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Hypoxia tolerance and preconditioning are not additive in the trout (*Oncorhynchus mykiss*) heart

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Summary

Research has shown that the trout heart is normally hypoxia-sensitive, and that it can be preconditioned. However, we have identified a group of rainbow trout *Oncorhynchus mykiss* that shows a surprising degree of myocardial hypoxia tolerance. In this study, we used *in situ* hearts from these fish as a comparative model to examine whether the cardioprotective effects afforded by hypoxic adaptation and preconditioning are additive. *In situ* trout hearts were exposed to severe hypoxia (perfusate P_{O_2} 5–10 mmHg) in the absence and presence of a transient hypoxic pre-exposure (preconditioning). The four groups studied were: (1) control (no hypoxia); (2) 5 min of severe hypoxia; (3) 30 min of severe hypoxia; and (4) 5 min of severe hypoxia (hypoxic preconditioning) followed 20 min later by 30 min of severe hypoxia. 30 min of severe hypoxia significantly decreased maximum cardiac output and stroke volume by 15–30%. However, hypoxic preconditioning failed to confer any protection against post-hypoxic myocardial dysfunction. This work shows that the protection afforded by inherent myocardial hypoxia tolerance and preconditioning are not additive in this population of trout, and strongly suggests that the relationship between hypoxic adaptation and preconditioning in fishes resembles that of the neonatal/immature, not adult, mammalian heart. Further, our results (1) indicate that stretch (volume loading) and chronic exposure to low levels of adrenaline (15 nmol l^{-1}) do not confer any protection against hypoxia-related myocardial dysfunction in this population, and (2) validate the use of the *in situ* trout heart as a comparative model for studying aspects of myocardial hypoxia tolerance and preconditioning in vertebrates.

Key words: rainbow trout, *Oncorhynchus mykiss*, myocardial hypoxia, stretch, hypoxia tolerance, functional recovery.

Introduction

Preconditioning is a phenomenon whereby exposure to brief periods of stress (e.g. hypoxia, ischaemia, stretch, heat shock), or certain biochemical and pharmacological agents, make tissues resistant to damage caused by a subsequent period of ischaemia and reperfusion. Preconditioning has been extensively studied in the hypoxia-intolerant mammalian heart (Lawson and Downey, 1993; Yellon et al., 1998; Okubo et al., 1999; Nakano et al., 2000; Yellon and Downey, 2003), has been documented in birds (Rischard and McKean, 1998), and there is direct experimental evidence to support its existence in hypoxia-sensitive trout (Gamperl et al., 2001). Thus, it appears that preconditioning is an inherent ability of the hypoxia/ischemia sensitive heart to protect itself that appeared early in the evolution of vertebrates.

However, the importance and indeed existence of preconditioning in hypoxia-tolerant vertebrate hearts is unclear. Ischaemic preconditioning failed to improve contractile function following 40 min of global ischaemia in hypoxia-tolerant neonatal rat hearts (1 or 4 days post-partum), and only slightly (by 7%) improved contractile function in relatively hypoxia-sensitive rat hearts tested 7 days post-partum (Ostadalova et al., 1998). Further, Baker et al. (1999) showed that hearts from 7–10-day-old rabbits that were reared in a hypoxic environment (12% oxygen) developed a degree of myocardial ischaemia-tolerance (60% recovery of contractile function following 40 min of ischaemia), and no longer experienced increased functional recovery in response to preconditioning. In contrast, both Tajima et al. (1994) and Neckář et al. (2002) demonstrated that although hearts from chronically hypoxic adult rats had increased resistance to ischaemia-related damage, preconditioning conferred an additional amount of protection. These results question whether long-term and short-term mechanisms of ischaemic protection in the mammalian heart share the same signal transduction pathways and end-effectors, and whether the relationship between inherent hypoxia/ischaemia tolerance and preconditioning is fundamentally different between life stages.

The rainbow trout is generally considered to be a hypoxia-

sensitive fish species (Gesser, 1977; Dunn and Hochachka, 1986; Gamperl et al., 2001). However, Faust et al. (2004) recently identified a group of rainbow trout with a significant degree of inherent myocardial hypoxia tolerance. For example, hearts from these trout required twice the duration of severe hypoxia (15 min *vs* 30 min) and 5 times the workload during hypoxia (output pressure 1 kPa *vs* 5 kPa) to get the same amount of post-hypoxic loss (25%) of function as in Gamperl et al. (2001). In the present study, we used an *in situ* heart preparation as a comparative model to investigate whether (1) hypoxic preconditioning can improve post-hypoxic myocardial functional recovery in these trout, i.e. whether the protection afforded by inherent hypoxia tolerance and preconditioning are additive; and (2) whether stretch and exposure to low levels of adrenaline confer any protection against hypoxia-related myocardial dysfunction.

