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The effects of sustained exercise and hypoxia upon oxygen tensions in the red muscle of rainbow trout

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Summary

Teleost fish possess discrete blocks of oxidative red muscle (RM) and glycolytic white muscle, whereas tetrapod skeletal muscles are mixed oxidative/glycolytic. It has been suggested that the anatomy of RM in teleost fish could lead to higher intramuscular O_2 partial pressures (P_{O_2}) than in mammalian skeletal muscles. This study provides the first direct experimental support for this suggestion by using novel optical fibre sensors to discover a mean (± S.E.M., N=6) normoxic steady-state red muscle P_{O_2} ($P_{RM O_2}$) of 61±10 mmHg (1 mmHg=133.3 Pa) in free-swimming rainbow trout *Oncorhynchus mykiss*. This is significantly higher than literature reports for mammalian muscles, where the P_{O_2} never exceeds 40 mmHg. Aerobic RM powers sustained swimming in rainbow trout. During graded incremental exercise, $P_{RM O_2}$ declined from 62±5 mmHg at the lowest swim speed down to 45±3 mmHg at maximum rates of aerobic work, but then rose again to 51±5 mmHg at exhaustion. These measurements of $P_{RM O_2}$ during exercise indicated, therefore, that O_2 supply to the RM was not a major limiting factor at exhaustion in trout. The current study found no evidence that teleost haemoglobins with a Root effect cause extremely elevated O_2 tensions in aerobic tissues. Under normoxic conditions, $P_{RM O_2}$ was significantly lower than arterial P_{O_2} (119±5 mmHg), and remained lower when the arterial to tissue P_{O_2} gradient was reduced by exposure to mild hypoxia. When two sequential levels of mild hypoxia (30 min at a water P_{O_2} of 100 mmHg then 30 min at 75 mmHg) caused Pa_{O_2} to fall to 84±2 mmHg then 61±3 mmHg, respectively, this elicited simultaneous reductions in $P_{RM O_2}$, to 51±6 mmHg then 41±5 mmHg, respectively. Although these hypoxic reductions in $P_{RM O_2}$ were significantly smaller than those in Pa_{O_2}, the effect could be attributed to the sigmoid shape of the trout haemoglobin–O_2 dissociation curve.

Key words: O_2-sensitive optode, Root effect, O_2 partial pressure, arterial blood O_2 content, O_2 consumption, swimming.

Introduction

The anatomy of the skeletal musculature in teleost fish differs significantly from that of the tetrapod vertebrates. Teleosts possess distinct blocks of highly vascularised oxidative slow-twitch fibres ('red' muscle, RM), arranged alongside blocks of less vascularised glycolytic fast-twitch fibres ('white' muscle, WM), whereas tetrapod muscles all comprise a mixture of oxidative slow-twitch and glycolytic fast-twitch fibres (Bone, 1978; Young, 1981). The different anatomical arrangement of the oxidative RM of teleost fish has led to the suggestion that the partial pressures of oxygen (P_{O_2}) in their red muscle fibres ($P_{RM O_2}$) may be significantly higher than typically found in skeletal muscles of mammals (Egginton, 2002). Recent measurements of the P_{O_2} in various skeletal muscles of mammals never seem to exceed approximately 40 mmHg under resting conditions in normoxia (Hutter et al., 1999; Jung et al., 1999; Behnke et al., 2001; Suttner et al., 2002). Although arterial and venous blood $P_{RM O_2}$ values are known for teleost fishes such as the rainbow trout *Onchorhynchus mykiss* (Holeton and Randall, 1967; Stevens and Randall, 1967; Kiceniuk and Jones, 1977; Thomas and Hughes, 1982; Thomas et al., 1987; Farrell and Clutterham, 2003), we are unaware of any $P_{RM O_2}$ measurements that have tested this prediction. In fact, measurements of the P_{O_2} of muscle appear to be limited to those of Jankowsky (1966), who reported a very low value of <5 mmHg in the glycolytic WM of eels (*Anguilla* sp.).

