The Journal of Experimental Biology 210, 2574-2584
Published by The Company of Biologists 2007
doi:10.1242/jeb.004028

Cardiac remodelling in rainbow trout *Oncorhynchus mykiss* Walbaum in response to phenylhydrazine-induced anaemia

Danielle L. Simonot and Anthony P. Farrell*

Department of Biological Sciences, 8888 University Drive, Simon Fraser University, Burnaby, BC, V5A 1S6, Canada

*Author for correspondence at present address: Department of Zoology and Faculty of Land and Food Systems, 6270 University Boulevard, University of British Columbia, Vancouver, BC, V6T 1Z4, Canada (e-mail: farrellt@interchange.ubc.ca)

Accepted 2 May 2005

Summary

We examined the nature, extent and timing of cardiac ventricular remodelling in response to chronic, chemically induced anaemia in warm- and cold-acclimated rainbow trout *Oncorhynchus mykiss*. Chronic anaemia was induced by bi-weekly injections of phenylhydrazine hydrochloride (PHZ) and resulted in transient but large decreases in haematocrit (Hct) and haemoglobin concentration. After 2 weeks of anaemia, relative ventricular mass (rM_V) in warm-acclimated rainbow trout had already increased significantly and, by the eighth week of anaemia, rM_V was 58% greater than in the sham-injected control fish. Temperature modulated the anaemia-induced ventricular remodelling and erythropoietic responses, as indicated by cold-acclimation reducing the extent of the cardiac remodelling and slowing erythropoietic recovery. For example, in cold-acclimated fish, PHZ reduced Hct to 8.8±1.9% (ranging from 4–16%) and increased rM_V by 15% over a 4-week period, whereas the same treatment in warm-acclimated fish reduced Hct to only 17.4±2.1% (ranging from 6–29%) and yet increased rM_V by 28%. Cold-acclimated fish also recovered more slowly from anaemia. In addition, warm-acclimated fish maintained compact myocardium between 32% and 37% during anaemia, while cold-acclimated fish responded with an increase in compact myocardium (from 29% to 37%). Routine cardiac output ($\dot{Q}$) was continuously monitored following a single PHZ injection to examine the initial cardiac response to anaemia. Contrary to expectations, acute anaemia did not produce an immediate, proportionate increase in routine $\dot{Q}$. In fact, $\dot{Q}$ did not increase significantly until Hct had decreased to 10%, suggesting that rainbow trout may initially rely on venous oxygen stores to compensate for a reduced arterial oxygen-carrying capacity. Thus, we conclude that myocardial oxygenation, acclimation temperature and cardiac work load could all influence anaemia-induced cardiac remodelling in rainbow trout.

Key words: cardiac remodelling, compact myocardium, anaemia, temperature acclimation, heart.

Introduction

The more than tenfold variability observed in the relative ventricular mass (0.03% to 0.4% of body mass) (Santer, 1985) among fishes is unusually large among vertebrates. This interspecific variability can be linked directly to both cardiac workload and volume output of the heart. For example, while the large ventricle in tuna supports a high cardiac output ($\dot{Q}$) and a high ventral aortic blood pressure (Farrell, 1991; Brill and Bushnell, 2001), the proportionally similarly sized ventricle of haemoglobin-free Antarctic icefish supports an equally high $\dot{Q}$ but only a low ventral aortic blood pressure (Axelsson, 2005).

Fish hearts also show remarkable plasticity (Gamperl and Farrell, 2004). For salmonids, cardiac remodelling (changes in mass, geometry and composition) (Smits et al., 1991) occurs in response to exercise-training (Hochachka, 1961; Greer-Walker and Emerson, 1978), sexual maturation (Franklin and Davie, 1992b; Davie and Thorarensen, 1997; Clark and Rodnick, 1999), acute anaemia (McClelland et al., 2005) and cold acclimation (Farrell et al., 1988; Graham and Farrell, 1989; Taylor et al., 1996). In fact, sexual maturation in male rainbow trout *Oncorhynchus mykiss* imparts a very strong remodelling signal that can double relative ventricular mass (rM_V) (Franklin and Davie, 1992b; Clark and Rodnick, 1999). By comparison, cold-acclimation, exercise-training and acute anaemia can increase rM_V by 10–50% (Hochachka, 1961; Greer-Walker and Emerson, 1978; Farrell et al., 1988; Graham and Farrell, 1989; Davie and Thorarensen, 1997; McClelland et al., 2005). In addition to ventricular enlargement, the relative proportions of the two muscle types in the salmonid ventricle (outer compact and inner spongy myocardium) can change during cardiac remodelling. For example, the percentage of outer compact myocardium in the ventricle increases preferentially during sexual maturation and warm acclimation (Tota et al., 1983; Farrell et al., 1988; Graham and Farrell, 1992; Clark and Rodnick, 1998), as well as during the early stages of growth in Atlantic salmon *Salmo salar* (Poupa et al., 1974). Ventricular geometry can also change, for example salmonids respond to a culture environment with a more rounded (see Gamperl and

Farrell, 2004) and smaller ventricle (Graham and Farrell, 1989) compared with their wild counterparts.

Here we perform the first comprehensive characterisation of the nature, extent and timing of ventricular remodelling in rainbow trout during chronic anaemia and therefore extend a previous acute study in which phenylhydrazine-induced anaemia produced a 30% increase in rM_V (McClelland et al., 2005). Given that rM_V doubles with chronic anaemia in juvenile rats and sexual maturation in rainbow trout (Poupa et al., 1974; Franklin and Davie, 1992b; Clark and Rodnick, 1999), we suspect that the full extent of anaemia-induced ventricular remodelling has yet to be established for rainbow trout. We also performed the first direct measurements of $\dot{Q}$ during acute anaemia. This was important for two reasons. Foremost, previous studies have relied on the Fick Principle to estimate $\dot{Q}$ (Cameron and Davis, 1970; Holeton, 1971; Wood et al., 1979). Second, while anaemia reduced arterial oxygen-carrying capacity sixfold, this was accompanied by only a threefold increase in the estimated $\dot{Q}$ (Cameron and Davis, 1970). Therefore, we wished to determine whether the initial increase in $\dot{Q}$ was proportionate to the initial decline in haematocrit (Hct).

Materials and methods

Animal acquisition and care

An acute study of the responses of $\dot{Q}$, Hct and haemoglobin concentration ([Hb]) to a single injection of phenylhydrazine hydrochloride (PHZ) on rainbow trout *Oncorhynchus mykiss* Walbaum was conducted at the University of British Columbia. Rainbow trout were obtained from Aquafarm JV (Fort Langley, BC, Canada) and held in indoor fiberglass tanks with flow-through dechlorinated municipal water at 6°C for several months prior to the study. Body mass (M_b; mean ± s.e.m.) was 1045±113 g, fork length (FL) was 40.1±1.5 cm and the condition factor [$(M_b/FL^3)100$] was 1.51±0.07 (N=5 fish).

Studies of chronic anaemia, using repeated injections of PHZ, focused on cardiac remodelling and recovery from anaemia in both warm- and cold-acclimated rainbow trout. Rainbow trout were obtained from Sun Valley Trout Hatchery (Mission, BC, Canada) and held at Simon Fraser University in indoor fiberglass tanks with flow-through, dechlorinated municipal water. The study with warm-acclimated fish took place from August through October, when the water temperature during the experimental anaemia period averaged 17.6°C (ranging between 17.0°C and 18.3°C), followed by a seasonal decrease from 15°C to 12°C during the last 4 weeks of the experiment while the fish were recovering from anaemia. M_b was 135.9±2.9 g, FL was 21.1±0.1 cm and condition factor was 1.43±0.02 (N=144 fish). The study with cold-acclimated fish took place from February through April, when the water temperature averaged 6.4°C (ranging between 6.0°C and 7.0°C). M_b was 96.6±2.6 g, FL was 19.1±0.2 cm and condition factor was 1.35±0.02 (N=90 fish).

All fish were fed a maintenance diet of trout pellets (Aquafeed Limited; Chilliwack, BC, Canada) and were kept on a seasonal light:dark cycle. The experimental protocols were approved by the respective Animal Care Committees at Simon Fraser University and the University of British Columbia in accordance with the Canadian Council on Animal Care.