Materials and methods

Fish husbandry

Female rainbow trout *Oncorhynchus mykiss* Walbaum (mass 380–750 g; mean 550±15 g) were purchased from Clear Creek Rainbow Ranch (Oregon City, OR, USA) and transported in insulated tanks to the Aquatic Vertebrate Facility at Portland State University (PSU). Once at PSU, these fish were transferred to 1000 liter indoor tanks where they were held for a minimum of 10 days before experimental use. The 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 a concentrated sodium thiosulfate solution (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 liters each day. In addition, 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 anaesthetized 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 a maintenance level of oxygenated and 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). Briefly, an input cannula was introduced into the sinus venosus through a hepatic vein and perfusion with heparinized (10 i.u. ml^{-1}) saline containing 5 or 15 nmol l^{-1} adrenaline (see Experimental protocols) was begun immediately. Silk thread (3–0) was then used to secure the input cannula in place, and to occlude any remaining hepatic veins. An output cannula was inserted into the ventral aorta at a point confluent with the bulbus arteriosus and firmly tied in place with 1-O silk thread. Finally, silk ligatures (1 silk) were tied around each ductus Cuvier to occlude these veins and to crush the cardiac branches of the vagus nerve. This procedure left the pericardium intact, while isolating the heart in terms of saline and autonomic nervous inputs and outputs.

The saline used to perfuse the heart contained (in mmol l^{-1}): 124 NaCl; 3.1 KCl; 0.93 $MgSO_4 7H_2O$; 2.52 $CaCl_2 2H_2O$; 5.6 glucose; 6.4 TES salt; and 3.6 TES acid (Keen et al., 1993). These chemicals were purchased from Fisher Scientific (Fair Lawn, NJ, USA), with the exception of the TES salt, which was purchased from Sigma Chemical Co. (St Louis, MO, USA). The TES buffer system was used to simulate the buffering capacity of trout plasma, and the normal change in blood pH with temperature (pK_a/dT=0.016 pH units $°C^{-1}$) (Keen et al., 1993). Adrenaline bitartrate (5 or 15 nmol l^{-1}; Sigma Chemical Co.) was added to the perfusate throughout the experiments, 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 shows 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 the 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 (1 mmHg=0.133 kPa). 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 hypoxia.

Experimental protocols

Experiment 1: Can hypoxia-tolerant trout hearts be preconditioned?

This experiment examined whether 5 min of hypoxic pre-exposure could improve myocardial functional recovery following 30 min of severe hypoxia, and each treatment protocol was separated into three main sections: (1) stabilization and maximum cardiac function test 1 ($\dot{Q}_{MAX}1$); (2) the experimental period; and (3) recovery and $\dot{Q}_{MAX}2$. All cardiovascular variables (input pressure, P_{IN}; output pressure, P_{OUT}; cardiac output, $\dot{Q}$) were manipulated in an identical manner during the initial and final portions of each treatment. However, the treatments were unique in terms of the number of