Measurements of O_2 tensions in the skeletal musculature of teleost fish would be particularly informative for two other reasons. One of these is to investigate the extent to which convective O_2 supply might be a limiting factor in the

performance of sustained aerobic exercise. During sustained exercise in tetrapods, increased muscle O_2 demand relative to rates of supply causes a reduction in muscle P_{O_2} (Jung et al., 1999; Behnke et al., 2001), and fatigue is associated with a severe decline in intramuscular O_2 tension (Molé et al., 1999; Howlett and Hogan, 2001). Fish support sustained swimming activity with their RM, while WM powers the faster, unsteady sprint and burst swimming activities (Bone, 1978). Therefore, measurements of P_{RMO_2} during swimming would provide insight into whether RM O_2 supply is a factor limiting the performance of sustained swimming, that is, whether exhaustion is associated with a profound decline in P_{RMO_2}.

Another reason why P_{RMO_2} of teleosts might be particularly interesting relates to a unique characteristic of some teleost haemoglobins, the Root effect (Root, 1931). When blood pH drops, haemoglobins with a Root effect exhibit a markedly reduced capacity to bind O_2, and hence will release bound O_2 (Root, 1931; Randall, 1998; Pelster and Randall, 1998). A well established physiological role for the Root effect is found in specialised vascular beds (retes), where high rates of lactic acid and CO_2 production by specialised cells generate low pH, resulting in localised P_{O_2} values that are considerably higher than in arterial blood leaving the gills, due to unloading of O_2 from haemoglobin. In particular, the choroid rete ensures that photoreceptors in the retina are well oxygenated, while the rete mirabilis provides O_2 to inflate the swimbladder and maintain buoyancy as fish descend in the water column (Jensen et al., 1998; Pelster and Randall, 1998).

Theoretically, the Root effect may also promote the release of O_2 from haemoglobin at other respiring tissues. This is because *in vitro* evidence shows that diffusion of respiratory CO_2 into the teleost erythrocyte, and its carbonic anhydrase-catalysed hydration to HCO_3^- and H^+, occurs more rapidly than diffusional release of O_2 from haemoglobin in response to a P_{O_2} gradient (Brauner and Randall, 1998; Pelster and Randall, 1998). Consequently, a transient drop in erythrocyte pH following the catalysed hydration of CO_2 could elicit a Root effect and generate high P_{O_2} values in well-vascularised aerobic tissues. The presence and extent of this effect in tissues other than retes, such as RM, has not been studied. If measurements of P_{RMO_2} revealed that it was higher than the P_{O_2} of arterial blood leaving the gills (P_{aO_2}), then this would be dramatic evidence that the Root effect influences O_2 tensions in aerobic tissues.

In the current study, novel O_2-sensitive optical fibre sensors ('micro-optodes') were used to measure the P_{RMO_2} of conscious free-swimming rainbow trout, a species with a pronounced Root effect (Binotti et al., 1971). Measurements were made under three regimes: during normoxia, to compare with data reported for mammalian skeletal muscles (Hutter et al., 1999; Jung et al., 1999; Suttner et al., 2002; Behnke et al., 2001) and to investigate whether the Root effect contributes to elevated P_{RMO_2}; during graded sustained exercise, to gain insights into RM O_2 supply during an increase in demand, and also during mild hypoxia, to investigate whether reducing the arterial-to-tissue P_{O_2} gradient would expose an impact of the Root effect upon P_{RMO_2}.

Materials and methods

Experimental animals

Rainbow trout *Oncorhynchus mykiss* Walbaum with a mean (± S.D.) mass of 697±152 g and fork length of 36±2 cm, were transported from Sun Valley Trout Farm (Mission, BC, Canada) to Simon Fraser University, where they were held outside in 1000 l circular fibreglass tanks provided with a flow of fresh water at seasonal temperatures of 13–15°C (mean temperature 13.8±0.4°C). Fish were acclimated to these conditions for at least 2 weeks, and fed daily. Individual trout were starved for 24 h prior to surgery.

Surgical preparation and measurement of red muscle P_{O_2}

Fish were anaesthetised in 0.1 mg l^{-1} MS-222 buffered with 0.1 mg l^{-1} $NaHCO_3$, and then transferred to an operating table where their gills were irrigated with aerated water containing diluted anaesthetic (0.05 mg l^{-1} MS-222 and $NaHCO_3$). A small incision was made in the skin just dorsal to the lateral line to reveal the underlying RM sheet. A blunted surgical needle (15G Terumo, Leuven, Belgium) was then advanced under the skin for approximately 1 cm, with the foremost end of the blunted needle bevel against the underside of the skin. Great care was taken to avoid penetrating the underlying musculature. An oxygen-sensitive optical chemical fibre sensor (PreSens; Precision Sensing GmbH, Regensburg, Germany), with a tapered Teflon-coated tip (diameter <10 μm), was inserted into the bore of the needle and advanced until the tip reached the end of the needle. The needle was then angled at approximately 45° to the skin such that the bevelled end rested flat against the musculature and the tip of the optode advanced gently, at the prevailing angle of 45°, for approximately 3 mm into the underlying sheet of RM. The needle was then withdrawn along the optode lead, and the optode secured in position with sutures to the skin. Trout were then cannulated in the dorsal aorta (DA) using the technique described by Soivio et al. (1975).