Considerations for PHZ injections

An intraperitoneal injection of 10.0–12.5 μg PHZ g^{-1} M_b causes a well-characterized transient haemolytic anaemia in fish, with haematocrit decreasing by 75–80% within 2–4 days (Smith et al., 1971; Chudzik and Houston, 1983; McClelland et al., 2005). Recovery occurs within a week at a high temperature, but not necessarily at low temperatures (Cameron and Davis, 1970; Byrne and Houston, 1988; McClelland et al., 2005). We are unaware of any study that has induced chronic anaemia with repeated PHZ injections. Therefore, we conducted a pilot study with three rainbow trout to evaluate the erythropoietic response to weekly PHZ injections (10 μg g^{-1} dissolved in 100 μl kg^{-1} M_b of physiological saline). Hct partially recovered with weekly PHZ injections and the sensitivity to PHZ decreased following several injections, requiring PHZ dosage to be doubled to create a comparable decrease in Hct. Given the concern that weekly injections could cause undue stress and the need to progressively increase the PHZ dosage, we used a bi-weekly injection protocol and doubled the PHZ dosage, as needed, to generate a state of functional anaemia for 1–2 months (i.e. overall Hct was <18% but fluctuated). Hct values for normocythemic rainbow trout are reported to range from 17% to 44% (Wells and Weber, 1991), but rainbow trout are functionally anaemic when Hct is <22% (based on a depression of swimming performance) (Gallaugher et al., 1995).

Experimental protocols

Effects of acute anaemia on cardiac output

$\dot{Q}$, Hct and [Hb] were monitored in rainbow trout before and for up to 4 days following a single injection of PHZ. Fish were prepared for these experiments by placing a Transonic flowprobe (Transonic Systems, Ithaca, NY, USA) on the ventral aorta (Farrell and Clutterham, 2003) to measure $\dot{Q}$. This method makes a small incision in the isthmus anterior of the pericardium, thereby leaving the pericardium intact. Also, a polyethylene (PE50) cannula was inserted into the dorsal aorta for blood sampling. Fish were placed in mesh tubes within a 200 l tank to recover and restrict activity. Following a 24 h recovery period, $\dot{Q}$ and heart rate (f_H) were recorded on Labview software (National Instruments, Ottawa, Ontario, Canada) for 10 min in the morning, and then again at noon and in late afternoon. Cardiac stroke volume (V_S) was calculated from $\dot{Q}$ and f_H measurements. Cardiovascular variables were averaged for each 10 min period and these data were pooled to generate a value representative of the normocythemic, routine cardiac status of each fish (excluding data measurements for visibly active fish during the recording period). Following the final recording for normocythemic fish, a blood sample (200 μl) was withdrawn from the cannula into a heparinized syringe and replaced with 200 μl of heparinized physiological saline. Hct was determined (as described below) and the remaining blood was frozen in heparinized vials for later [Hb] determination by standard cyanmethaemoglobin assay (Sigma Diagnostics, St Louis, USA). Fish were lightly anaesthetized in 0.1 g l^{-1} of buffered MS-222 (0.1 g l^{-1} sodium bicarbonate and 0.1 g l^{-1} tricaine methanosulfonate) and quickly injected intra-peritoneally with PHZ (20 μg g^{-1} M_b in 100 μl kg^{-1} M_b in physiological saline). The injection procedure lasted less than 2 min. Cardiac variables, [Hb] and Hct were monitored for up

to 4 days while Hct decreased. At the termination of the experiment, fish were euthanized by anaesthetic overdose and the ventricle excised and measured (see below).

Effects of chronic anaemia in warm-acclimated fish

There were four experimental treatment groups of warm-acclimated (17°C) rainbow trout: sham-injected fish; PHZ-treated fish; chased sham-injected fish; and chased PHZ-treated fish. Each group of fish was placed into an adjacent 140 l tank for 2 weeks prior to starting injection and chasing protocols. Fish were injected bi-weekly over an 8-week period (on four occasions) and were then allowed to recover for a further 4 weeks. Each fish was individually netted and anaesthetized in 0.1 g l^{-1} of buffered MS-222, weighed and injected intraperitoneally with either PHZ (10 μg g^{-1} M_b in 100 μl kg^{-1} M_b) or physiological saline (100 μl kg^{-1} M_b of physiological saline). Because anaemic fish could respond differently to the stress of handling than normocythemic fish, two of the groups (PHZ-injected and sham-injected) were additionally chased daily to deliberately increase their level of stress and activity. The chasing protocol followed the procedures described by Perry et al. (Perry et al., 1996). Fish were individually netted, placed in a separate tank, where they were chased with a net (and with additional prodding as needed because the fish quickly became accustomed to the net), before being placed into a holding tank while the remainder of the group was chased. Chasing was intended to increase cardiac work and not necessarily exhaust the fish, therefore it lasted either 2 min or until the fish became refractory to prodding. This protocol was approximately five-times shorter than routine protocols used to induce complete exhaustion (Milligan, 1996). Anaemic fish were notably less willing (or able) than normocythemic fish to swim vigorously for 2 min.

Fish were injected at weeks 0, 2, 4 and 6. Following the first PHZ injection, fish displayed a yellow discolouration of the body, as previously reported by Smith et al. (Smith et al., 1971). The first two PHZ injections were at a dose of 10 μg g^{-1} M_b, while the last two were 20 μg g^{-1} M_b. Following week 8, fish were allowed a 4-week recovery period without any Hct sampling or chasing. Final sampling occurred at week 12. There were 13 mortalities among the anaemic fish (six PHZ-treated fish and seven chased PHZ-treated fish) during the first 3 weeks of the experiment, but none thereafter. One sham-injected fish jumped from its tank and died. A water supply problem prematurely terminated the entire chased PHZ-treated group just prior to week 12, precluding any data for the recovery of this group.

Effects of chronic anaemia in cold-acclimated fish

The PHZ and sham injections with cold-acclimated fish (6°C) replicated those performed on warm-acclimated fish. However, given that chasing produced no additive effects in warm-acclimated fish and that significant cardiac remodelling had occurred after 4 weeks of anaemia (see Results), animal care considerations required us to reduce fish usage by eliminating the chased fish groups and restrict the chronic anaemic period to 4 weeks followed by a 4-week recovery period. Sample size was increased to N=10 fish per sample date per test group. Fish were sampled at weeks 0 (pre-treatment control), 4 and 8. In view of the sham injection eliciting a modest cardiac response in warm-acclimated fish, we also included an untreated group of fish that were not handled whatsoever, except for blood and tissue sampling. One fish died following the first PHZ injection.

Tissue sampling procedures during the chronic studies

Fish were sacrificed for cardiac and blood samples at the same time as the injections were performed, i.e. at weeks 0 (pre-treatment control), 2, 4, 6 and 8, and at the termination of the experiment, i.e. weeks 8 and 12. To monitor Hct between injections, blood was sampled 3–7 days later. On days when cardiac tissue was sampled, fish from each group were individually netted and anaesthetized. A blood sample (100 μl) was withdrawn by caudal puncture and Hct determinations (Readacrit centrifuge, Becton Dickinson, NJ, USA) were made in triplicate. Given the large number of fish used in the study, the difficulty of maintaining an indwelling cannula clot-free much beyond a week, and the unknown chronic effects of heparin on cardiac remodelling and erythopoiesis, we opted to obtain blood by caudal puncture. While this blood sampling method can increase Hct due to red blood cell swelling and splenic release of red blood cells (Perry and Gilmour, 1996), the effect is small relative to the level of experimental anaemia that was induced and represented a consistent overestimate of Hct throughout the study. Following blood sampling, fish were euthanized by cervical dislocation, weighed and measured. The ventricle, spleen and gonads were removed through a mid-ventral incision along the abdomen between the pelvic and pectoral girdles. The ventricle was blotted dry, wet mass determined to a precision of 0.1 mg and placed into 70% ethanol for later dissection. The spleen was weighed immediately and discarded, while gonads were inspected for sexual maturity. As peak anaemic response is reported to occur 2–3 days following PHZ injection (Smith et al., 1971; Chudzik and Houston, 1983; Byrne and Houston, 1988; McClelland et al., 2005), three fish were individually netted and anaesthetized as a sub-sample from each treatment group to monitor the Hct after the PHZ injection. The blood sample was replaced with 100 μl of heparinized saline (100 IU ml^{-1}), the fish was marked with a pectoral fin clip to prevent repeated sampling of any individual, revived and returned to the tank.

The ventricle was separated into its compact and spongy myocardial layers using blunt dissection under a dissection microscope, a simple procedure after tissue fixation with ethanol because of the presence of a thin fibrous membrane between the two tissue layers (Poupa and Carlsten, 1973; Farrell et al., 2007). Separated tissue samples were then desiccated to a constant mass (3 days at 65°C) and weighed to a precision of 0.1 mg. Compact myocardial mass was expressed as a percentage of the total dry mass of the ventricle. Percentage water content of the ventricle was determined from the difference between the wet and dry masses. Relative ventricular and myocardial masses (both wet and dry) are reported as a percentage of body mass.

Statistical analysis

All data are reported as a mean ± standard error of the mean (s.e.m.). In the cardiac remodelling experiments, comparisons with the pre-treatment control for each treatment group were

analyzed by ANOVA and a Dunnett's *post-hoc* test. Time-matched comparisons between PHZ-treated fish and sham-injected and untreated control fish also used ANOVA. Cardiorespiratory variables during acute anaemia were compared using repeated-measures ANOVA and a Tukey–Kramer multiple-range test. The level of significance for all statistical analyses was $P<0.05$. All statistical analyses were conducted using JMP 5.0 software (SAS Institute Inc., Cary, NC, USA), except the repeated-measures ANOVA, which was conducted on SigmaStat 3.0 software (SPSS, Chicago, IL, USA).