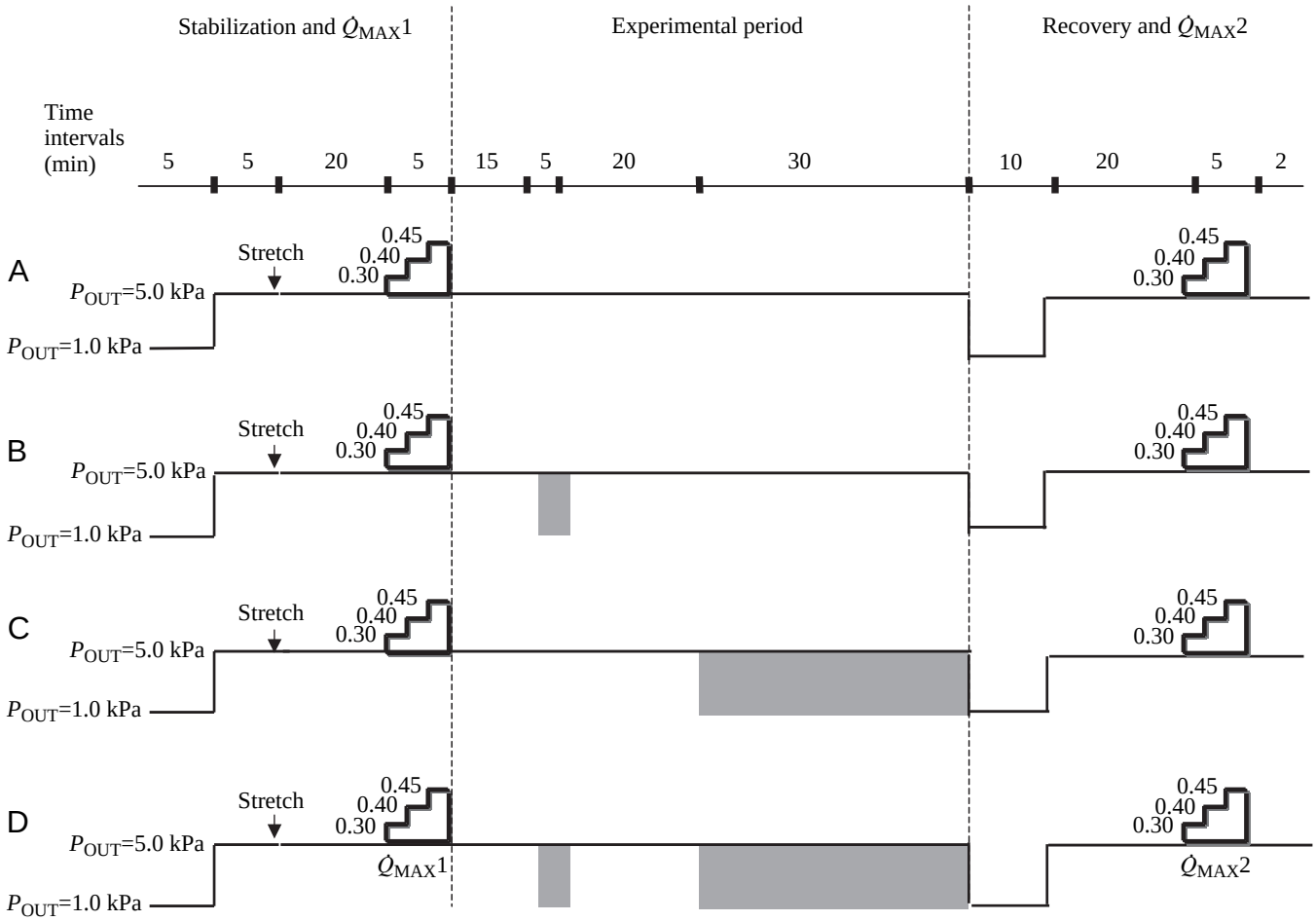

Fig. 1. Protocols used in Experiment 1. Hearts were exposed to one of four treatments: (A) control, (B) 5 min of severe hypoxia, (C) 30 min of severe hypoxia or (D) 5 min of severe hypoxia (preconditioning) followed by 30 min of severe hypoxia. The solid line represents the pressure development of the ventricle as determined by the height of the output pressure (P_{OUT}) head, which was set to either a physiologically relevant level of 5.0 kPa, or a sub-physiological level of 1.0 kPa. 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 bold steps mark the maximum cardiac output tests ($\dot{Q}_{max}$), where P_{IN} was raised sequentially from 0.3 kPa to 0.4 kPa, and finally to 0.45 kPa. The shaded rectangles represent periods of 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 ml min^{-1} kg^{-1}, by adjusting P_{IN} as needed.

severe hypoxia periods administered during the experimental period (see Fig. 1).

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 1 kPa for 5 min. Thereafter, P_{OUT} was raised to 5 kPa, a level comparable to *in vivo* arterial pressures (Kiceniuk and Jones, 1977). After allowing the heart to stabilize at a P_{OUT} of 5 kPa 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 0.3 kPa increase in P_{IN} to reach a $\dot{Q}$ of 30 ml min^{-1} kg^{-1}, and were assumed to have poor cannula placement, cannula obstruction or cardiac 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 $\dot{Q}$ to 0.3 kPa, to 0.4 kPa, and finally to 0.45 kPa (Fig. 1). Each stepwise increase in P_{IN} was 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 $\dot{Q}_{MAX}1$ was measured, hearts were randomly assigned to a treatment group.

Experimental period

In situ trout hearts were exposed to one of four experimental

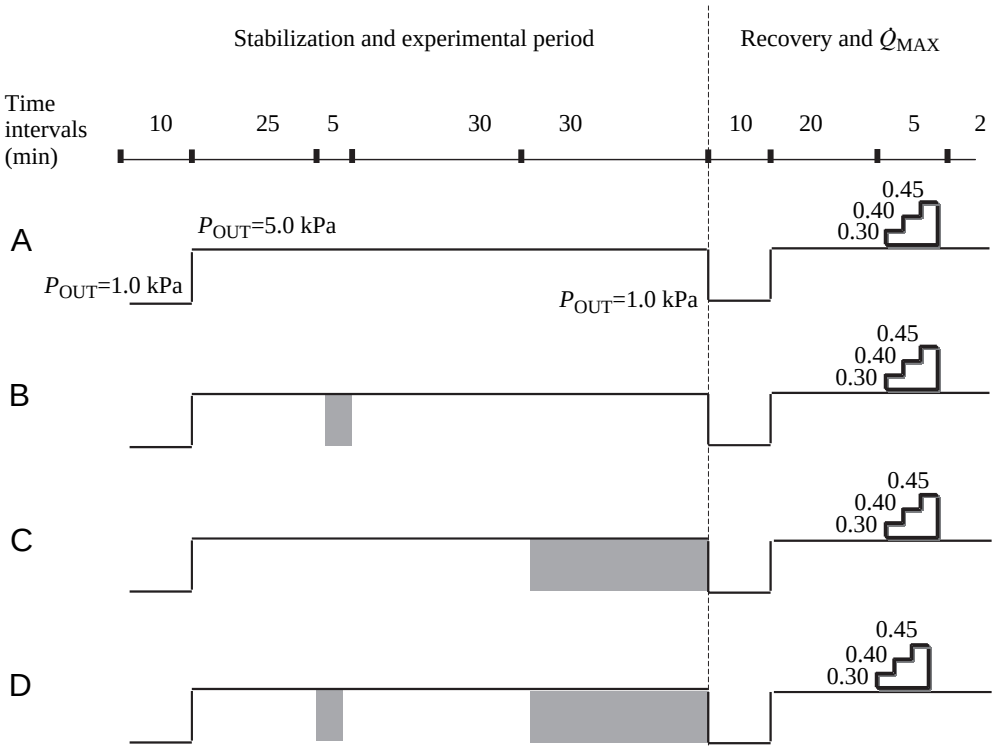

Fig. 2. Protocols used in Experiment 2. Hearts were exposed to one of four treatments: (A) control, (B) 5 min of severe hypoxia, (C) 30 min of severe hypoxia or (D) 5 min of severe hypoxia (preconditioning) followed by 30 min of severe hypoxia. All other details are described in Fig. 1.

