmon (*Oncorhynchus nerka*). *Am. Zool.* **11**, 99-113.

Bushnell, P. G., Brill, R. W. and Bourke, R. E. (1990). Cardiorespiratory responses of skipjack tuna (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus albacares*), and bigeye tuna (*Thunnus obesus*) to acute reductions of ambient oxygen. *Can. J. Zool.* **68**, 1857-1865.

Cartee, G. D., Douen, A. G., Ramlal, T., Klip, A. and Holloszy, J. O. (1991). Stimulation of glucose transport in skeletal muscle by hypoxia. *J. Appl. Physiol.* **70**, 1593-1600.

Dickson, I. W. and Kramer, R. H. (1971). Factors influencing scope for activity and active and standard metabolism of rainbow trout (*Salmo gairdneri*). *J. Fish. Res. Bd Can.* **28**, 587-596.

Diederichs, F. (1997). A decrease of both $[Ca^{2+}]e$ and $[H^+]e$ produces cell damage in the perfused rat heart. *Cell Calcium* **22**, 487-496.

Driedzic, W. R. and Gesser, R. H. (1994). Energy metabolism and contractility in ectothermic vertebrate hearts: hypoxia, acidosis, and low temperature. *Physiol. Rev.* **74**, 221-258.

Driedzic, W. R., Gesser, H. and Johansen, H. (1985). Effects of hypoxic adaptation on myocardial performance and metabolism of *Zoarces viviparous*. *Can. J. Zool.* **63**, 821-923.

Driedzic, W. R., Phleger, C. F., Fields, J. H. A. and French, C. (1978). Alterations in energy metabolism associated with the transition from water to air breathing in fish. *Can. J. Zool.* **56**, 730-735.

Driedzic, W. R., Stewart, J. M. and Scott, D. L. (1982). The protective effect of myoglobin during hypoxic perfusion of isolated fish hearts. *J. Mol. Cell. Cardiol.* **14**, 673-677.

Dunn, J. F. and Hochachka, P. W. (1986). Metabolic responses of trout (*Salmo gairdneri*) to acute environmental hypoxia. *J. Exp. Biol.* **123**, 229-242.

Farrell, A. P., Johansen, J. A. and Suarez, R. K. (1991). Effects of exercise-training on cardiac performance and muscle enzymes in rainbow trout, *Oncorhynchus mykiss*. *Fish Physiol. Biochem.* **9**, 303-312.

Farrell, A. P., MacLeod, K. R. and Chancey, B. (1986). Intrinsic mechanical properties of the perfused rainbow trout heart and the effects of catecholamines and extracellular calcium under control and acidotic conditions. *J. Exp. Biol.* **125**, 319-345.

Ferrari, R., Ceconi, C., Curello, S., Percoco, G., Toselli, T. and Antonioli, G. (1999). Ischemic preconditioning, myocardial stunning, and hibernation: Basic aspects. *Am. Heart J.* **138**, S61-S68.

Gamperl, A. K., Todgham, A. E., Parkhouse, W. S., Dill, R. and Farrell, A. P. (2001). Recovery of trout myocardial function following anoxia: preconditioning in a non-mammalian model. *Am. J. Physiol.* **281**, R1775-R1763.

Gesser, H. (1977). The effects of hypoxia and reoxygenation on force development in myocardia of carp and rainbow trout: protective effects of CO_2/HCO_3^-. *J. Exp. Biol.* **69**, 199-206.

Ghosh, S., Standen, N. B. and Galinanes, M. (2000). Preconditioning the human myocardium by simulated ischemia: studies on the early and delayed protection. *Cardiovasc. Res.* **45**, 339-350.

Graham, M. S. and Farrell, A. P. (1989). The effect of temperature acclimation and adrenaline on the performance of a perfused trout heart. *Physiol. Zool.* **62**, 38-61.

