

Recovery of trout myocardial function following anoxia: preconditioning in a non-mammalian model

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Gamperl, A. K., A. E. Todgham, W. S. Parkhouse, R. Dill, and A. P. Farrell. Recovery of trout myocardial function following anoxia: preconditioning in a non-mammalian model. *Am J Physiol Regulatory Integrative Comp Physiol* 281: R1755–R1763, 2001.—Studies with mammals and birds clearly demonstrate that brief preexposure to oxygen deprivation can protect the myocardium from damage normally associated with a subsequent prolonged hypoxic/ischemic episode. However, is not known whether this potent mechanism of myocardial protection, termed preconditioning, exists in other vertebrates including fishes. In this study, we used an in situ trout (*Oncorhynchus mykiss*) working heart preparation at 10°C to examine whether prior exposure to 5 min of anoxia ($P_{O_2} \leq 5$ mmHg) could reduce or eliminate the myocardial dysfunction that normally follows 15 min of anoxic exposure. Hearts were exposed either to a control treatment (oxygenated perfusion) or to one of three anoxic treatments: 1) anoxia with low P_{out} [15 min of anoxia at an output pressure (P_{out}) of 10 cmH₂O]; 2) anoxia with high P_{out} [10 min of anoxia at a P_{out} of 10 cmH₂O, followed by 5 min of anoxia at $P_{out} = 50$ cmH₂O]; and 3) preconditioning [5 min of anoxia at $P_{out} = 10$ cmH₂O, followed after 20 min of oxygenated perfusion by the protocol described for the anoxia with high P_{out} group]. Changes in maximum cardiac function, measured before and after anoxic exposure, were used to assess myocardial damage. Maximum cardiac performance of the control group was unaffected by the experimental protocol, whereas 15 min of anoxia at low P_{out} decreased maximum stroke volume ($V_{s\ max}$) by 15% and maximum cardiac output (\dot{Q}_{max}) by 23%. When the anoxic workload was increased by raising P_{out} to 50 cmH₂O, these parameters were decreased further (by 23 and 38%, respectively). Preconditioning with anoxia completely prevented the reductions in $V_{s\ max}$ and \dot{Q}_{max} that were observed in the anoxia with high P_{out} group and any anoxia-related increases in the input pressure (P_{in}) required to maintain resting \dot{Q} (16 ml·min⁻¹·kg⁻¹). Myocardial levels of glycogen and lactate were not affected by any of the experimental treatments; however, lactate efflux was sevenfold higher in the preconditioned hearts. These data strongly suggest that 1) a preconditioning-like mechanism exists in the rainbow trout heart, 2) increased anaerobic glycolysis, fueled by exogenous glucose, was associated with anoxic preconditioning, and 3) preconditioning represents a fundamental mechanism of cardioprotection that appeared early in the evolution of vertebrates.

myocardium; hypoxia; metabolites; anaerobic glycolysis; cardiac output; *Oncorhynchus mykiss*; glycogen; lactate

IN 1986, MURRY ET AL. (33) reported that prior exposure of the dog heart to short periods of ischemia greatly diminished the degree of myocardial necrosis that resulted from exposure to 30 min of ischemia and reperfusion. This phenomenon, termed preconditioning, has been the focus of intense research over the past 15 years. Ischemic preconditioning has been demonstrated in nearly every mammalian species studied, including humans, and most recently in birds (39). It appears that a wide variety of stimuli have the ability to trigger preconditioning-like effects in the mammalian heart, and researchers have made significant progress in understanding which signaling pathways and end-effectors are central to the infarct-limiting ability of preconditioning (9, 34, 35).

In contrast to the extensive work that has been conducted on preconditioning and myocardial protection against acute hypoxia/ischemia in mammals, scant research has been conducted on lower vertebrates. In the fishes, a vertebrate group containing over 20,000 species, there exists a wide range of myocardial sensitivity to oxygen-limiting conditions. For example, although the tuna heart is extremely sensitive to even modest decreases in environmental oxygen (6) and the in situ trout heart fails rapidly when required to maintain routine cardiac work under severely hypoxic conditions (1), the hearts of eel (23) and hagfish (2, 22) are able to maintain cardiac function during prolonged periods of hypoxia and/or anoxia. Given the scope of hypoxia sensitivity in fishes, it could be concluded that mechanisms that mediate anoxia/hypoxia tolerance are absent in fishes in which myocardial performance and viability are compromised by acute exposure to conditions of limited oxygen availability. However, it is also possible that the hearts of hypoxia-intolerant fishes, like those of mammals, only require biochemical or other signals to trigger the protective mechanisms that are inherent in hypoxia/anoxia-tolerant fishes. To our knowledge, there is no

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experimental evidence to suggest that mechanisms of myocardial protection can be triggered in hypoxia-intolerant lower vertebrates or that a mechanism similar to preconditioning exists in fishes. If preconditioning can be confirmed in the fish heart, this would strongly suggest that this phenomenon developed early in vertebrate evolution and would be a very important discovery in the context of cardiovascular/integrative biology.

The present study examined whether a prior 5-min anoxic exposure could reduce the degree of myocardial dysfunction that results when the *in situ* rainbow trout (*Oncorhynchus mykiss*) heart is exposed to 15 min of anoxia. In addition, lactate efflux and myocardial levels of glycogen and lactate were measured to investigate whether any improvements in myocardial function were associated with changes in carbohydrate metabolism. We selected the *in situ* rainbow trout heart as a model for studying myocardial preconditioning in fishes for several reasons. Foremost, the largest knowledge base on cardiac anatomy, biochemistry, and physiology exists for this species. Second, the *in situ* trout heart can perform at workloads equivalent to maximum *in vivo* levels (29, 47). This makes it an extremely tractable preparation for examining anoxic preconditioning. Third, previous experiments (1, 19) demonstrated that the hearts of this species are normally extremely hypoxia intolerant. Last, the time course and magnitude of the loss of myocardial function during acute anoxia are similar in the trout and rabbit at 20°C (19, 29). These data suggest that the hypoxia/anoxia sensitivity of the trout and mammalian heart is comparable.