Results

Effects of acute anaemia on cardiac output

The cardiovascular effects of a single PHZ injection on $\dot{Q}$ and Hct are summarized in Fig. 1 and Table 1. There was a linear correlation between [Hb] and Hct over a wide range of Hct values (Fig. 1 inset). However, $\dot{Q}$ did not increase proportionally as Hct decreased (Fig. 1). While Hct and [Hb] were reduced sixfold at the termination of these acute recordings, $\dot{Q}$ had barely doubled (Table 1). In fact, $\dot{Q}$ did not increase significantly until Hct fell below 10% (Table 1) and the increase in $\dot{Q}$ lagged behind the decrease in Hct during the first 40 h of anaemia. In some individuals, Hct decreased by as much as 50% within 9 h of the PHZ injection without a major increase in $\dot{Q}$ (Fig. 1). Anaemia did not alter f_H (Table 1) and the final compensatory increase in $\dot{Q}$ was through increased V_S, which would have increased ventricular stretch because rainbow trout increase V_S by increasing end-diastolic volume rather than decreasing end-systolic volume (Franklin and Davie, 1992a). The rM_V was 0.146±0.010% and the percentage of compact myocardium was 40.1±1.5%.

Effects of chronic anaemia on warm-acclimated trout

Haematology

For sham-injected, warm-acclimated fish, Hct averaged 33.2±1.1% (N=49 fish; Fig. 2) for the entire 12-week experiment. Daily chasing had no significant effect on this Hct (31.1±1.0%; N=49 fish; bi-weekly data are not shown). Pooling these two data sets yielded a control Hct of 32.1±0.8% (N=98 fish) for the 12-week experiment.

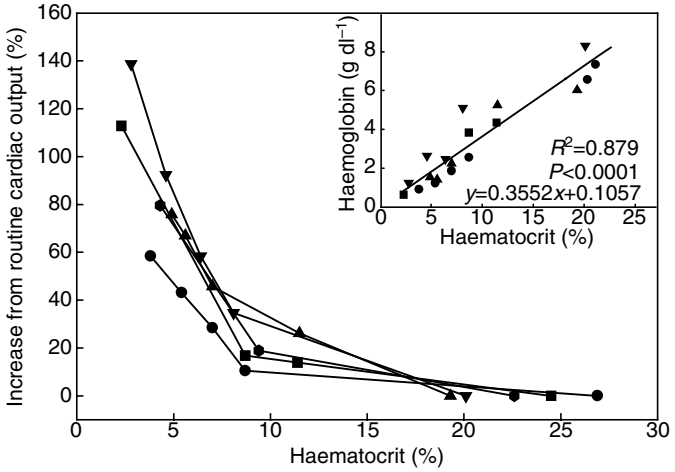

Fig. 1. Acute effect of phenylhydrazine hydrochloride injections on haematocrit (Hct), cardiac output ($\dot{Q}$) and haemoglobin concentrations ([Hb]) in cold-acclimated rainbow trout. Each line and matched symbol represent data for an individual rainbow trout (N=5). Data were collected for up to a 5-day period following injection of phenylhydrazine hydrochloride.

Similar to the acute anaemia experiment, a PHZ injection depressed Hct with a partial recovery after 2 weeks (Fig. 2). Bi-weekly PHZ injections then resulted in an 8-week period of anaemia such that Hct averaged 17.7±2.0% (N=49 fish) for PHZ-treated fish during this treatment period. Again, chasing had no significant effect on Hct of PHZ-treated fish (17.0±2.1%; N=37 fish; data are not shown). Pooling these two data sets yielded an Hct of 17.4±1.8% for PHZ-treated fish (N=86 fish), a value almost half the control Hct ($P<0.05$).

The recovery period (between weeks 8 and 12) resulted in Hct reaching 28.6±1.7% (N=6 fish) in PHZ-treated fish, a value that was not significantly different ($P>0.05$) from the control Hct at week 12 (29.0±1.6%; N=6 fish; Fig. 2; Table 2). Similar results were obtained for the chased fish groups (data are not shown). These recovery values at week 12 were marginally 10% lower ($P<0.05$) than the Hct at the outset of the experiment.

Ventricular remodelling

PHZ injections in warm-acclimated fish resulted in significant ($P<0.05$) increases in rM_V at all bi-weekly sample dates when compared with sham-injected fish (Table 2). In fact, by the second week of anaemia, rM_V was 40% greater than sham-injected fish. After 8 weeks of chronic anaemia, rM_V was 58% greater than the sham-injected fish and 84% greater than the pre-treatment control fish (Fig. 3A). Compared with the pre-treatment control value of 0.086±0.002%, sham injections significantly ($P<0.05$) increased rM_V at week 4 (by 20%) but at no other time (Fig. 3A). As with Hct, chasing of anaemic fish had no significant effect on rM_V of either sham-injected or PHZ-treated fish (data not shown).

Relative ventricular mass of PHZ-treated fish remained significantly elevated ($P<0.05$) compared with sham-injected fish at week 12 (Table 2). Thus, the recovery of rM_V post-anaemia (Fig. 3A) appeared to lag behind that of Hct (Fig. 2) such that warm-acclimated fish still had an enlarged ventricle when Hct was normal.

Cardiac water content remained unchanged throughout treatment and recovery [87.8±0.2% and 87.2±0.2% for PHZ-treated (N=30 fish) and sham-injected (N=30 fish) groups, respectively] compared with pre-treatment control fish (87.6±0.6%; N=6 fish). As a result, a significant ($P<0.05$) linear relationship existed between wet rM_V and dry rM_V (Fig. 3B). Thus, alterations in ventricular water content cannot explain the considerable ventricular remodelling observed with chronic PHZ-induced anaemia. In fact, the changes in dry rM_V (Fig. 4) paralleled those observed for wet rM_V, increasing significantly ($P<0.05$) by 40% after 8 weeks of chronic anaemia and being unchanged in sham-injected fish. Chasing fish had no effect the responses of dry rM_V (data not shown).

At the end of the 8-week anaemic period, the proportion of compact myocardium in PHZ-treated fish (37.3±1.5%) was the same as both the pre-treatment control (32.2±2.1%) and the sham-injected control (34.6±1.5%) values (Table 2). Therefore, the ventricular enlargement in warm-acclimated anaemic fish occurred as a result of equivalent growth of the compact and

Table 1. *Acute effects of a single intraperitoneal phenylhydrazine injection on cardiorespiratory variables in cold-acclimated rainbow trout*

	Haematocrit (%)	Haemoglobin (g dl^{-1})	Cardiac output (ml min^{-1} kg^{-1})	Heart rate (min^{-1})	Stroke volume (ml kg^{-1})
Normocythemia	22.7±1.4[a]	6.6±0.7[a]	18.3±3.2[a]	51.0±3.0	0.37±0.06
Anaemic	9.4±0.6[b]	4.0±0.5[b]	21.7±3.5[a]	47.9±4.2	0.45±0.06
Severely anaemic	3.6±0.5[c]	1.1±0.2[c]	33.3±3.9[b]	53.2±4.2	0.62±0.06

As there was some variability in the time for the onset of anaemia among fishes, sampling interval varied for each fish such that the anaemic and severe anaemic data were generally collected 24–72 h and 72–96 h, respectively, after injection. The maximal period for data collection post-injection for any fish was 4 days.

Values are means ± s.e.m. (N=5). Significant differences among data for normocythemic, anaemic and severely anaemic statesare indicated by different letters, as determined by repeated-measures ANOVA and Tukey–Kramer multiple-range test (P<0.05).

spongy myocardia (Fig. 4), with the exception of weeks 2 and 4 when growth of compact myocardium predominated.

Effects of chronic anaemia on cold-acclimated fish

Haematology

For cold-acclimated fish, PHZ injection significantly (P<0.05) decreased Hct from 38.6±1.1% to 12.0±2% after 3 days, and Hct averaged 8.8±1.9% for the 4-week period. In untreated and sham-injected fish, Hct remained unchanged except for a modest 10% decrease (P<0.05) at week 8. Hct recovered almost fully in PHZ-treated fish (28.1±1.5%) after the 4–week recovery period, but was about 20% lower than the final Hct for sham-injected and untreated fish (Table 3). This incomplete and reduced rate of recovery for Hct was a clear difference between the cold-acclimated and warm-acclimated fish.

While splenic mass increased significantly (P<0.05) after 4 weeks in the untreated and sham-injected groups, this increase was significantly larger in anaemic fish and represented a doubling of splenic mass compared with the outset of the experiment (Table 3). Consistent with a lag in the recovery of Hct, the enlarged splenic mass persisted in the PHZ-treated fish during recovery (Table 3).