treatments: (A) control (oxygenated perfusion) (N=7), (B) 5 min of severe hypoxia (N=7), (C) 30 min of severe hypoxia (N=8) or (D) 5 min of severe hypoxia (preconditioning) followed 20 min later by 30 min of severe hypoxia (N=8) (Fig. 1). Throughout the experimental period, P_{OUT} was set at 5.0 kPa. A period of 30 min, with P_{OUT} left at 5 kPa, was chosen as the main hypoxic insult because Faust et al. (2004) showed that this results in an approx. 25% reduction in maximum cardiac function ($\dot{Q}$; stroke volume, V_S) in this population of trout, and we wanted post-hypoxic myocardial function after the main hypoxic period to be similar to that experienced by the hearts in Gamperl et al. (2001). This similarity in post-hypoxic myocardial function allowed for a direct comparison on the preconditioning effects of 5 min of hypoxia in hypoxia-sensitive (Gamperl et al., 2001) *vs* hypoxia-tolerant (present study) trout hearts. During all periods of oxygenated perfusion $\dot{Q}$ was maintained at a resting level of 16–17 ml min^{-1} kg^{-1} body mass by adjusting P_{IN}. However, P_{IN} was not increased to maintain $\dot{Q}$ during severe hypoxia because Faust et al. (2004) showed that the *in situ* hearts failed to regain contractile function when an attempt was made to maintain pre-hypoxic workloads.

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

Immediately following the 30 min 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 (1 kPa) and gradually increasing P_{IN}. Following this 10 min period of reduced after-load, P_{OUT} was restored to 5.0 kPa and the heart was allowed to recover for 20 min before the final maximum cardiac output test ($\dot{Q}_{MAX}2$) was administered (Fig. 1). This test was performed using the same procedures as described for the $\dot{Q}_{MAX}1$ test.

Experiment 2: Validation of preconditioning protocol.

In mammalian studies it has been shown that the stimulation of α- and β-adrenergic receptors (Bankwala et al., 1994; Lochner et al., 1999; Yabe et al., 1998) and stretch (Ovize et al., 1994) can precondition the myocardium. Because the hearts in Experiment 1 were volume-loaded prior to the preconditioning stimulus (at the stretch and during $\dot{Q}_{MAX}1$, see Fig. 1), and the perfusate contained 15 nmol l^{-1} adrenaline, we wanted to ensure that the lack of preconditioning in these hypoxia-tolerant hearts was not due to inadvertent preconditioning (Kloner et al., 1995). Therefore, we determined the minimum adrenaline concentration at which myocardial viability could be maintained long-term (5 nmol l^{-1}), and then repeated the preconditioning experiment without the initial stretch or $\dot{Q}_{MAX}1$. In this experiment (Fig. 2), each treatment protocol was only divided into two main sections: (1) stabilization and experimental period and (2) recovery and $\dot{Q}_{MAX}$. All other methodological details were the same as in Experiment 1.

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

Statistics

All statistical analyses were performed using StatView Software (SAS Institute Inc., Cary, NC, USA). One-way 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 $\dot{Q}_{MAX}1$ in Experiment 1; (3) maximum cardiac function ($\dot{Q}$, V_S and f_H) at $\dot{Q}_{MAX}1$ in Experiment 1; (4) the percentage change in maximum cardiac performance ($\dot{Q}_{MAX}2$ *vs* $\dot{Q}_{MAX}1$) in Experiment 1; (5) the percentage change in resting P_{IN} prior to $\dot{Q}_{MAX}1$ *vs* $\dot{Q}_{MAX}2$ in Experiment 1; and (6) maximum cardiac function ($\dot{Q}$, V_S and f_H) at $\dot{Q}_{MAX}$ in Experiment 2. Repeated-measures ANOVAs were performed for comparisons of (1) maximum myocardial performance ($\dot{Q}_{MAX}1$ *vs* $\dot{Q}_{MAX}2$) within each treatment group in Experiment 1; (2) the loss of cardiac function ($\dot{Q}$ and f_H) during 30 min of severe hypoxia between the treatment groups in Experiment 1; and (3) resting P_{IN} (prior to $\dot{Q}_{MAX}2$ *vs* prior to $\dot{Q}_{MAX}1$) within each treatment group in Experiment 1. All percentage data were arc-sine transformed prior to running any statistical tests. The level of statistical significance used in each analysis was $P<0.05$, and data reported in the text, figures and tables represent means ± S.E.M.

Results

Ventricular mass and relative ventricular mass [(heart mass/body mass)×100] ranged from 0.33 to 0.65g (mean 0.510±0.016 g) and from 0.08 to 0.13% (mean 0.094±0.002%), respectively, but did not differ ($P>0.05$) between groups in either experiment.

Experiment 1

Initial cardiac function under oxygenated conditions

Prior to $\dot{Q}_{MAX}1$, resting $\dot{Q}$ was maintained at 16.9±0.2 ml min^{-1} kg^{-1} (Table 1), using a P_{IN} of –0.15±0.03 kPa (Fig. 3). At this resting $\dot{Q}$, f_H was 66.4±3.4 beats min^{-1} and V_S was 0.26±0.01 ml kg^{-1} (Table 1). *In situ* hearts in the 30 min of severe hypoxia treatment had a significantly higher resting V_S (by 0.05–0.07 ml kg^{-1}) as compared with the other treatment groups, probably due to their marginally lower f_H ($P<0.09$) (Table 1). However, there were no significant differences in $\dot{Q}$ (55.5±2.8 ml min^{-1} kg^{-1}), V_S (0.99±0.05 ml kg^{-1}), or f_H (56.5±2.3 beats min^{-1}) between groups at $\dot{Q}_{MAX}1$ (Fig. 5A).