METHODS

Experimental animals. Rainbow trout (*O. mykiss*), 498 ± 28 g, were obtained from a local supplier (West Creek Trout Farms, Aldergrove, BC, Canada) and maintained in 2,000-liter fiberglass tanks supplied with dechlorinated Vancouver tap water. These fish were held under natural photoperiod at a temperature of 10 ± 1°C, and fed commercial trout pellets *ad libitum* daily.

Surgical procedures. Fish were anesthetized in an oxygenated and buffered solution of tricaine methane sulfonate (0.1 g/l MS-222; 0.1 g/l sodium bicarbonate) and transferred to an operating table where their gills were irrigated with oxygenated and buffered anesthetic (0.05 g/l MS-222; 0.05 g/l sodium bicarbonate) at 4–6°C. Fish were injected with 1.0 ml of heparinized (100 IU/ml) saline via the caudal vessels, and an *in situ* heart preparation was obtained from the trout as detailed in Farrell et al. (17). Briefly, an input cannula was introduced into the sinus venosus through a hepatic vein and perfusion with heparinized (10 IU/ml) saline containing 15 nmol/l epinephrine was begun immediately. Silk thread (3–0) was used to secure the input cannula and to occlude any remaining hepatic veins. The output cannula was inserted into the ventral aorta at a point confluent with the bulbus arteriosus and firmly tied in place with 1 silk thread. Finally, 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) 12.4 NaCl, 3.1 KCl, 0.93 MgSO₄·7H₂O, 2.52 CaCl₂·2H₂O, 5.6 glucose, 6.4 TES salt, and 3.6 TES acid (28). These chemicals were purchased from Fisher Scientific (Fair Lawn, NJ), with the exception of the TES salt, which was purchased from Sigma (St. Louis, MO). The TES buffer system was used to simulate the buffering capacity of trout plasma and the normal change in blood pH with temperature ($\Delta pK_a/dT = 0.016$ pH units/°C) (28). Epinephrine bitartrate (15 nmol/l; Sigma) was added to the perfusate throughout the experiment to ensure the long-term viability of the perfused fish heart (21). As epinephrine is light sensitive and deteriorates over time, it was added to a fresh perfusate bottle every 20 min.

To make our preconditioning experiments with the trout heart as comparable as possible to mammalian/avian studies, the saline was bubbled with 100% O₂ for a minimum of 45 min before use. Although the coronary circulation was not perfused in our preparations, research suggests that this level of oxygenation can supply a sufficient amount of O₂ to the outer myocardial layer. Maximum performance of *in situ* trout hearts perfused with normobaric hyperoxia (95–100% O₂) is comparable (17) and perhaps even higher (16) than that measured *in vivo*. The compact myocardium in these trout was only ~0.4- to 0.5-mm thick, and data from J. Altimiras and H. Gesser (unpublished) show that P_{O₂} in the middle of a 1-mm-thick trout myocardial slice would still be ~350 mmHg (0.6 of perfusate P_{O₂}) when myocardial O₂ consumption was equal to that measured in working hearts performing at basal levels. Furthermore, Braunlin et al. (5) showed that a saline P_{O₂} of 450 mmHg was sufficient to meet the metabolic requirements of cat papillary muscles (0.7 mm thick) that were contracting isometrically (24 times/min) at 30°C. For the anoxic exposures, the perfusate was saturated with 100% N₂ for a minimum of 2 h before experimental use to ensure that P_{O₂} was ≤5 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% N₂ into the bath beginning 5 min before the onset of anoxia.

Experimental protocols. Once surgery was completed (~15–20 min), the fish was immersed in a temperature-controlled saline bath at 10°C. The input cannula was attached to an adjustable constant-pressure reservoir, and the output cannula was connected to a constant pressure head. Output pressure (P_{out}) was initially set to 50 cmH₂O to simulate resting *in vivo* blood pressure (47), and input pressure (P_{in}) was adjusted to give a physiologically realistic cardiac output (\dot{Q} ; 16 ml·min⁻¹·kg⁻¹; Ref. 31). The heart was maintained at this initial control level of performance for a period of 10 min to allow it to recover from surgery and for equilibration of the saline bath. Thereafter, P_{in} was gradually increased until \dot{Q} reached 25 ml·min⁻¹·kg⁻¹. This brief cardiac stretch cleared any air bubbles from within the heart and provided an initial assessment of cardiac viability. Hearts that required more than a 2-cmH₂O increase in P_{in} to reach a \dot{Q} of 25 ml·min⁻¹·kg⁻¹ were discarded and assumed to have poor cannula placement, cannula obstruction, or myocardial damage.