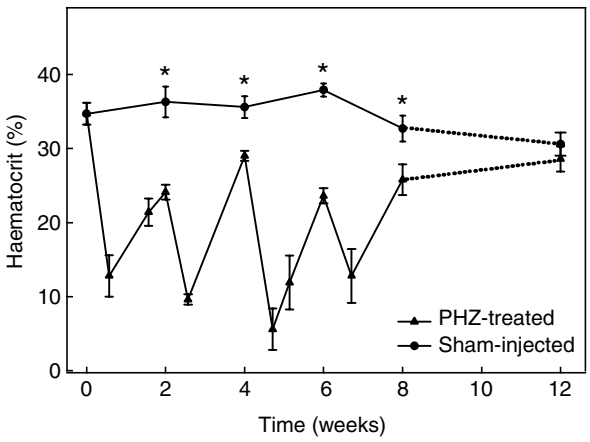

Fig. 2. Changes in haematocrit in warm-acclimated rainbow trout induced by four phenylhydrazine hydrochloride injections (syringe symbol) at weeks 0, 2, 4 and 6 (solid line and triangles) and compared with sham-injected control fish receiving saline (solid line and circles). The dotted lines represent the recovery periods. Mean values ± s.e.m. at weeks 0, 2, 4, 6, 8 and 12 represent N=6 fish and all other sample times represent N=3 fish. *Significant difference (P<0.05) between a time-matched PHZ-treated and sham-injected group, as determined by Student's t-test statistic (P<0.05).

Ventricular remodelling

For sham-injected fish, rM_V increased (P<0.05) by 18% after 4 weeks compared with the outset of the experiment, but returned to the control level during the 4-week recovery period (Fig. 5A). For PHZ-treated fish, rM_V increased 17% more than sham-injected controls (P<0.05) and was 35% larger (P<0.05) than the pre-treatment control fish (Fig. 5A). This PHZ-induced ventricular enlargement persisted during the recovery period (Fig. 5A). For untreated control fish, both wet and dry rM_V remained unchanged during the experiment (Fig. 5A and Fig. 6).

Again, alterations in water content did not account for the ventricular remodelling observed in cold-acclimated fish. Ventricular water content at the outset of the experiment (87.8±0.9%) was similar (P>0.05) to that at the end of each treatment (86.7±0.3%, 86.3±0.3% and 87.3±0.2% for untreated, sham-injected and PHZ-treated fish, respectively). Wet and dry rM_V were linearly related (Fig. 5B) and the PHZ-induced changes in wet rM_V were similar to those for dry rM_V (Fig. 6).

In cold-acclimated anaemic fish, ventricular remodelling after 4 weeks was largely accounted for by a significant (P<0.05) increase in compact myocardium (Fig. 6). As a result, the percentage of compact myocardium significantly (P<0.05) increased from 29.4±1.8% to 37.0±1.5% after 4 weeks of anaemia (Table 3). Consequently, while cold-acclimated rainbow trout normally have a lower (P<0.05) percentage of compact myocardium than warm-acclimated rainbow trout (32.2 and 37.3%, respectively), chronic anaemia eliminated this difference.

Discussion

Phenylhydrazine hydrochloride has been widely used to study experimental anaemia in a range of animals (Itano et al., 1976), including fish (Cameron and Wohlschlag, 1969; Cameron and Davis, 1970; Jones, 1971; Smith and Jones, 1982; Chudzik and Houston, 1983; Houston and Keen, 1984; Murad et al., 1990; Marinsky et al., 1990; Gilmour and Perry, 1996; McClelland et al., 2005). PHZ causes oxidative haemolysis of red blood cells and, despite its widespread usage in piscine studies, has no reported side effects (Cameron and Wohlschlag, 1969; Cameron

Table 2. *The chronic effects of two intraperitoneal phenylhydrazine injections (at weeks 0 and 2) on the haematocrit, relative ventricular mass, percentage compact myocardium and splenic mass in warm-acclimated rainbow trout*

Week	Treatment	Haematocrit (%)	Relative ventricular mass	Compact composition (%)	Relative spleen mass
0	Pre-treatment	34.7±1.5	0.086±0.006	32.2±2.3	NA
2	Sham-injected	36.3±2.1	0.0816±0.005	36.1±1.3	0.116±0.012
	PHZ-injected	24.1±1.0*+	0.126±0.008*+	40.5±1.3*+	0.191±0.012+
4	Sham-injected	35.6±1.5	0.105±0.003+	33.1±2.0	0.104±0.009
	PHZ-injected	29.3±0.7*+	0.122±0.005*+	35.9±1.4	0.188±0.015+
6	Sham-injected	37.9±0.9	0.097±0.003	34.6±1.6	0.100±0.110
	PHZ-injected	23.6±1.0*+	0.146±0.011*+	34.1±1.2	0.173±0.007+
8	Sham-injected	32.7±1.8	0.094±0.002	34.6±1.2	0.108±0.011
	PHZ-injected	25.8±2.1*+	0.158±0.006*+	37.3±1.7	0.262±0.013+
12	Sham-injected	30.6±1.6	0.115±0.007*	30.8±1.8	0.134±0.015
	PHZ-injected	28.1±1.7	0.141±0.005*+	32.0±1.2	0.214±0.060+

N=12 for each sample date and treatment, except PHZ-injected Week 12 (*N*=6). NA, not available.

*Significant difference compared with the pre-treatment value (mean ± s.e.m.), as determined by ANOVA and Dunnett's test ($P<0.05$); +significant difference between PHZ-injected and time-matched control groups.

and Davis, 1970; Smith et al., 1971; Chudzik and Houston, 1983; Houston and Keen, 1984; Murad et al., 1990; Marinsky et al., 1990; Gilmour and Perry, 1996; McClelland et al., 2005), other than those directly related to the anaemia itself. A single study on anaemic rats reported that the observed increase in coronary flow during cardiac contractions following PHZ treatment was prevented by an antioxidant, as well as reducing focal lesions in the muscle and the degree of hypertrophy (Meerson and Evsevieva, 1985). Also in rats, a single PHZ injection caused leukocytosis, perhaps as a result of a PHZ stimulation of lymphoid blastogenesis, which then could have served to more rapidly remove from the circulation red blood cells whose membranes had been damaged by PHZ (Dornfest et al., 1990). In view of this information, we assume here that the cardiac remodelling was in response to chronic anaemia and side-effects were minimal.

Injection of PHZ requires fish handling, which we controlled for by using untreated fish, sham-injections and a parallel set of experiments in which fish were additionally chased to increase the level of handling stress. Given the large number of fish that were used, the long durations of the experiments and the small size of many fish studied (<200 g), it was not feasible to cannulate blood vessels to sample blood except in the acute experiments that measured $\dot{Q}$. Given that caudal puncture can overestimate Hct compared with Hct values derived from cannulated fish (Gallaugher and Farrell, 1998), the level of chronic anaemia we have reported here likely overestimated the true Hct of the fish in a small and consistent manner.

The present study demonstrated that repeated PHZ injections can be used to produce chronic anaemia, which was then associated with cardiac remodelling in both warm- and cold-acclimated rainbow trout. An earlier study with warm-acclimated rainbow trout demonstrated that a single injection of PHZ, causing temporary anaemia (Hct=10%), increased rM_V by 30% after 2–4 weeks (McClelland et al., 2005). In the present study we established that, after about 8 weeks of chronic

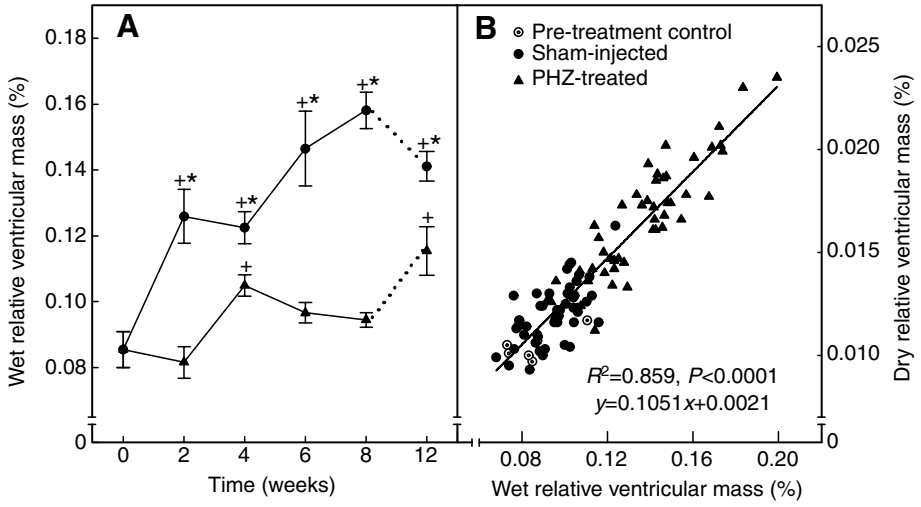

Fig. 3. (A) Change in relative ventricular mass in warm-acclimated rainbow trout induced by four phenylhydrazine hydrochloride injections at weeks 0, 2, 4 and 6 (solid line and triangles) compared with sham-injected control fish receiving saline (solid line and circles). The dotted lines represent the recovery periods. Mean values ± s.e.m. at weeks 0, 2, 4, 6, 8 and 12 represent *N*=6 fish. +Significant difference ($P<0.05$) from the pre-treatment control value at week 0, as detected by ANOVA and Dunnett's test. *Significant difference ($P<0.05$) between a time-matched PHZ-treated and sham-injected group, as determined by Student's *t*-test statistic ($P<0.05$). (B) A linear relationship exists between wet and dry rM_V, indicating constant water content among treatment groups.