While the trout were still under anaesthesia, the optode was connected to a Microx 1 oxygen meter (PreSens), connected in turn *via* a serial port to a PC with dedicated software, which displayed P_{RMO_2} at the optode tip every 1 s and saved a measure of P_{RMO_2} every 1 min in an ASCII file. Prior to surgery, each optode was calibrated in oxygen-free and air-saturated water, and the tip soaked for 10 min in 100 i.u. ml^{-1} heparin (Farrell and Clutterham, 2003). The position of the probe in the RM was confirmed *post-mortem* by careful dissection under a binocular microscope. Data are reported only for those experiments where the probe could be recalibrated, *post-mortem*, to correct for any drift, according to the manufacturer's instructions. In one case where blood clotting and tissue damage were visible around the tip of the probe, the results were disregarded.

Fish were recovered for approximately 42 h in normoxic

water while swimming gently at a speed equivalent to 0.5 body lengths s^{-1} (BL s^{-1}) in the Brett-type swimming respirometer described in Gallaugher et al. (1995), and P_{RMO_2} was measured every 1 min throughout. The DA cannula was flushed every 24 h with heparinised (10 i.u. ml^{-1}) teleost saline. Measurements of control normoxic P_{RMO_2} values were made while the animals were swimming gently so as to establish a constant level of muscular work and consequent O_2 demand and to reduce spontaneous changes in activity level, thus minimising variability in P_{RMO_2} (see Fig. 1).