After the cardiac stretch, \dot{Q} was reset to 16 ml·kg⁻¹·min⁻¹ for 25 min, and the hearts were randomly assigned to one of the four experimental protocols ($n = 7$ or 8). These protocols (schematically illustrated in Fig. 1) were identical in duration (~1.5 h) and were designed to examine the effect of anoxic workload and anoxic preexposure (preconditioning) on the recovery of resting and maximum cardiac function. The groups were 1) control: these hearts were exposed to oxygen-

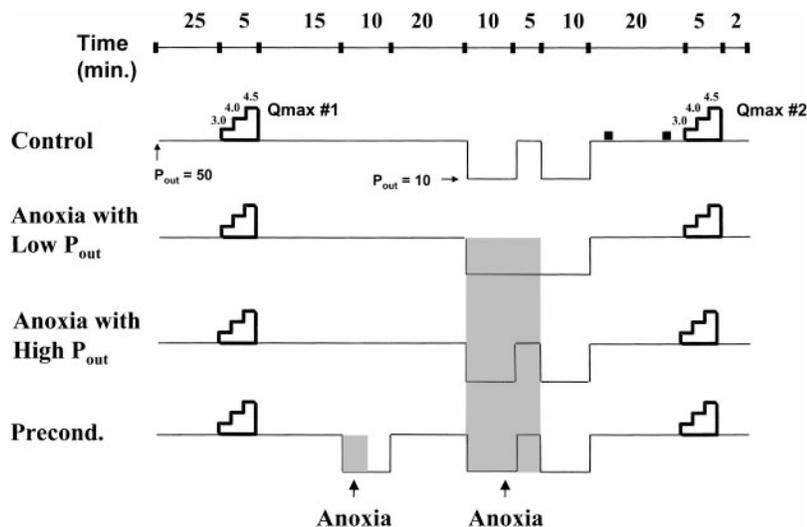


Fig. 1. Experimental protocols used to examine the effect of anoxic workload on the recovery of cardiac performance and whether preconditioning exists in the trout heart. The solid line represents the pressure development of the ventricle as determined by the height of the output pressure (P_{out}) head, which was set to either a physiological level of 50 cmH_2O or a subphysiological level of 10 cmH_2O . The solid steps mark the maximum cardiac output tests (\dot{Q}_{max}), where P_{in} was raised sequentially from 3 to 4 cmH_2O and finally to 4.5 cmH_2O . The shaded rectangles represent periods of anoxia ($PO_2 \leq 5$ mmHg). During anoxia, 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 $16 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ by adjusting P_{in} as needed. ■, Points at which perfusate samples were collected for biochemical analysis. $1 \text{ cmH}_2O = 0.736 \text{ mmHg}$.

ated saline, and changes in P_{out} were identical to those for the anoxia with high P_{out} group; 2) anoxia with low P_{out} : 15 min of anoxia (saline $PO_2 \leq 5$ mmHg) at a P_{out} of 10 cmH_2O ; 3) anoxia with high P_{out} : 10 min of anoxia at a P_{out} of 10 cmH_2O followed by 5 min of anoxia at $P_{out} = 50 \text{ cmH}_2O$; and 4) preconditioning: 5 min of anoxia at $P_{out} = 10 \text{ cmH}_2O$ followed after 20 min of oxygenated perfusion by the protocol described for the anoxia with high P_{out} group. For all hearts, maximum \dot{Q} (\dot{Q}_{max}) was measured before and 30 min after anoxic exposure, and resting cardiac function was recorded before $\dot{Q}_{max 1}$, before the 15-min anoxic period and before $\dot{Q}_{max 2}$. In this way, each heart acted as its own control. \dot{Q}_{max} was measured by increasing P_{in} to 3.0 cmH_2O , then to 4.0 cmH_2O , and finally to 4.5 cmH_2O (Fig. 1). These levels of P_{in} were reached by gradually increasing P_{in} over ~ 1 min, and P_{in} was maintained at 3.0, 4.0, and 4.5 cmH_2O for ~ 20 s to ensure that cardiac function had stabilized.

Several preliminary experiments showed that hearts failed to regain contractile function when P_{in} was increased in an effort to maintain resting \dot{Q} during the anoxic challenge. Therefore, P_{in} was not adjusted and \dot{Q} was allowed to fall during periods of anoxia (or during identical periods for the control group). Although \dot{Q} was quickly returned to $16 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ following all anoxic periods, P_{out} was maintained at 10 cmH_2O following anoxic exposure for 5 or 10 min. (see Fig. 1). Reducing P_{out} following anoxia facilitated the functional recovery of the hearts by 1) reducing the amount of pressure development required by the heart to produce positive flow and 2) reducing the time before oxygenated perfusion was restored to the myocardium.

Following $\dot{Q}_{max 2}$, the heart was stabilized at a resting \dot{Q} of $16 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ for 2 min. Thereafter, the fish was quickly moved to the surgical table, and isolation of the heart from the experimental bath was verified by connecting a 3-ml syringe to the input cannula and ensuring that a negative pressure was created when the plunger was retracted. The beating heart was then quickly removed from the pericardium, weighed, and freeze-clamped with aluminum tongs.

Perfusate samples were collected 15 and 30 min after the main anoxic period to determine the rate of myocardial lactate efflux (Fig. 1). Perfusate samples were collected for 1 min with \dot{Q} set at $16 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$. Heart tissue and perfusate samples were stored at -80°C before biochemical analyses were performed.

Instrumentation and data analysis. An in-line electromagnetic flow probe (Zepeda Instruments, Seattle, WA) was used

to record \dot{Q} , and pressure transducers (Narco Life Sciences, Houston, TX) were used to measure P_{in} and P_{out} through saline-filled sidearms. Before experimentation, pressure changes due to cannula resistance were calculated at known flow rates. These values were then used to adjust P_{in} and P_{out} to those in the sinus venosus and bulbus arteriosus, respectively. Pressure transducers were calibrated daily against a static water column and were referenced to the saline level in the experimental bath. Pressure and flow signals were amplified and displayed on a four-channel chart recorder (Gould, Cleveland, OH), which was coupled to a microcomputer running Labtech Notebook (Laboratory Technologies, Wilmington, MA). Data were continuously collected at 5 Hz, and block averages were calculated every 5 s. Heart rate (f_H) was measured by counting the number of systolic peaks recorded during a 10-s period.

$$\text{Stroke volume was calculated as: } V_s = (\dot{Q}/f_H)/M_b$$

where \dot{Q} is measured in milliliters per minute and M_b is body mass in kilograms.