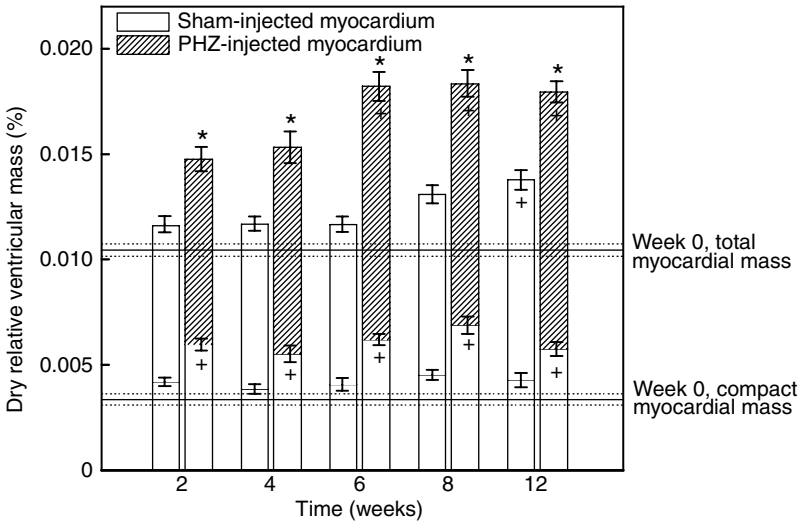

Fig. 4. Change in dry relative ventricular masses for compact (lower portion) and spongy (upper) myocardium in warm-acclimated rainbow trout induced by four phenylhydrazine hydrochloride injections at weeks 0, 2, 4 and 6 (hatched columns) compared with sham-injected control fish receiving saline (open columns) and followed by a recovery period to week 12. Values are means ± s.e.m., N=6. The horizontal lines denote mean values for the pre-treatment (day 0) controls (the dotted lines represent ± s.e.m.). $^{+}$Significant difference (P<0.05) from the pre-treatment control value at week 0, as detected by ANOVA and Dunnett's test. *Significant difference (P<0.05) between the dry relative myocardial masses (compact, spongy and total) of time-matched PHZ-treated and sham-injected groups, as determined by Student's t-test statistic (P<0.05).

anaemia, the ventricle of warm-acclimated rainbow trout was enlarged by nearly 60%, i.e. twice the growth observed previously. Thus, the present study suggests that the full extent of ventricular remodelling to anaemia was not revealed in earlier studies. Whether more stable and longer anaemic periods than used here would double rM_V, as reported for sexual maturation in male rainbow trout, will need further study.

Absent from an earlier study (McClelland et al., 2005) were appropriate controls to eliminate the possibility of changes in cardiac water content and injection effects accounting for the ventricular enlargement. In the present study, we eliminated the possibility of significant changes in cardiac water content affecting the estimate of ventricular enlargement based solely on wet mass with the finding that there were similar changes in cardiac dry and wet masses during ventricular remodelling. However, we did discover a modest but significant sham-injection effect, which could mean that McClelland et al. overestimated the extent of cardiac remodelling due to anaemia *per se* (McClelland et al., 2005). The nature of this sham injection effect on rM_V is unclear since chasing fish did not alter the response to anaemia. Increased activity in mammals similarly has been shown not to alter the response to anaemia (Magosso and Ursino, 2004).

The stimulatory effect of cold-acclimation on rM_V is well recognized for rainbow trout (Farrell et al., 1988; Graham and Farrell, 1989; Taylor et al., 1996) and other fishes (Kent and Prosser, 1985; Tsukuda et al., 1985; Goolish, 1987). McClelland et al. (McClelland et al., 2005) observed a 24% increase in rM_V in rainbow trout during a seasonal decrease in water temperature from 12°C to 2°C. However, cold-acclimation also completely attenuated erythropoiesis in goldfish, since Hct recovery was absent after several months at 7.5°C and yet took only several days at 30°C (Chudzik and Houston, 1983). The present study demonstrates that both erythropoiesis and cardiac remodelling were attenuated by cold-acclimation since equivalent PHZ injections in cold-acclimated fish resulted in a lower average Hct 3 days following injection (8.8±1.9%) and a smaller increase in rM_V within 4 weeks (17% compared with sham-injected fish) compared with warm-acclimated fish. In the present study we showed that Hct completely recovered after 4 weeks in warm-acclimated fish, but only partially recovered in cold-acclimated rainbow trout even though splenic enlargement persisted. Attenuation of anaemia-induced cardiac remodelling in cold-acclimated rainbow trout is a novel finding that was surprising given that the level of anaemia was about twice that in warm-acclimated fish. This diminished cardiac remodelling response could reflect temperature-related reductions in whole animal metabolism and protein synthesis (i.e. the ability to

Table 3. *The chronic effects of two intraperitoneal phenylhydrazine injections (at weeks 0 and 2) on the haematocrit, relative ventricular mass, percentage compact myocardium and splenic mass in cold-acclimated rainbow trout*

Week	Treatment	Haematocrit (%)	Relative ventricular mass (%)	Percentage compact myocardium (%)	Relative splenic mass (%)
0	Pre-treatment	38.6±1.1	0.087±0.003	29.4±1.8	0.108±0.01
4	Untreated	38.6±1.9	0.095±0.003	31.1±1.4	0.156±0.01*
	Sham-injected	36.0±2.1	0.103±0.005*	31.2±1.2	0.159±0.03*
	PHZ-treated	15.6±1.5*†	0.118±0.004*†	37.0±1.5*$^{+}$	0.199±0.01*$^{+}$
8	Untreated	34.1±1.0	0.088±0.003	29.3±1.4	0.110±0.01
	Sham-injected	34.9±1.3	0.092±0.003	30.3±1.8	0.068±0.006
	PHZ-treated	28.1±1.5*†	0.113±0.005*†	34.2±1.4	0.210±0.04*$^{+}$

Values are means ± s.e.m. (N=10 for each sample date and treatment). *Significant difference compared with the pre-treatment value, as determined by ANOVA and Dunnett's test (P<0.05); †significant difference between PHZ-injected and time-matched control groups.

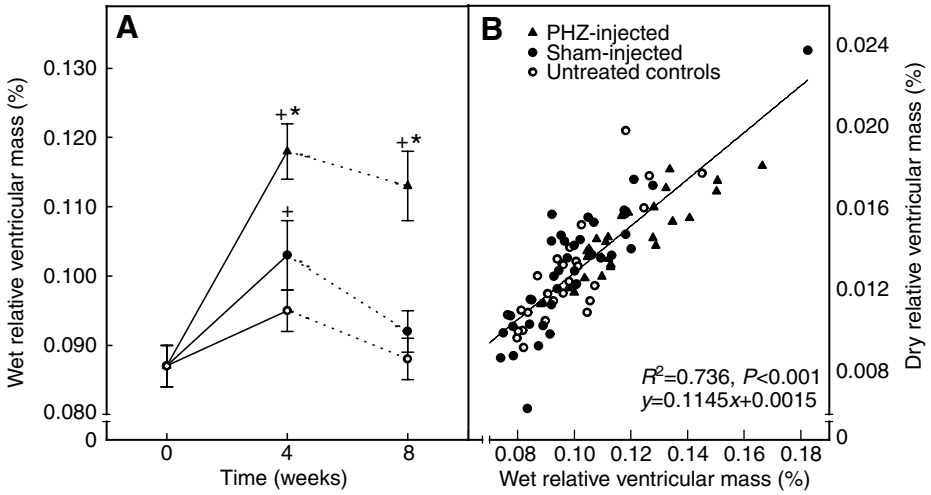

Fig. 5. (A) Change in relative ventricular mass in cold-acclimated rainbow trout induced by phenylhydrazine hydrochloride injections at weeks 0 and 2 (solid line and triangles) compared with sham-injected control fish receiving saline (solid line and circles) and untreated fish (solid line and open circles). The dotted lines represent the recovery periods. Mean values (± s.e.m.) at weeks 0, 4 and 8 represent N=10 fish. $^{+}$Significant difference (P<0.05) from the pre-treatment control value at week 0, as detected by ANOVA and Dunnett's test. *Significant difference (P<0.05) between a time-matched PHZ-treated and sham-injected group, as determined by Student's t-test statistic (P<0.05). (B) A linear relationship exists between wet and dry rM_V, indicating constant water content among treatment groups.

remodel tissues). Thus, it may take cold-acclimated, anaemic rainbow trout more than 4 weeks to increase rM_V beyond 0.11%, or alternatively the anaemic signal may not be strong enough for the ventricle to reach the rM_V (0.15%) observed for warm-acclimated rainbow trout.