Table 1. *Resting cardiac function prior to $\dot{Q}_{MAX}1$, Experiment 1*

Treatment	f_H (beats min^{-1})	$\dot{Q}$ (ml min^{-1} kg^{-1})	V_S (ml kg^{-1})
Control (N=7)	72.9±3.3	16.9±0.1	0.24±0.01[a]
Preconditioning only (N=7)	66.2±2.0	16.9±0.2	0.26±0.01[a]
30 min of hypoxia (N=8)	58.8±5.6	17.0±0.2	0.31±0.03[b]
Preconditioning +30 min of hypoxia (N=8)	67.5±2.9	16.9±0.2	0.25±0.01[a]

Preconditioning, 5 min of hypoxic pre-exposure (see text for details).

Values are means ± S.E.M., and were analyzed using one-way ANOVA. Dissimilar letters indicate a significant difference in V_S between the treatments ($P<0.05$).

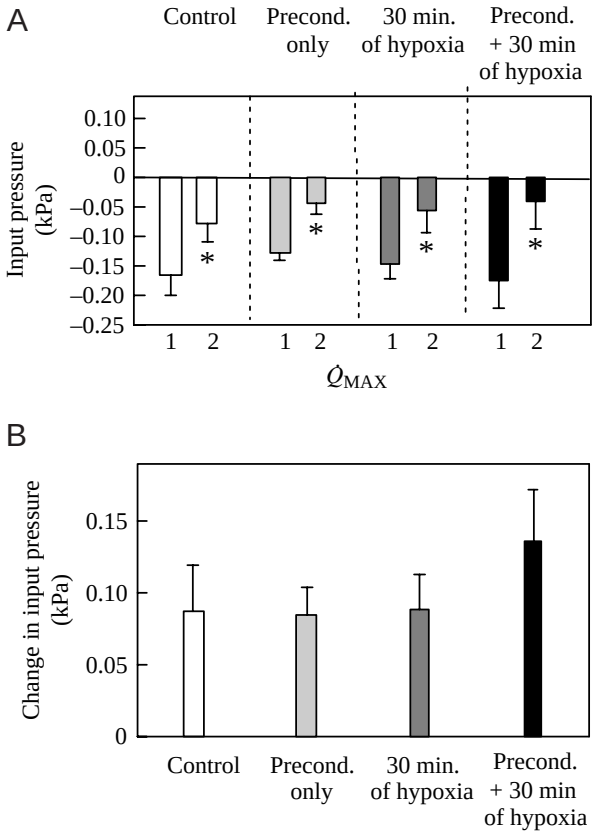

Fig. 3. The effect of pre-exposure to 5 min of severe hypoxia (preconditioning) on (A) resting input pressure (P_{IN}) prior to $\dot{Q}_{max}1$ (1) and $\dot{Q}_{max}2$ (2) and (B) the increase in resting P_{IN} between $\dot{Q}_{max}2$ and $\dot{Q}_{max}1$. *Significant differences ($P<0.05$), identified using repeated-measures ANOVA. One-way ANOVA did not identify any significant differences between the treatments. Values are means ± S.E.M. (N=7–8 in each group).

Cardiac function during severe hypoxia

Cardiac output decreased by 34.5% (Fig. 4) during the 5 min of hypoxic pre-exposure (preconditioning). However, 5 min of hypoxic pre-exposure (preconditioning) had no effect on the

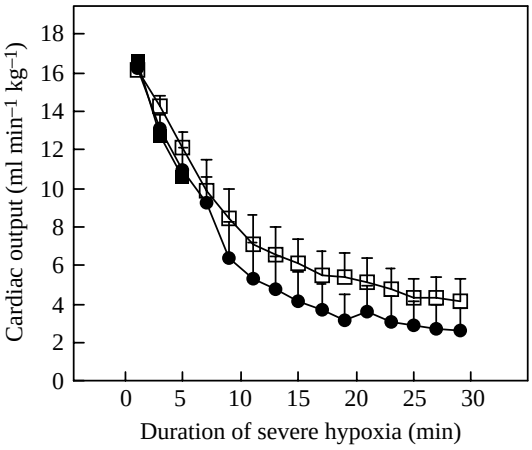

Fig. 4. Effect of hypoxic preconditioning (5 min of severe hypoxia) on the loss of cardiac output ($\dot{Q}$) during a subsequent 30 min hypoxic exposure (N=7–8 in each group): 5 min of hypoxic-preconditioning (filled squares); 30 min of severe hypoxia (filled circles); and 30 min of severe hypoxia following 5 min of hypoxic preconditioning (open squares). Repeated-measures ANOVA indicated that pre-exposure to 5 min of hypoxia did not affect the rate at which $\dot{Q}$ fell when hearts were exposed to 30 min of severe hypoxia. Values are means ± S.E.M.

loss of cardiac function during the subsequent 30 min period of severe hypoxia. Resting $\dot{Q}$ decreased by 79.5% (Fig. 4), and f_H and V_S fell by 41% and 61.4%, respectively (data not shown). These data indicate that: (1) myocardial function during the main hypoxic challenge was not altered by 5 min of hypoxic pre-exposure; and (2) any effects of hypoxic pre-exposure on post-hypoxic maximum myocardial function were not the result of differences in cardiac workload during severe hypoxia.