Biochemical measurements. Whole hearts were powdered under liquid N_2 using a precooled mortar and pestle and added to ice-cold 0.6 N perchloric acid (PCA). The PCA extracts were homogenized on ice for 2×15 s at maximum speed with a tissue homogenizer (Ultraturax). An aliquot was immediately frozen for determination of glycogen. The remaining PCA extract was then centrifuged at 13,000 rpm at 4°C in a microcentrifuge. A known volume of the supernatant was removed and immediately transferred to another Eppendorf tube and neutralized with saturated Tris. The neutralized extracts were stored at -80°C until analysis.

Glycogen was digested with amyloglucosidase, and glucose levels were determined as described by Parkhouse et al. (37). Muscle lactate was also determined as described by Parkhouse et al. (37). Perfusate lactate concentration was determined on 100- μl aliquots, and lactate efflux rate was calculated as

$$\text{Lactate efflux rate (nmol} \cdot \text{ventricle}^{-1} \cdot \text{min}^{-1}) = \frac{\text{perfusate lactate conc (nmol/ml)} \times \text{cardiac output (ml/min)}}{\text{ventricular mass (g)}}$$

Statistics. All statistical analyses were performed using StatView Software (SAS Institute, Cary, NC). One-way

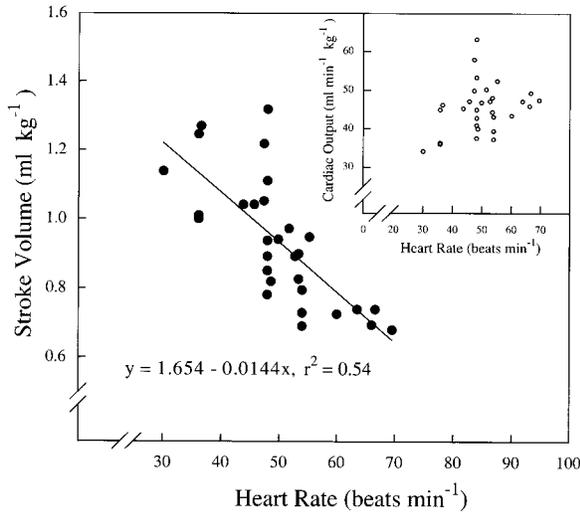


Fig. 2. Relationship between maximum stroke volume ($V_{s\max}$) and heart rate (f_H) before anoxic exposure. Each point represents an individual fish, and fish from all groups were included in the analysis ($n = 30$). The negative relationship between $V_{s\max}$ and f_H was significant ($P < 0.05$); however, there was no relationship between f_H and \dot{Q}_{\max} (inset).

ANOVAs were used to compare parameters between the treatment groups, including 1) body and ventricular mass, 2) the percent change in maximum cardiac performance (before anoxia vs. after), and 3) differences in lactate efflux rate and heart biochemical levels. Repeated-measures ANOVAs were performed for comparison of 1) maximum cardiac performance (before anoxia vs. after) within a specific treatment group and 2) resting P_{in} (before $\dot{Q}_{\max 1}$, anoxia, and $\dot{Q}_{\max 2}$) within and between treatment groups. A repeated-measures ANOVA could not be used to examine treatment differences in \dot{Q} during the 15-min anoxic challenge because of highly significant interaction ($P < 0.001$) between treatment and within-treatment (time) effects. Therefore, separate repeated-measures ANOVAs were performed for the first 10 min of anoxia (all groups at $P_{out} = 10$ cmH₂O) and for the last 5 min of anoxia when anoxia with high P_{out} and preconditioned hearts were exposed to a $P_{out} = 50$ cmH₂O. Between-group differences were identified using Fisher's least-significant difference post hoc test, and within-treatment effects were examined using Dunnett's post hoc tests or multiple contrasts. All percentage data were arc-sine transformed before any statistical tests were run. $P < 0.05$ was used as the level of statistical significance in all analyses.

RESULTS

Fish mass (498 ± 28 g) and ventricular mass (0.516 ± 0.02 g) were not significantly different ($P > 0.05$) among the treatment groups. In addition, there were no significant differences in resting cardiovascular parameters between the groups at the start of the experiment. A mean P_{in} of -0.39 ± 0.02 cmH₂O was required to maintain the resting \dot{Q} of 16 ml·min⁻¹·kg⁻¹ and, at this \dot{Q} , V_s and f_H were 0.33 ± 0.02 ml/kg and 56.6 ± 4.3 beats/min, respectively. There were also no differences in \dot{Q} (45.4 ± 2.2 ml·min⁻¹·kg⁻¹), V_s (0.93 ± 0.07 ml/kg), or f_H (50.0 ± 3.3 beats/min) between groups at $\dot{Q}_{\max 1}$. f_H varied considerably among the 30 preparations at $\dot{Q}_{\max 1}$. However, this had no

apparent effect on $\dot{Q}_{\max 1}$, because $V_{s\max}$ and f_H were negatively correlated. $V_{s\max}$ decreased by ~ 0.14 ml/kg with every 10 beat/min increase in f_H (Fig. 2).

\dot{Q} fell gradually in the anoxia with low P_{out} group, reaching 10.1 ± 1.2 ml·min⁻¹·kg⁻¹ by the 15th min of anoxia (Fig. 3). The rate of decline in \dot{Q} during the first 10 min was comparable to that measured in the other two groups exposed to anoxia (anoxia with high P_{out} and preconditioning). However, \dot{Q} immediately fell by 50% when P_{out} was raised to 50 cmH₂O and remained at ~ 5 – 7 ml·min⁻¹·kg⁻¹ until the heart was reoxygenated. Preconditioning had no significant effect on the decreases in \dot{Q} that were associated with the duration of anoxia or an increase in cardiac workload (P_{out}). These results indicate that anoxic duration and workload, but not preconditioning, significantly influenced cardiac function during anoxia.