Cardiac remodelling is thought to be of prime physiological importance to compensate for changes in cardiac work (Tota, 1983). Therefore, given that the principle compensatory adjustment to anaemia in fishes is an elevation of $\dot{Q}$ (Cameron and Davis, 1970) with blood pressure either remaining unchanged (Cameron and Davis, 1970) or decreasing (Wood et al., 1979), chronic anaemia is an interesting experimental perturbation to examine the physiological triggers for ventricular remodelling. These triggers, with the exception of the role played by reproductive hormones (testosterone and 17-α methyltestosterone) (Davie and Thorarensen, 1997), are poorly understood in fishes. Theoretically, anaemia-induced cardiac remodelling could be triggered by increased cardiac flow work (through increased $\dot{Q}$), increased cardiac stretch (through increased cardiac stroke volume), reduced cardiac pressure work, or some combination. However, direct measurements of $\dot{Q}$ have never been measured in fish during anaemia. Also, hypoxia could act as a potential trigger for anaemia-induced cardiac remodelling because tissue oxygen extraction increases appreciably during anaemia, lowering the venous oxygen tension encountered by the heart (Wood et al., 1979), while whole animal oxygen uptake and arterial oxygen saturation are maintained (Cameron and Davis, 1970; Wood et al., 1979; Gilmour and Perry, 1996). The present observations allow us to examine to what extent cardiac stretch and cardiac hypoxia might have contributed to ventricular remodelling.

The present study is the first to directly measure the temporal lag between the decrease in Hct and the compensatory increase in $\dot{Q}$. Hct had decreased to below 10% (i.e. a threefold decrease in Hct) before $\dot{Q}$ increased significantly. Moreover, the nearly twofold increase in $\dot{Q}$ could not fully account for a sixfold decrease in Hct after 4 days of anaemia. This finding is entirely consistent with an earlier study using starry flounder, which showed that progressive bleeding over a 4–14 day period did not trigger an increase in the Fick estimate of $\dot{Q}$ until Hct fell below 5% (Wood et al., 1979). Thus, the increase in cardiac V_S that was observed here during acute anaemia (with no change in f_H) is not only consistent with earlier observations of acute anaemia in fish (Cameron and Davis, 1970; Wood et al., 1979) (but see Holeton, 1971), but also consistent with cardiac stretch (through an increase in end-diastolic volume) being a trigger for remodelling. Mechanical stretch associated with volume-loading of the heart through increasing $\dot{Q}$ by V_S is thought to be major stimulus for ventricular remodelling in mammals (Delcayre et al., 1988) and fish (Clark and Rodnick, 1998). All the same, the initial cardiorespiratory response to anaemia was not an increase in V_S and yet ventricular remodelling occurred rapidly (more than half of the ventricular enlargement occurred within 2 weeks; Fig. 3A).

Given that the increase in $\dot{Q}$ after 5 days of anaemia never fully accounted for the decrease in Hct in terms of arterial oxygen transport {Note: Hct and [Hb] are linearly related (Cameron and Wohlschlag, 1969); present study} and whole animal oxygen uptake does not change during anaemia [(Cameron and Wohlschlag, 1969; Cameron and Davis, 1970; Wood et al., 1979; Gilmour and Perry, 1996) but see Holeton (Holeton, 1971) for CO-induced anaemia], we conclude that tissue oxygen extraction likely increased threefold. This change would decrease venous oxygen tension considerably. A large decrease in venous oxygen tension is a very important consideration for any fish because the oxygen supply of spongy myocardium is derived from venous blood. In fact, the anemia-induced decrease in venous oxygen tension in anaemic flounder (Wood et al., 1979) and rainbow trout (Holeton, 1971) to around 1 kPa resulted in the venous oxygen tension approaching the

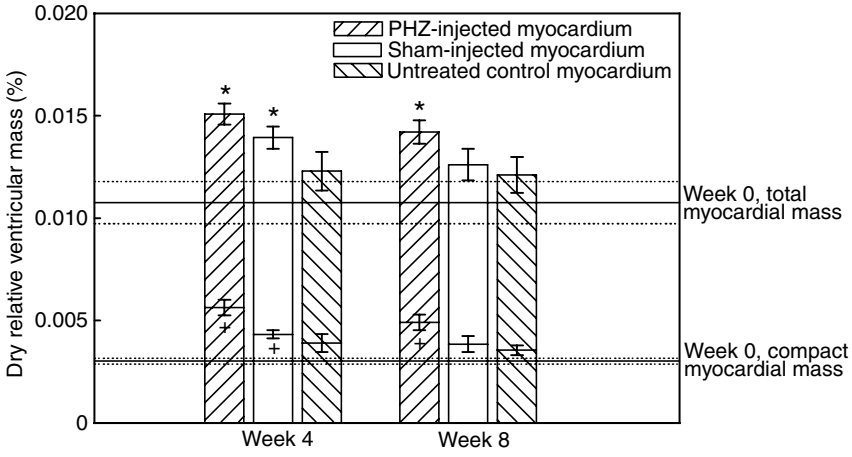

Fig. 6. Change in dry relative ventricular masses for compact (lower portion) and spongy (upper) myocardium in cold-acclimated rainbow trout induced by phenylhydrazine hydrochloride injections at weeks 0 and 2 (hatched columns) compared with sham-injected control fish receiving saline (open columns) and untreated control fish (reverse hatched columns), and followed by a recovery period to week 8. Values are means ± s.e.m., N=6. The horizontal lines denote mean values for the pre-treatment (day 0) controls (the dotted lines represent ± s.e.m.). $^{+}$Significant difference (P<0.05) from the pre-treatment control value at week 0, as detected by ANOVA and Dunnett's test. *Significant difference (P<0.05) between a time-matched PHZ-treated and sham-injected group, as determined by Student's t-test statistic (P<0.05).

predicted minimum gradient for adequate oxygen delivery to ventricular trabeculae (Davie and Farrell, 1991). Furthermore, hypoxia has emerged as a principle stimulus evoking erythropoiesis and haemoglobin accumulation in fish (Tun and Houston, 1986). Therefore, future studies should consider cardiac hypoxia as a potential trigger for anaemic remodelling despite the expectation that mechanical stretch is the major stimulus.

If chronic anaemia has the potential to create a chronic hypoxic condition for the spongy myocardium, chasing fish may have exacerbated this problem by periodically reducing venous oxygen tension (Farrell and Clutterham, 2003) even further. However, we did not observe disproportionate cardiac remodelling in warm-acclimated rainbow trout, but then anaemic fish were more lethargic than normocythemic fish. Similarly, a hypoxic signal in anaemic fish might trigger disproportionate growth of the compact myocardium because the arterial blood supply to the compact myocardium has a higher oxygen partial pressure. Conversely, the lower myocardial oxygen demands of spongy myocardium, combined with its higher activities of oxidative enzymes than compact myocardium (Tota et al., 1983; Gamperl et al., 1994), could compensate for the different levels of hypoxia experienced by the compact and spongy myocardia. Interestingly, cold-acclimated rainbow trout differed from warm acclimated fish by disproportionately increasing the percentage of compact myocardium from 29% to 37%. Therefore cold-acclimated fish reached the percentage of compact myocardium normally associated with warm-acclimated rainbow trout. Therefore, it is possible that the more severe anaemia (8% Hct *vs* 17% Hct) in cold-acclimated fish compared with the warm-acclimated fish triggered a hypoxic response, as well as a mechanical response in the compact myocardium. Studies on molecular signals in cardiac tissues during anaemia would provide greater insight in this matter.