Cardiac function following severe hypoxia

The P_{IN} required to maintain a resting $\dot{Q}$ of 16–17 ml min^{-1} kg^{-1} increased significantly in all groups (by 0.08 kPa to 0.14 kPa) over the duration of the experiment (Fig. 3A). Maximum f_H also increased slightly following the control treatment (by 3.4±1.4 beats min^{-1}; Fig. 5A). However, there were no significant differences between the changes in resting P_{IN} or maximum f_H when all groups were compared (Figs 3B and 5B, respectively). Maximum V_S fell slightly over the course of the experiment in both the control (by 0.05±0.01 ml kg^{-1}) and the 5 min of hypoxic pre-exposure (preconditioning) treatments (by 0.09±0.02 ml kg^{-1}) (Fig. 5).

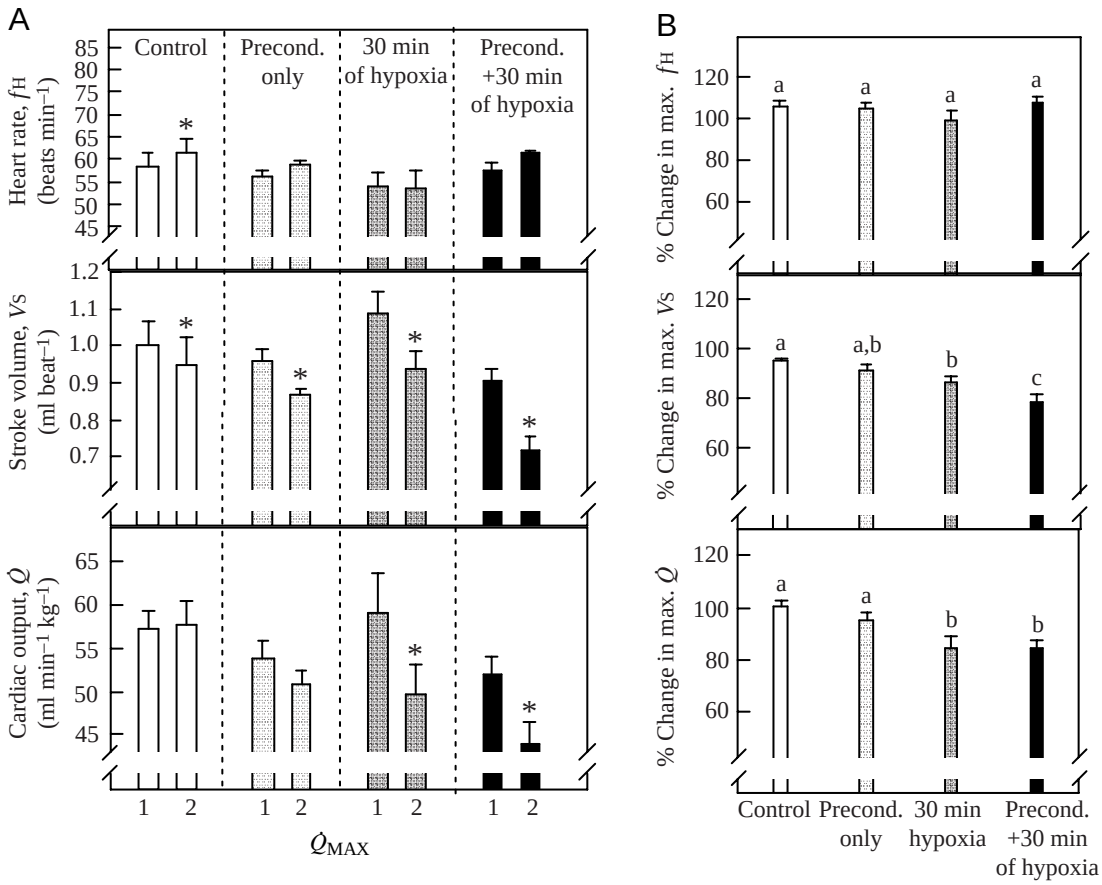

Fig. 5. Effect of hypoxic preconditioning (5 min of severe hypoxia) on the recovery of maximum cardiac performance of *in situ* rainbow trout hearts exposed to 30 min. of severe hypoxia (N=7–8 in each group). (A) Comparison of maximum cardiac performance at $\dot{Q}_{max}1$ and $\dot{Q}_{max}2$. (B) The percentage change in cardiac performance, $\dot{Q}_{max}2$ relative to $\dot{Q}_{max}1$. *Significant difference (P<0.05), as determined using repeated-measures ANOVA. Dissimilar letters represent a significant difference (P<0.05) between treatment groups, as determined using one-way ANOVA. Values are presented as means ± S.E.M.

However, it is unlikely that these changes were the result of reduced myocardial function because the reduction in maximum V_S was slight (approx. 5–9%), and there was no significant decrease in $\dot{Q}_{MAX}$ following either of these two treatments. 30 min of severe hypoxia significantly decreased $\dot{Q}_{MAX}$ (by approx. 15–20%), independent of whether the hearts were pre-exposed to 5 min of severe hypoxia (Fig. 5). Further, the decrease in maximum V_S in the group pre-exposed to 5 min of severe hypoxia was significantly greater (by 7.5%) when compared with the group only exposed to 30 min of severe hypoxia.