\dot{Q}_{\max} , $V_{s\max}$, and f_H were not affected by the control protocol. In contrast, 15 min of anoxia with low P_{out} significantly ($P < 0.05$) decreased $V_{s\max}$ (by 0.14 ml·min⁻¹·kg⁻¹; 15%) and \dot{Q} (by 9.8 ml·min⁻¹·kg⁻¹; 23%; Fig. 4). Again, it was clear that myocardial workload during anoxia significantly influenced cardiac function. Increasing P_{out} to 50 cmH₂O for 5 min during the anoxic period caused a further reduction in both $V_{s\max}$ (by 23%) and \dot{Q}_{\max} (by 38%). Preconditioning completely eliminated the reductions in $V_{s\max}$ and \dot{Q}_{\max} that were associated with anoxic exposure. When the P_{in} required to maintain resting \dot{Q} was used as an index of cardiac function, similar effects of anoxic cardiac workload and preconditioning were observed (Fig. 5). P_{in} increased by ~ 0.4 cmH₂O in the control and anoxia with low P_{out} groups, and by 0.8 cmH₂O in the anoxia with high P_{out} group, during the last half of the experiment. However, P_{in} was unchanged in preconditioned hearts over this time period.

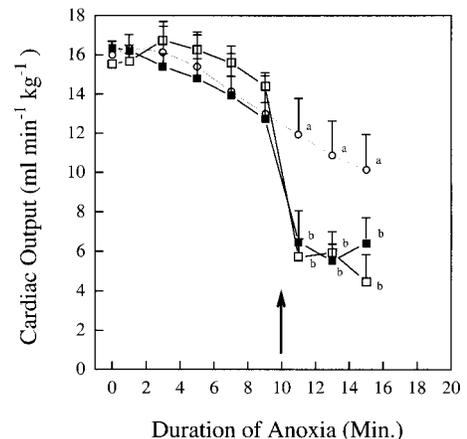


Fig. 3. \dot{Q} of in situ hearts ($n = 7$ or 8 per group) during 15 min of anoxia: \circ , output pressure (P_{out}) maintained at 10 cmH₂O; \square , P_{out} increased from 10 to 50 cmH₂O at 10 min; \blacksquare , preconditioned hearts, increase in P_{out} from 10 to 50 cmH₂O at 10 min. \dot{Q} was set to 16 ml·min⁻¹·kg⁻¹ at time 0 by adjusting P_{in} , thereafter, \dot{Q} was allowed to fall as cardiac function diminished. The arrow indicates when P_{out} was raised from 10 to 50 cm H₂O. At each time point, dissimilar letters indicate groups that are significantly ($P < 0.05$) different. Data for control animals are not presented because \dot{Q} was maintained at 16 ml·min⁻¹·kg⁻¹ in this group. 1 cmH₂O = 0.736 mmHg.

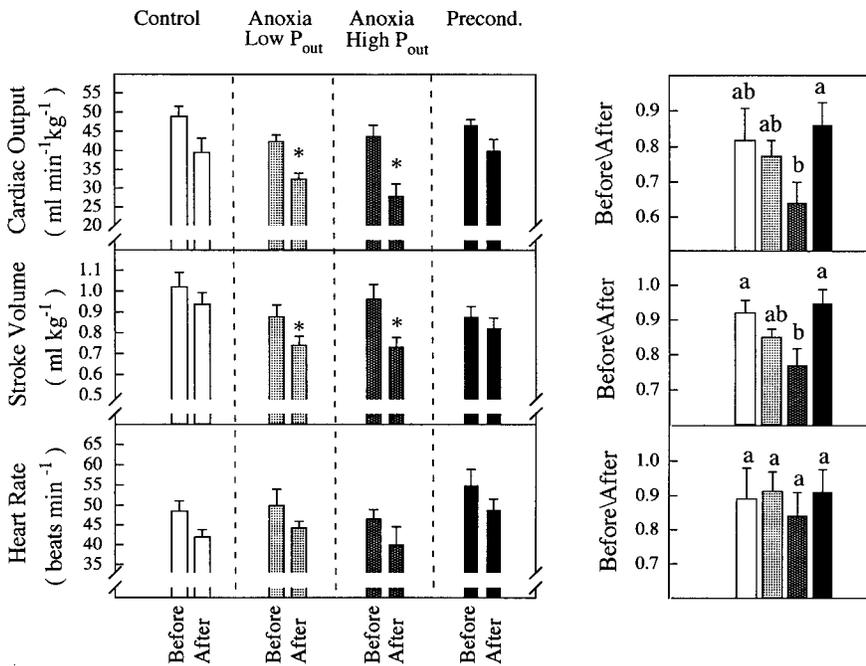


Fig. 4. Maximum performance of in situ working rainbow trout hearts ($n = 7$ or 8 per group) measured before and after exposure to anoxia. *Significant decrease ($P < 0.05$) as determined by repeated-measures ANOVA. Dissimilar letters indicate before/after values that were significantly different from each other. Significant differences between groups were identified using ANOVA and Fisher's least-significant difference (LSD) tests.