Quantitatively, the compensatory changes associated with anaemia have theoretical limits. Normocythemic rainbow trout can increase both routine $\dot{Q}$ and tissue oxygen extraction by about threefold without reaching either maximum $\dot{Q}$ or compromising oxygen supply to the spongy myocardium (Farrell, 1984; Farrell, 2002; Farrell and Clutterham, 2003). Thus, the maximum decrease in Hct could be as much as ninefold before routine oxygen uptake becomes compromised. Previously, routine $\dot{Q}$ tripled during severe anaemia in rainbow trout and flounder (Cameron and Davis, 1970; Holeton, 1971; Wood et al., 1979). However, direct measurements of $\dot{Q}$ are needed to verify these earlier Fick estimates because some of the $\dot{Q}$ values were unrealistically high (see Cameron and Davis, 1970). Here, routine $\dot{Q}$ (18.3 ml min^{-1} kg^{-1}) was comparable to literature values for rainbow trout at similar water temperature (17.6–18.0 ml min^{-1} kg^{-1}) (Kiceniuk and Jones, 1977; Gamperl et al., 1994) and nearly doubled with a sixfold decrease in Hct. Also, we do not know to what extent the anaemia-induced reduction in venous oxygen tension might compromise maximum $\dot{Q}$. In perfused rainbow trout hearts, hypoxia compromises maximum performance (Hanson et al., 2006), and a hot temperature increases the hypoxic threshold (Hanson and Farrell, 2007). Such constraints might help explain why PHZ injections caused some mortality in warm- but not cold-acclimated rainbow trout, and made fish lethargic when chased. Holeton (Holeton, 1977) similarly found that tolerance to anaemia was inversely related to water temperature and Cameron and Davis (Cameron and Davis, 1970) found that extreme levels of anaemia compromised swimming activity, just as we saw here with the chasing protocol.

While a reasonable upper limit can be placed on the compensatory changes to anaemia for routine $\dot{Q}$ and tissue oxygen extraction, the limit to cardiac plasticity in rainbow trout is less clear. A doubling of rM_V is certainly possible with sexual maturation for male rainbow trout and rM_V has reached 0.18–0.27% (Davie and Thorarensen, 1997; Clarke et al., 2004). Also, implants of 17-α methyltestosterone induced a 70% increase in ventricular mass and a 60% increase in atrial mass of mixed sex juvenile rainbow trout [testosterone implants had a somewhat reduced effect (Davie and Thorarensen, 1997)]. Why the hearts of both sexes respond to androgenic implants and to anaemia (the present experiments also combined males and females), but only male rainbow trout hearts respond to sexual maturation is unclear. However, mature male rainbow trout are hypervolemic, have a resting bradycardia and show systolic hypotension (Clark and Rodnick, 1999). Thus, for rainbow trout, mechanical factors (including stretch and afterload), androgens and venous hypoxemia are all potential triggers for cardiac growth, but how they interact is unclear even though it has been thought that anaemia and androgens may have additive effects on cardiac remodelling (Davie and

Thorarensen, 1997). Temperature clearly has a modulating influence.

A large ventricle, high V_S and low Hct are features normally found in other fishes. The haemoglobin-free notothenioids have an exceedingly high rM_V (0.4%) (Tota et al., 1991; Axelsson, 2005), which is similar to that of small mammals. Other notothenioids such a *Pagothenia borchgrevenski* maintain a low Hct, which can double during strenuous exercise (Axelsson, 2005). Thus, in an evolutionary context, anaemia, together with low temperature, may have been contributing factors in shaping the variability seen in cardiac morphology among fishes. Recently, Sidell and O'Brien suggested that the unusual cardiorespiratory modifications seen in Antarctic fishes result from NO-stimulated morphogenesis because there is no Hb to sequester NO (Sidell and O'Brien, 2006). Experiments are currently underway to test whether NO inhibitors restrict cardiac enlargement in anaemic rainbow trout. Even so, flounder are similarly characterized by a low Hct compared with rainbow trout, ranging from 4.3–34.6% (mean 20%, mode 26%) in wild populations (Wood et al., 1979; Cech et al., 1976) and although they have an unusually large cardiac V_S, their rM_V is about half that found in rainbow trout (Joaquim et al., 2004). Clearly, much remains to be discovered regarding the factors determining genotypic and phenotypic variability in ventricular size among fishes, in addition to determining the exact triggers and extent of cardiac remodelling possible.

List of abbreviations

[Hb]	haemoglobin concentration
f_H	heart rate (min^{-1})
FL	fork length (cm)
Hct	haematocrit
M_b	body mass
PHZ	phenylhydrazine hydrochloride
$\dot{Q}$	cardiac output (ml min^{-1} kg^{-1})
rM_V	relative ventricular mass (ventricular mass:body mass ratio)
V_S	stroke volume

This research was support by the Natural Sciences and Engineering Research Council of Canada.

References

Axelsson, M. (2005). The circulatory system and its control. In *The Physiology of Polar Fishes* (*Fish Physiology Series*). Vol. 22 (ed. A. P. Farrell and J. F. Steffensen), pp. 239-280. San Diego: Academic Press.

Brill, R. W. and Bushnell, P. G. (2001). The cardiovascular system of tunas. In *Tuna: Physiology, Ecology and Evolution* (*Fish Physiology Series*). Vol. 19 (ed. B. A. Block and E. D. Stevens), pp. 79-120. San Diego: Academic Press.

Byrne, A. P. and Houston, A. H. (1988). Use of phenylhydrazine in the detection of responsive changes in haemoglobin isomorph abundances. *Can. J. Zool.* **66**, 758-762.

Cameron, J. N. and Davis, J. C. (1970). Gas exchange in rainbow trout (*Salmo gairdneri*) with varying blood oxygen capacity. *J. Fish. Res. Board Can.* **27**, 1068-1085.

Cameron, J. N. and Wohlschlag, D. E. (1969). Respiratory response to experimentally induced anemia in the pinfish (*Lagadon rhomboids*). *J. Exp. Biol.* **50**, 307-317.

Cech, J. J., Bridges, D. W., Rowell, D. M. and Blazer, P. J. (1976). Cardiovascular responses of winter flounder, *Pseudopleuronectes americanus* (Walbaum), to acute temperature increase. *Can. J. Zool.* **54**, 1383-1388.

Chudzik, J. and Houston, A. H. (1983). Temperature and erythropoiesis in goldfish. *Can. J. Zool.* **61**, 1322-1325.

Clark, R. J. and Rodnick, K. J. (1998). Morphometric and biochemical characteristics of ventricular hypertrophy in male rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **201**, 1541-1552.

Clark, R. J. and Rodnick, K. J. (1999). Pressure and volume overloads are associated with ventricular hypertrophy in male rainbow trout. *Am. J. Physiol.* **277**, R938-R946.

Clarke, J. J., Clark, R. J., McMinn, J. T. and Rodnick, K. J. (2004). Microvascular and biochemical compensation during ventricular hypertrophy in male rainbow trout. *Comp. Biochem. Physiol.* **139B**, 695-703.

Davie, P. S. and Farrell, A. P. (1991). The coronary and luminal circulations of the myocardium of fishes. *Can. J. Zool.* **69**, 1993-2001.

Davie, P. S. and Thorarensen, H. (1997). Heart growth in rainbow trout in response to exogenous testosterone and 17-α methyltestosterone. *Comp. Biochem. Physiol.* **117A**, 227-230.

Delcayre, C., Samuel, J. L., Marcotte, F., Best-Bellepomme, C., Mercardier, J. J. and Rapaport, L. (1988). Synthesis of stress proteins in rat cardiac myocytes 2-4 days after imposition of hemodynamic overload. *J. Clin. Invest.* **82**, 460-468.

Dornfest, B. S., Bush, M. E., Lapin, D. M., Adu, S., Fulop, A. and Naughton, B. A. (1990). Phenylhydrazine as a mitogen and activator of lymphoid cells. *Ann. Clin. Lab. Sci.* **20**, 353-370.

Farrell, A. P. (1984). A review of cardiac performance in the teleost heart: intrinsic and humoral regulation. *Can. J. Zool.* **62**, 523-536.

Farrell, A. P. (1991). From hagfish to tuna: a perspective on cardiac function in fish. *Physiol. Zool.* **64**, 1137-1164.

Farrell, A. P. (2002). Cardiorespiratory performance in salmonids during exercise at high temperature, insights into cardiovascular design limitations in fishes. *Comp. Biochem. Physiol.* **132A**, 797-810.

Farrell, A. P. and Clutterham, S. M. (2003). On-line venous oxygen tensions in rainbow trout during graded exercise at two acclimation temperatures. *J. Exp. Biol.* **206**, 487-496.

Farrell, A. P., Hammons, A. M., Graham, M. S. and Tibbits, G. F. (1988). Cardiac growth in rainbow trout, *Salmo gairdneri*. *Can. J. Zool.* **66**, 2368-2373.

Farrell, A. P., Simonot, D. L., Seymour, R. S. and Clark, T. D. (2007). A novel technique for estimating the compact myocardium in fish reveals surprising results for an athletic air-breathing fish, the Pacific tarpon. *J. Fish Biol.* **71**. In press.

Franklin, C. E. and Davie, P. S. (1992a). Dimensional analysis of the ventricle of an *in situ* perfused trout heat using echocardiography. *J. Exp. Biol.* **166**, 47-60.

Franklin, C. E. and Davie, P. S. (1992b). Sexual maturity can double heart mass and cardiac power output in male rainbow trout. *J. Exp. Biol.* **171**, 139-148.