Hess, M. L., Okabe, E., Poland, J., Warner, M., Stewart, J. R. and Greenfield, L. J. (1983). Glucose, insulin, potassium protection during the course of hypothermic global ischemia and reperfusion: a new proposed mechanism by the scavenging of free radicals. *J. Cardiovasc. Pharmacol.* **5**, 35-43.

Janier, M. F., Vanoberschelde, J. J. and Bergmann, S. R. (1994). Ischemic preconditioning stimulates anaerobic glycolysis in the isolated rabbit heart. *Am. J. Physiol.* **267**, H1353-H1360.

Kawabata, K., Osada, M., Netticadan, T. and Dhalla, N. S. (1998). Beneficial effects of ischemic preconditioning on Ca^{2+} paradox in the rat heart. *Life Sci.* **63**, 685-692.

Keen, J. E., Vianzon, D.-M., Farrell, A. P. and Tibbitts, G. F. (1993). Thermal acclimation alters both adrenergic sensitivity and adrenoceptor density in cardiac tissue of rainbow trout. *J. Exp. Biol.* **181**, 27-41.

Kiceniuk, J. W. and Jones, D. R. (1977). The oxygen transport system in trout (*Salmo gairdneri*) during sustained exercise. *J. Exp. Biol.* **69**, 247-260.

Legate, N. J. N., Bailey, J. R. and Driedzic, W. R. (1998). Oxygen consumption in myoglobin-rich and myoglobin-poor isolated fish cardiomyocytes. *J. Exp. Zool.* **280**, 269-276.

McKean, T. and Landon, R. (1982). Comparison of the response of muskrat, rabbit, and guinea pig heart muscle to hypoxia. *Am. J. Physiol.* **243**, R245-R250.

McKean, T. and Mendenhall, R. (1996). Comparison of the responses to hypoxia, ischaemia and ischaemic preconditioning in wild marmot and laboratory rabbit hearts. *J. Exp. Biol.* **199**, 693-697.

Overgaard, J., Stecyk, J. A. W., Gesser, H., Wang, T., Gamperl, A. K. and Farrell, A. P. (2004). Preconditioning stimuli do not benefit the myocardium of hypoxia-tolerant rainbow trout (*Oncorhynchus mykiss*). *J. Comp. Physiol. B*, in press.

Piironen, J. and Holopainen, R. (1986). A note on seasonality in anoxia tolerance of crucian carp (*Carassius carassius* (L.)) in the laboratory. *Ann. Zool. Fennici.* **23**, 335-338.

Podrabsky, J., Javillonar, C., Hand, S. C. and Crawford, D. L. (2000). Intraspecific variation in aerobic metabolism and glycolytic enzyme expression in heart ventricles. *Am. J. Physiol.* **279**, R2344-R2348.

Rodnick, K. J., Bailey, J. R., West, J. L., Rideout, A. and Driedzic, W. R. (1997). Acute regulation of glucose uptake in cardiac muscle of the American eel *Anguilla rostrata*. *J. Exp. Biol.* **200**, 2871-2880.

Runnman, E. M., Lamp, S. T. and Weiss, J. N. (1990). Enhanced utilization of exogenous glucose improves cardiac function in hypoxic rabbit ventricle without increasing total glycolytic flux. *J. Clin. Invest.* **86**, 1222-1233.

Stokke, M., Gronvold, T., Siebke, A. M., Skjaeggestad, O. and Stromme, J. H. (1998). Serial measurements of cardiac markers to rule in or out acute myocardial damage less than 3 h after admission in acute chest pain patients without ECG-signs of acute myocardial infarction. *Scand. J. Clin. Lab. Invest.* **1998**, 331-338.

Wolff, R. A., Chien, G. L. and Van Winkle, D. M. (2000). Propidium iodide compares favorably with histology and triphenyl tetrazolium chloride in the assessment of experimentally-induced infarct size. *J. Mol. Cell. Cardiol.* **32**, 225-232.