In the control group, the rate of lactate efflux rate was 24.5 and $13.7 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{g ventricle}^{-1}$ at time points equivalent to 15 and 30 min postanoxia (Table 1). The rate of lactate efflux was highly variable in the three anoxic groups at 15 min postanoxia. Thus, although lactate efflux values for these groups were 1.5 - to 3 -fold greater compared with the control group, these differences were not significant ($P = 0.32$). Lactate efflux was significantly elevated (~ 7 -fold) in the preconditioned hearts at 30 min postanoxia compared with the other three groups (Table 1). Myocardial concentrations of lactate and glycogen at the end of the experiment were not significantly different between groups (Table 1).

DISCUSSION

In our experiments, we showed that 5 min of anoxic preexposure completely eliminated the loss of maxi-

mum and resting cardiac function that normally followed 15 min of anoxia ($P_{O_2} \leq 5 \text{ mmHg}$). These data strongly suggest that a preconditioning-like mechanism exists in the trout heart. This is a novel finding, the implications of which are discussed below.

In situ trout hearts: performance and anoxia tolerance. In our experiments at 10°C , physiological levels of resting \dot{Q} (27) were achieved at slightly negative filling pressures (P_{in} ; approximately $-0.4 \text{ cmH}_2\text{O}$), and maximum values of \dot{Q} and V_s were $45.4 \pm 2.2 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ and $0.93 \pm 0.07 \text{ ml/kg}$, respectively. These values compare very well with those measured in other in situ experiments ($15, 16, 27$) and with direct measurements of cardiac performance in trout during intense exercise (47). Collectively, these favorable comparisons indicate that the trout hearts were not damaged during surgery and that our findings with regard to myocardial hypoxia tolerance and preconditioning

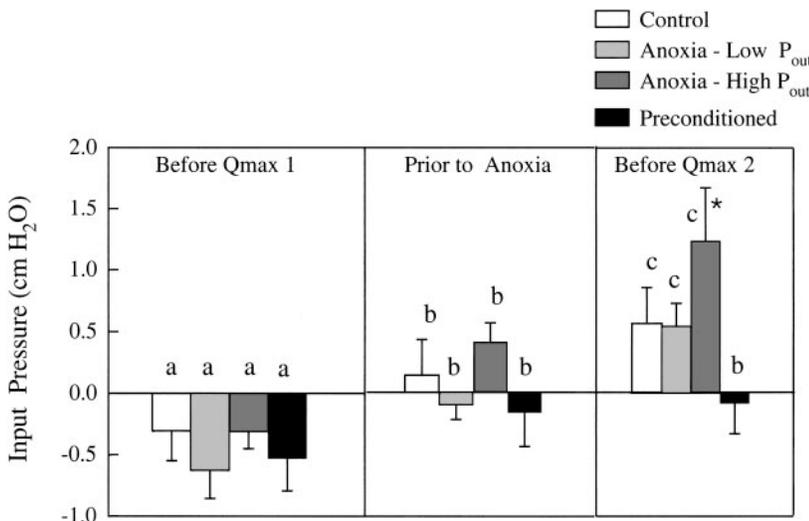


Fig. 5. P_{in} required to maintain resting \dot{Q} ($16 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) at various points during the experimental protocol. *Significant difference ($P < 0.05$) from all other groups within the "before \dot{Q}_{max2} " measurement period identified using repeated-measures ANOVA and Fisher's LSD tests. Dissimilar letters indicate input pressure values that were significantly different within each group ($n = 7$ or 8) as determined by repeated-measures ANOVA and multiple contrasts. $1 \text{ cmH}_2\text{O} = 0.736 \text{ mmHg}$.

Table 1. *Myocardial biochemistry and lactate efflux in trout hearts exposed to anoxic or oxygenated saline*

	Lactate Efflux, nmol·g ⁻¹ ·min ⁻¹		Myocardial Concentrations, μM/g	
	15 min	30 min	Lactate	Glycogen
Control	24.5 ± 5.0	13.7 ± 2.7*	4.50 ± 0.07	51.5 ± 3.7
Anoxia low P _{out}	37.4 ± 21.9	14.3 ± 2.0*	3.64 ± 0.5	55.3 ± 7.6
Anoxia high P _{out}	42.2 ± 10.5	12.8 ± 3.6*	3.89 ± 0.4	58.0 ± 5.1
Precond	72.9 ± 24.7	80.0 ± 31.9†	4.03 ± 0.7	46.9 ± 3.6

Values are means ± SE; *n* = 7 or 8 hearts. Hearts were sampled for biochemical analysis 2 min after the second maximal cardiac output (\dot{Q}_{\max}) test. Dissimilar symbols indicate group values that were significantly different from each other ($P < 0.05$) as determined by ANOVA and Fisher's least-significant difference tests. P_{out}, output pressure.

are relevant to the whole animal. Despite the good health of the preparations used in these experiments, an increase in P_{in} (~0.8 cm H₂O) was needed during the 2-h experiment to maintain resting \dot{Q} (Fig. 5). This small change in perfusion conditions likely reflects a diminished myocardial sensitivity to the tonic levels of epinephrine in the perfusate (15 nM). Epinephrine has positive chronotropic and inotropic effects on the trout heart (12, 17, 21), and the slight fall in f_H during the experiments (~8%) would necessitate an increase in resting P_{in} to elevate V_s and restore \dot{Q} .

Previous studies with working in situ heart preparations strongly suggest that the trout heart is intolerant of anoxia or severe hypoxia. Farrell et al. (18) showed that the threshold P_{O₂} for maintaining maximum power output is between 46 and 67 mmHg. Arthur et al. (1) demonstrated that the severely hypoxic (P_{O₂} < 5 mmHg) perfused trout heart failed rapidly when cardiac workload was raised to in vivo levels. A similar conclusion was reached using isolated trout myocardial strips where only 40% of initial force development is recovered after 30 min of anoxia (19). We made several observations that confirm the anoxia/hypoxia sensitivity of the in situ trout heart: 1) in preliminary experiments, hearts made to perform at resting in vivo physiological pressure (50 cmH₂O) for 10 min during anoxia could not be recovered (data not shown), 2) \dot{Q} fell immediately by ~50% when P_{out} was raised to 50 cmH₂O during anoxia (Fig. 3), and 3) increasing workload during anoxia resulted in greater decreases in $V_{s\max}$ and \dot{Q}_{\max} (Fig. 4). In view of this hypoxia intolerance, we consider the rainbow trout heart to be a suitable non-mammalian model for preconditioning studies.