Gallaugher, P. and Farrell, A. P. (1998). Haematocrit and blood oxygen-carrying capacity. In *Fish Respiration* (*Fish Physiology Series*). Vol. 17 (ed. S. F. Perry and B. Tufts), pp. 185-227. San Diego: Academic Press.

Gallaugher, P., Thorarensen, H. and Farrell, A. P. (1995). Haematocrit in oxygen transport and swimming in rainbow trout (*Oncorhynchus mykiss*). *Respir. Physiol.* **102**, 279-292.

Gamperl, A. and Farrell, A. P. (2004). Cardiac plasticity in fishes: environmental influences and intraspecfici differences. *J. Exp. Biol.* **207**, 2539-2550.

Gamperl, A., Pinder, A. and Boutlier, R. (1994). Effect of coronary ablation and adrenergic stimulation on *in vivo* cardiac performance in trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **186**, 127-143.

Gilmour, K. M. and Perry, S. F. (1996). The effects of experimental anemia on CO_2 excretion *in vitro* in rainbow trout, *Oncorhynchus mykiss*. *Fish Physiol. Biochem.* **15**, 83-94.

Goolish, E. M. (1987). Cold acclimation increases the ventricle size of carp, *Cyprinus carpio*. *J. Therm. Biol.* **12**, 203-206.

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.

Graham, M. S. and Farrell, A. P. (1992). Environmental influences on cardiovascular variables in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Biol.* **41**, 851-858.

Greer-Walker, M. and Emerson, I. (1978). Sustained swimming speeds and myotomal muscle function in the trout, *Salmo gairdneri*. *J. Fish Biol.* **13**, 475-481.

Hanson, L. M. and Farrell, A. P. (2007). The hypoxic threshold for maximum cardiac performance in rainbow trout (*Oncorhynchus mykiss*) during simulated exercise conditions at 18°C. *J. Fish Biol.* In press.

Hanson, L. M., Obradovich, S., Mouniargi, J. and Farrell, A. P. (2006). The role of adrenergic stimulation in maintaining maximum cardiac performance

in rainbow trout (*Oncorhynchus mykiss*) during hypoxia, hyperkalemia and acidosis at 10°C. *J. Exp. Biol.* **209**, 2442-2451.

Hochachka, P. W. (1961). The effect of physical training on oxygen debt and glycogen reserves in trout. *Can. J. Zool*. **127**, 565-587.

Holeton, G. F. (1971). Oxygen uptake and transport by the rainbow trout during exposure to carbon monoxide. *J. Exp. Biol.* **54**, 239-254.

Holeton, G. F. (1977). Constancy of arterial blood pH during CO-induced hypoxia. *Can. J. Zool*. **55**, 1010-1013.

Houston, A. H. and Keen, J. E. (1984). Cadmium inhibition of erythropoiesis in goldfish, *Carassius auratus*. *Can. J. Fish. Aquat. Sci.* **41**, 1819-1834.

Itano, H. A., Hosokawa, K. and Hirota, K. (1976). Induction of haemolytic anemia by substituted phenylhydrazines. *Br. J. Haematol*. **32**, 99-104.

Joaquim, N., Wagner, G. N. and Gamperl, A. K. (2004). Cardiac function and critical swimming speed of the winter flounder (*Pleuronectes americanus*) at two temperatures. *Comp. Biochem. Physiol.* **138A**, 277-285.

Jones, D. R. (1971). The effect of hypoxia and anaemia on the swimming performance of rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* **55**, 541-551.

Kent, J. D. and Prosser, C. L. (1985). Protein hypertrophy in liver and heart following cold acclimatization and acclimation in channel catfish. *Am. Zool*. **25**, 134.

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.

Magosso, E. and Ursino, M. (2004). Modeling study of the acute cardiovascular response to hypocapnic hypoxia in healthy and anaemic subjects. *Med. Biol. Eng. Comput*. **42**, 158-166.

Marinsky, C. A., Houston, A. H. and Murad, A. (1990). Effect of hypoxia on haemoglobin isomorph abundances in rainbow trout, *Salmo gairdneri*. *Can. J. Zool.* **68**, 884-888.

McClelland, G. B., Dalziel, A. C., Fragoso, N. M. and Moyes, C. D. (2005). Muscle remodeling in relation to blood supply, implications for seasonal changes in mitochondrial enzymes. *J. Exp. Biol.* **208**, 515-522.

Meerson, F. Z. and Evsevieva, M. E. (1985). Disturbances of the heart structure and function in chronic haemolytic anemia, their compensation with increased coronary flow, and their prevention with ionol, an inhibitor of lipid peroxidatoin. *Adv. Myocardiol*. **5**, 201-211.

Milligan, C. L. (1996). Metabolic recovery from exhaustive exercise in rainbow trout. *Comp. Biochem. Physiol.* **113A**, 51-60.

Murad, A., Houston, A. H. and Samson, L. (1990). Hematological response to reduced oxygen-carrying capacity, increased temperature and hypoxia in goldfish, *Carassius auratus* L. *J. Fish Biol*. **36**, 289-305.

Perry, S. and Gilmour, K. (1996). Consequences of catecholamine release on ventilation and blood oxygen transport during hypoxia and hypercapnia in an elasmobranch *Squalus acanthias* and a teleost *Oncorhynchus mykiss*. *J. Exp. Biol.* **199**, 2105-2118.

Perry, S., Reid, S. and Salama, A. (1996). The effects of repeated physical stress on the β-adrenergic response of the rainbow trout red blood cell. *J. Exp. Biol.* **199**, 549-562.

Poupa, O. and Carlsten, A. (1973). Experimental cardiomyopathies in poikilotherms. *Recent Adv. Stud. Cardiac Struct. Metab*. **2**, 321-351.

Poupa, O., Gesser, H., Jonsson, S. and Sullivan, L. (1974). Coronary-supplied compact shell of ventricular myocardium in salmonids: growth and enzyme pattern. *Comp. Biochem. Physiol*. **48A**, 85-95.

Santer, R. M. (1985). Morphology and innervation of the fish heart. *Adv. Anat. Embryol. Cell Biol*. **89**, 1-99.

Sidell, B. D. and O'Brien, K. D. (2006). When bad things happen to good fish: the loss of haemoglobin and myoglobin expression in Antarctic icefishes. *J. Exp. Biol.* **209**, 1791-1802.

Smith, C. E., McLain, L. R. and Zaugg, W. S. (1971). Phenylhydrazine-induced anemia in chinook salmon. *Toxicol. Appl. Pharm*. **20**, 73-81.

Smith, F. M. and Jones, D. R. (1982). The effect of changes in blood oxygen carrying capacity on ventilation volume in the rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* **97**, 325-334.

Smits, J. F., Cleutjens, J. P., van Krimpen, C., Schoemaker, R. G. and Daemen, M. J. (1991). Cardiac remodeling in hypertension and following myocardial infarction: effects of arteriolar vasodilators. *Basic Res. Cardiol*. **86**, 133-139.

Taylor, S. E., Egginton, S. and Taylor, E. W. (1996). Seasonal temperature acclimatization of rainbow trout: cardiovascular and morphometric influences on maximal sustainable exercise level. *J. Exp. Biol.* **199**, 835-845.

Tota, B. (1983). Vascular and metabolic zonation in the ventricular myocardium of mammals and fishes. *Comp. Biochem. Physiol*. **76A**, 423-427.

Tota, B., Cimini, V., Salvatore, G. and Zummo, G. (1983). Comparative study of the arterial and lacunary systems of the ventricular myocardium of elasmobranch and teleost fishes. *Am. J. Anat*. **167**, 15-32.

Tota, B., Agnisola, C., Schioppa, M., Acierno, R., Harrison, P. and Zummo, G. (1991). Structural and mechanical characteristics of the heart of the icefish *Chionodraco hamatus* L. In *Biology of Antarctic Fishes* (ed. G. Di Prisco, B. Maresca and B. Tota), pp. 204-219. Berlin, Heidelberg, New York: Springer-Verlag.

Tsukuda, H., Liu, B. and Fujii, K.-I. (1985). Pulsation rate and oxygen consumption of isolated hearts of the goldfish, *Carassius auratus*, acclimated to different temperatures. *Comp. Biochem. Physiol*. **82A**, 281-283.

Tun, N. and Houston, A. H. (1986). Temperature, oxygen, photoperiod, and the haemoglobin system of the rainbow trout, *Salmo gairdneri*. *Can. J. Zool*. **64**, 1883-1888.

Wells, R. M. G. and Weber, R. E. (1991). Is there an optimal haematocrit for rainbow trout, *Oncorhynchus mykiss* (Walbaum). An interpretation of recent data based on blood viscosity measurements. *J. Fish Biol*. **38**, 53-65.

Wood, C. M., McMahon, B. R. and McDonald, D. G. (1979). Respiratory, ventilatory, and cardiovascular responses to experimental anemia in the starry flounder, *Platichthys stellatus*. *J. Exp. Biol.* **82**, 139-162.