Experiment 2

Prior to the determination of maximum cardiac output ($\dot{Q}_{MAX}$), the P_{IN} required to maintain resting cardiac output was not significantly different between treatments, and averaged –0.03±0.020 kPa. In the control group $\dot{Q}_{MAX}$, V_S and f_H at the end of the protocol averaged 62.5±4.3 ml min^{-1} kg^{-1}, 1.2±0.1 ml kg^{-1} and 51.4±2.0 beats min^{-1}, respectively. Exposure to 5 min of severe hypoxia (preconditioning) alone had no significant effect on any cardiovascular parameter. In addition, preconditioning with 5 min of hypoxia failed to prevent the approx. 25–30% decrease in both $\dot{Q}_{MAX}$ and V_S that followed the 30 min period of exposure to hypoxia (Fig. 6).

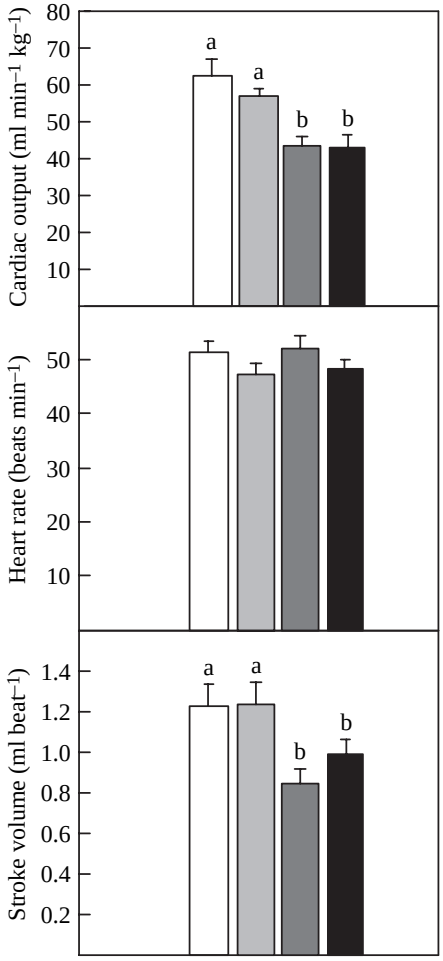

Fig. 6. Effect of 30 min of severe hypoxia, with and without 5 min of hypoxic preconditioning, on *in situ* maximum cardiac performance (N=7–8 per group). Control (open bars), 5 min hypoxic preconditioning (light gray bars), 30 min severe hypoxia (dark gray bars), 5 min hypoxic preconditioning followed by 30 min of severe hypoxia (black bars). Dissimilar letters represent a significant difference (P<0.05) between treatment groups, as determined using one-way ANOVA. Values are means ± S.E.M.

Discussion

The main objective of this study was to determine whether pre-exposure to a brief period of hypoxia can improve post-hypoxic myocardial functional recovery in these hypoxia-tolerant trout. 5 min of hypoxic pre-exposure (preconditioning) failed to reduce the myocardial dysfunction that normally follows 30 min of severe hypoxia (Figs 4 and 6). This finding directly conflicts with those of Gamperl et al. (2001), who showed that 5 min of hypoxia completely eliminated hypoxia-induced myocardial dysfunction in another group of trout with hypoxia-sensitive hearts, and strongly suggests that hypoxia-tolerant trout hearts cannot be preconditioned.

Two previous studies looking at neonatal/immature mammals have also shown that ischaemic pre-exposure (preconditioning) fails to protect the recovery of contractile function in hypoxia-tolerant hearts. First, preconditioning did not improve functional recovery in inherently hypoxia-tolerant neonatal (1–4 days *post partum*) rat hearts, following 40–60 min of ischaemia (Ostadalova et al., 1998). Second, preconditioning failed to improve contractile function, following 30 min of ischaemia, in neonatal rabbit hearts (7–10 days *post partum*) that were exposed to a hypoxic environment (12% O_2) from birth (Baker et al., 1999). The data from these two studies suggests that the cellular pathways and/or end-effectors that confer protection against ischaemic/hypoxic damage are maximally stimulated in hypoxia/ischaemia-tolerant hearts, and support the results for rainbow trout generated in this study. However, studies on adult mammalian hearts do not support this conclusion. Both Tajima et al., (1994) and Neckář et al. (2002) demonstrated that although hearts from chronically hypoxic adult rats had increased resistance to ischaemia-related damage, preconditioning conferred an increased amount of protection. Taken together, these results suggest that the relationship between inherent hypoxia tolerance and the ability to be preconditioned in lower vertebrates and neonatal/immature mammals is similar, but that these two groups differ as compared with adult mammals. Numerous mechanisms and signal transduction pathways appear to be involved in preconditioning the mammalian heart (Lawson and Downey, 1993; Parratt, 1995; Yellon et al., 1998; Baines et al., 1999; Okubo et al., 1999; Nakano et al., 2000; Yellon and Downey, 2003). Thus, it is possible that inherent hypoxia tolerance in lower vertebrates and neonatal/immature mammals is associated with stimulation of all the signal transduction pathways that are shared with preconditioning,

while in adult mammals only a portion of them are stimulated by exposure to chronic hypoxia. Clearly, this is a hypothesis that warrants further investigation.