Significant levels of lactate were detected in the perfusate leaving control hearts, and the rate of lactate efflux was greater at 15 min than at 30 min after these hearts were exposed to a P_{out} of 10 cmH₂O (Table 1). These results suggest that some anaerobic metabolism had occurred in the myocardium despite continuous perfusion of the heart lumen with oxygenated saline (P_{O₂} > 600 mmHg). We suspect that lowering P_{out} to 10 cmH₂O altered cardiac anatomy sufficiently to negatively impact O₂ delivery to the myocardium and that

the lactate that accumulated during this period was still washing out of the heart 15 min after P_{out} had been restored to 50 cmH₂O. It is unlikely, however, that the level of hypoxia experienced by control hearts was severe. The rate of lactate efflux (24.5 nmol·min⁻¹·g ventricle⁻¹) was <1% of that reported for severely hypoxic (P_{O₂} < 5 mmHg) in situ trout hearts performing at basal workloads (3.21 μmol·min⁻¹·g ventricle⁻¹; Ref. 1).

At $\dot{Q}_{\max 1}$, there was a significant negative relationship between f_H and V_s , but not between f_H and \dot{Q} (Fig. 2). These data are consistent with previous studies on cardiac function in hearts acclimated to and tested at different temperatures (13, 21, 27) and point to a strong interdependence between f_H and $V_{s\max}$ in the in situ trout heart that is not temperature dependent. Whether this effect is due to inadequate time for ventricular filling (decreased end-diastolic volume) or the inability of the ventricle to empty completely (increased end-systolic volume) awaits direct studies of heart chamber volumes during maximum cardiac function. Nonetheless, the results indicate that changes in f_H must be considered when interpreting the effect of experimental perturbations on $V_{s\max}$.

Preconditioning in the trout heart. The phenomenon of preconditioning, originally described by Murry et al. (33), has been shown to exist in almost all homeothermic vertebrates examined to date (for an exception, see Ref. 31). Preconditioning has been demonstrated in mice, dogs, pigs, rats, and rabbits, and evidence is now accumulating that preconditioning exists in the human heart (8, 20, 25, 50). In addition, Rischar and McKean (39) showed that the buffer-perfused pigeon heart could be preconditioned. In our study, we demonstrated that prior exposure to 5 min of anoxia was sufficient to eliminate the myocardial dysfunction that normally follows 15 min of anoxic exposure (Figs. 4 and 5). These data strongly suggest that preconditioning exists in the hypoxia-intolerant rainbow trout heart and that the phenomenon of preconditioning appeared early in the evolution of vertebrates.

We caution, however, that these results should not be interpreted as evidence that the entire trout heart was preconditioned by anoxic preexposure. The rainbow trout ventricle has two types of myocardia: an outer compact myocardium, which is normally perfused with highly oxygenated arterial blood supplied by the coronary artery, and an inner spongy myocardium, which is continuously exposed to a hypoxic microenvironment because it is perfused by the venous blood that percolates through its trabecular sinusoids. In rainbow trout of the size used in this study, we would expect 35–45% of the ventricle to be composed of compact myocardium (14). Thus it is feasible that the 38% reduction in \dot{Q}_{\max} following the high workload anoxic protocol was primarily or solely the result of damage to the compact myocardium and that this is the only myocardium that was salvaged by the preconditioning protocol. The mammalian literature contains evidence that both supports and refutes the idea that the trout's compact myocardium, but not its continuously hypoxic spongy myocardium, was precondi-

tioned. Hearts from newborn rabbits that were raised for 7–10 days in a hypoxic environment (12% O₂) could not be preconditioned (4). Furthermore, a decreasing tolerance of the rat heart to ischemia during postnatal life was counteracted by the ability to be preconditioned (36). In contrast, Tajima et al. (46) demonstrated that the protective effects of chronic hypoxia (3 wk, 10% O₂) and preconditioning on postischemic functional recovery of the adult rat heart were additive. Whether preconditioning in the fish heart is limited to compact myocardium that is supplied with oxygen-rich coronary arterial blood in vivo awaits further study. However, it is conceivable that preconditioning mechanisms are absent in the two-thirds of teleost fish species in which hearts are without a coronary circulation (42).

This is the first study of preconditioning in fish. Therefore, it is not known whether the pathways and end-effectors responsible for myocardial preconditioning in fish are similar to those proposed for mammals (34, 35, 50). The substantial increase in lactate efflux in preconditioned hearts following 30 min of anoxia (Table 1) suggests that increased anaerobic glycolysis is associated with anoxic preconditioning of the trout heart. This finding agrees with previous studies on rabbit hearts where preconditioning increased myocardial lactate production and reduced myocardial necrosis during low-flow ischemia (26). However, it contrasts with studies in the dog or rat that show that lactate efflux is decreased or unchanged in preconditioned hearts (7, 10, 33). The increased lactate production by preconditioned hearts could have been fueled by myocardial glycogen stores or by exogenous glucose. However, we believe that exogenous glucose was the predominant fuel source used by preconditioned trout hearts to enhance anaerobic glycolysis during the 15 min of anoxia. This conclusion is supported by recent experiments on the hypoxia-tolerant American eel (*Anguilla rostrata* L.) heart showing a requirement for extracellular glucose for anaerobic performance (3) and an upregulation of facilitated glucose transport during anoxia (40). Furthermore, studies on mammalian hearts indicate that 1) preconditioning-induced increases in lactate production during ischemia are paralleled by changes in exogenous glucose uptake (26), 2) ischemic preconditioning is associated with increased glucose uptake and increased glycolysis from glucose (10, 48), and 3) increased glycolytic rate and use of exogenous glucose during ischemia increase functional recovery (49). The use of exogenous glucose during ischemia may have additional benefits for the ischemic/hypoxic mammalian myocardium that are independent of increases in total glycolytic flux (41). Although there is no direct evidence that similar mechanisms of myocardial protection are exhibited by the fish heart, Driedzic et al. (11) demonstrated that tension development in myocardial strips from hypoxia-adapted *Zorares viviparous* could be restored to preanoxic levels if glucose, but not pyruvate, was provided in combination with 3 mM Ca²⁺.

While the glycolytic metabolism of extracellular glucose appears to be important in the preconditioning response of trout hearts, it is unlikely that alterations in cardiac function can explain the ability of our preconditioning protocol to improve functional recovery of the trout heart. \dot{Q} and cardiac work during the 15 min of anoxia were not significantly different between hearts exposed to preconditioning or anoxia with high P_{out} protocols (Fig. 3).

Limitations of the current study. In our study, we showed that the negative effects of 15 min of anoxia on performance of the in situ trout heart could be alleviated by prior exposure to a 5-min period of anoxia. Although this indicates that our protocol improved functional recovery of the trout myocardium following anoxia, the interpretation of whether this effect represents preconditioning is dependent on the definition used. In its strictest sense (termed “classical preconditioning”), preconditioning refers to the ability of short periods of ischemia and reperfusion (or other stimuli) to reduce or delay myocardial necrosis/infarction following a subsequent period of prolonged ischemia. Although we attempted to measure the release of creatine kinase (CK) as an index of myocardial necrosis (26) in these experiments, CK levels in the perfusate were below the detection limit of our spectrophotometric assay (<10 units). Therefore, we are unable to conclude whether the enhanced recovery of myocardial function in hearts preexposed to 5 min of anoxia was associated with a reduction in necrosis, the prevention of contractile dysfunction in viable myocardium (“stunning”), or both. Nevertheless, recent studies suggest that both a reduction in infarct size and the prevention of stunning are characteristic of myocardial preconditioning. First, Mosca et al. (32) showed that preconditioning with 5 min of ischemia prevented stunning but that the degree of necrosis was slight ($\leq 10\%$) and similar between preconditioned and control hearts. Second, Perez et al. (38) convincingly demonstrated that ischemic preconditioning prevents abnormalities in myofilament function that were previously shown to be associated with reversible postischemic contractile dysfunction. Consequently, we feel confident that our results provide direct evidence that preconditioning exists in the trout heart.

Hearts in these experiments were sampled 35 min after the end of anoxia and frozen ~20–30 s after perfusate flow to the heart was stopped. Although we report no differences in myocardial metabolite levels between groups at the end of the experiment, we do not know whether differences existed shortly after anoxia.

In these experiments, preconditioning prevented the small time-dependent decrease in resting cardiac function that was seen in control hearts (Fig. 5). We do not have an explanation for this observation at this time. However, we feel it is unlikely that the “preconditioning effect” we report is related to the prevention of hyperoxia-mediated myocardial injury, rather than protection against anoxia-mediated damage. First, if hyperoxic perfusion (free radical generation) was mediating the time-dependent loss of resting cardiac func-

tion and preconditioning diminished/prevented this effect, we would have expected P_{in} to increase less in the preconditioned group between “before $Q_{max 1}$ ” and “before anoxia.” This was not the case; the increase in P_{in} was the same in both groups. Second, although free radical generation is a major contributor to reperfusion-mediated myocardial damage in the mammalian heart, it is not clear whether hyperoxia increases this myocardial damage. Schnier et al. (43), using an opened-chest rabbit model, showed that hyperoxic reperfusion (arterial PO_2 550 mmHg) did not result in more myocardial necrosis following 45 min of ischemia compared with normoxic (arterial PO_2 96 mmHg) reperfusion. Sterling et al. (44) showed that hyperbaric oxygen (100% O_2 , 2.5 atm) reduced infarct size in the perfused rabbit heart compared with normobaric hyperoxia. In contrast, Hearse et al. (24) showed that reperfusion damage in crystalloid-perfused rat hearts increased with PO_2 .

Perspectives

The present study demonstrated that brief (5 min) anoxic preexposure completely eliminated the loss of cardiac function that normally follows a 15-min anoxic period and that this protection was associated with an increased rate of anaerobic glycolysis. These data provide the first evidence that myocardial preconditioning exists in fishes and suggest that preconditioning is a mechanism of myocardial protection that preceded the evolution of homeotherms. At present, it is not known whether preconditioning in the fish heart is restricted to myocardium that normally receives highly oxygenated arterial blood or whether the cellular mechanisms that mediate preconditioning (e.g., protein kinase C, ATP-sensitive K^+ channels, adenosine, etc.) in mammals are similar to those that mediate preconditioning and inherent myocardial hypoxia tolerance in the fish heart. These are intriguing questions, the answers to which have the potential to yield novel information about hypoxia tolerance of the fish myocardium and to provide further evidence that myocardial protection against ischemia/hypoxia-related damage in mammals and fishes is fundamentally similar. However, quantitative techniques for the measurement of myocardial necrosis in the perfused fish heart should be developed before these important experiments are conducted.

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