Although there appear to be differences in how inherently hypoxia/ischaemia tolerant fish, neonatal/immature mammal and adult mammal hearts respond to preconditioning, our results are in line with those of both Baker et al. (1999) and Neckář et al. (2002), who showed that the level of protection achieved by the combination of hypoxic adaptation and preconditioning in the mammalian heart was not additive (i.e. there was no increase in the total capacity of myocardial protective mechanisms). Thus, our results suggest that the non-additivity of these two forms of myocardial protection is a common feature of vertebrate hearts.

Alternative explanations for the failure of 5 min of hypoxic pre-exposure (preconditioning) to improve cardiac function following 30 min of severe hypoxia include: (1) 5 min of hypoxia was an insufficient period of time to elicit a preconditioning response in these trout hearts; and/or (2) these *in situ* hearts were inadvertently preconditioned due to myocardial stretch and/or exposure to adrenaline. However, we are confident that 5 min of hypoxia was a sufficient stimulus to precondition these *in situ* trout hearts. Several mammalian experiments have demonstrated that 5 min of either hypoxia or ischaemia are equipotent in their ability to protect the heart from ischaemia-induced contractile dysfunction (Lasley et al., 1993; Zhai et al., 1993) and cardiac infarction (Shizukuda et al., 1992). 5 min of hypoxic preconditioning completely eliminated the myocardial dysfunction that normally follows 15 min of hypoxic perfusion in hypoxia-sensitive *in situ* rainbow trout hearts (Gamperl et al., 2001). Overgaard et al. (2004) were unable to precondition hypoxia-tolerant trout hearts with 1× 5 min or even 2× 5 min of severe hypoxia. Finally, neither preconditioning with 1× 5 min or 3× 5 min of ischaemia improved the recovery of left ventricular developed pressure in immature rabbit hearts that were inherently hypoxia tolerant (Baker et al., 1999).

As discussed by Kloner et al. (1995), the meaningful interpretation of preconditioning experiments depends upon the exclusion of confounding stimuli that may inadvertently precondition the myocardium. Several mammalian studies have shown that adrenergic stimulation protects the ischaemic myocardium (Bankwala et al., 1994; Yabe et al., 1998; Hearse and Sutherland, 1999) and Ovize et al. (1994) showed that stretching the myocardium *via* volume overloading induces preconditioning in canine hearts. The *in situ* trout hearts used in Experiment 1 were perfused with saline containing 15 nmol l^{-1} of adrenaline. In addition, these hearts were volume-loaded twice before exposure to severe hypoxia (at stretch and at $\dot{Q}_{MAX}1$). Although it is possible that the *in situ* trout hearts in Experiment 1 were inadvertently preconditioned, it is difficult to reconcile this possibility with the results of Experiment 2 (this study) or those of Gamperl et al. (2001). Hypoxic pre-exposure did not improve cardiac performance following the main hypoxic period when the initial stretch and $\dot{Q}_{MAX}$ were eliminated, and the perfusate adrenaline concentration was reduced to 5 nmol l^{-1} [a level comparable to that measured in resting trout (1–4 nmol l^{-1}; Gamperl et al., 1994)] (Fig. 6). Further, the presence of both adrenergic stimulation (15 nmol l^{-1}) and a myocardial stretch ($\dot{Q}_{MAX}1$) did not prevent 5 min of hypoxia from completely ameliorating post-hypoxic myocardial dysfunction in hypoxia-sensitive *in situ* trout hearts (Gamperl et al., 2001). The inability of stretch (volume loading) to precondition hypoxia-tolerant trout hearts (Figs 5 *vs* 6) or to prevent hypoxic preconditioning in hypoxia-sensitive trout hearts (Gamperl et al., 2001) is in direct contrast to the results of Ovize et al. (1994) for the canine heart, but is not surprising. Fish hearts can increase stroke volume to a much greater degree than mammals, and venous pressure/the Starling response are primary determinants of changes in stroke volume (Farrell, 1991; Franklin and Davie, 1992). These results suggest that while the phenomenon of preconditioning is common to both vertebrate groups (fish and mammals), there may be fundamental differences in the type of stimuli that can trigger or promote the signal transduction pathways that mediate preconditioning.

Limitations of this study

In this study, 5 min of hypoxic pre-exposure (preconditioning) did not improve the recovery of trout cardiac function following 30 min of severe hypoxia. Some investigators might argue that it is unclear whether this represents an absence of myocardial preconditioning, because the recovery of myocardial function only provides an indirect assessment of myocardial viability. However, the recovery of contractile function has often been used as an index of myocardial preconditioning (Asimakis et al., 1992; Kolocassides et al., 1996; Gamperl et al., 2001) and recent studies indicate that improved cardiac function represents a specific end-point of preconditioning. First, Mosca et al. (1998) showed that ischaemic/hypoxic preconditioning improved contractility in rat hearts independently of reduced myocardial necrosis. Second, the results of Perez et al. (1999) strongly suggest that preconditioning improves the calcium responsiveness of individual myofilaments, which might in turn promote cardiac performance. Finally, at present we have been unable to show using a number of biochemical parameters that the *in situ* trout heart is irreversibly damaged when exposed to prolonged (<30 min) periods of severe hypoxia at 10°C (Gamperl et al., 2001; Faust et al., 2004; Overgaard et al., 2004).

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