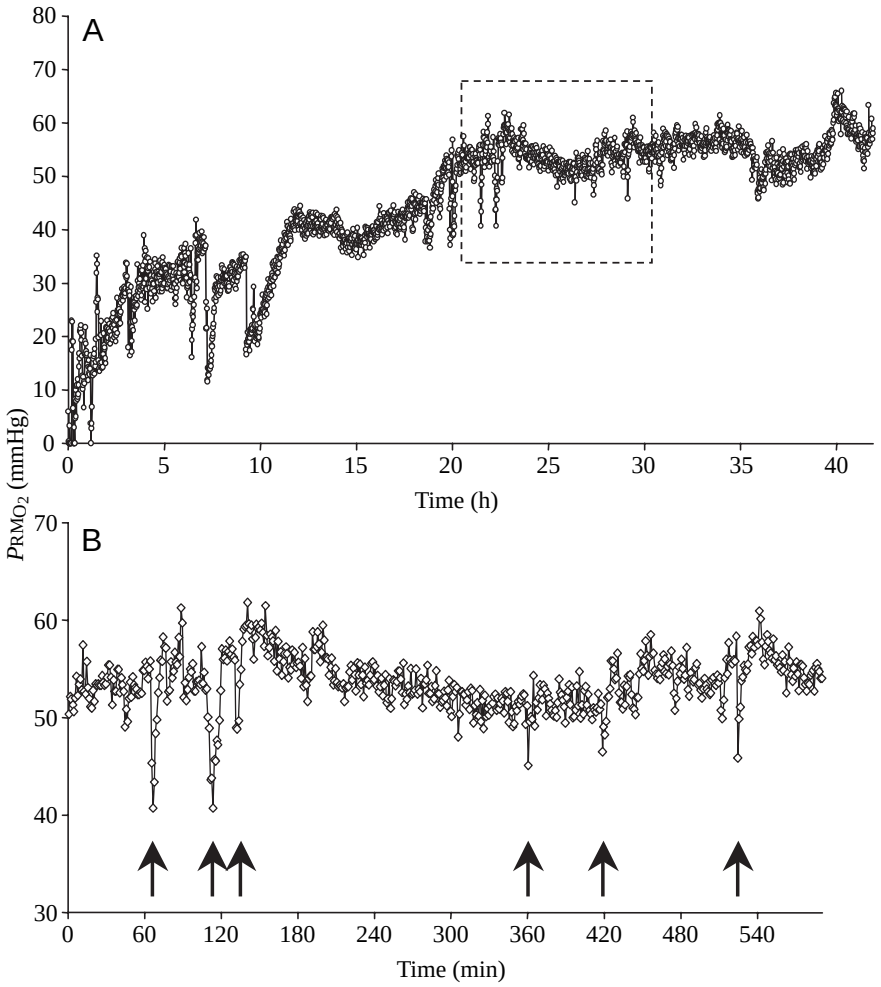

Fig. 1. (A) Representative trace of red muscle P_{O_2} (P_{RMO_2}) in a rainbow trout as measured every 1 min during approximately 42 h recovery from implantation of the micro-optode probe under anaesthesia. (B) Expanded view of the dotted box in A, with arrows indicating where the fish was observed to struggle violently in the respirometer.

Sustained exercise

Exercise performance was measured by exposing the fish to 0.5 BL s^{-1} increments in swimming speed every 30 min until fatigue. Maximum sustainable swimming speed (U_{crit}) was calculated as described by Brett (1964). The P_{RMO_2} was measured every 1 min throughout, while P_{aO_2}, arterial blood total O_2 content (C_{aO_2}) and arterial blood pH (pHa) were measured once at each swimming speed, at fatigue, and at 1 h and 2 h post-fatigue. The P_{aO_2} was measured by gently withdrawing blood along the DA catheter and into a glass cuvette (D616, Radiometer, Copenhagen, Denmark) containing an oxygen electrode (Radiometer E5046), thermostatted to the experimental temperature, with the signal displayed on a Radiometer PHM72 acid–base analyser. A subsample of this arterial blood was withdrawn (300 μl) and C_{aO_2} measured as described by Tucker (1967) using a Radiometer O_2 electrode thermostatted to 37°C, and pHa measured using a Radiometer BMS2 capillary pH electrode thermostatted to the same water temperature as the fish, with the signals displayed on a Radiometer PHM73 acid–base analyser. The remaining blood, plus 300 μl of saline, was returned to the animal. Water P_{O_2} (P_{wO_2}) was monitored continually using an oxygen-sensitive galvanic cell and associated meter (HO1G, Oxyguard, Birkerød, Denmark) with the signal displayed on a chart-recorder. The P_{wO_2} recording was used to measure oxygen consumption by the fish ($\dot{M}_{O_2}$, in mg kg^{-1} h^{-1}) in the sealed respirometer over 20 min at each swimming speed, then for a 30 min period centred around 1 h and 2 h recovery, using the techniques described in Gallaugher et al. (1995). For the analysis of the effects of exercise, mean values were derived for the measured variables under control conditions (i.e. exercising gently at 0.5 BL s^{-1}); for fish swimming at a common degree of sustained exercise (1 BL s^{-1}); for the maximum speed which the fish were able to sustain for a complete 30 min measurement interval (this ranged from 1 to 1.5 BL s^{-1}); immediately at exhaustion; and then at 1 h and 2 h of recovery. An indication of changes in blood O_2 supply during aerobic exercise was obtained by resolving the Fick equation:

$$\dot{M}_{O_2} = D_{O_2} \times (P_{aO_2} - P_{RMO_2}) , \quad (1)$$

where D_{O_2} is an index of rates of blood O_2 delivery. This index was resolved with the values of $\dot{M}_{O_2}$, P_{aO_2} and P_{RMO_2} measured at the lowest swim speed (0.5 BL s^{-1}) and then compared with those measured at maximum rates of oxygen uptake.

Exposure to hypoxia

While swimming gently at a speed of 0.5 BL s^{-1}, the trout were exposed to two levels of mild hypoxia, comprising 30 min at 100 mmHg, followed by 30 min at 75 mmHg, followed by 1 h recovery to normoxia (140 mmHg). The P_{RMO_2} was monitored every 1 min throughout the exposure

protocol. Water P_{O_2} was monitored continually, and water entering the respirometer made hypoxic by passing it counter-current to a flow of compressed 100% N_2 in a gas-exchange column. The $P_{W O_2}$ recording was used to measure $\dot{M}_{O_2}$ in the sealed respirometer for 30 min in normoxia, for 30 min at both levels of hypoxia, and for 30 min centred upon 1 h recovery to normoxia. A measurement of $P_{a O_2}$ was made every 5 min by gently withdrawing blood along the DA catheter and into the O_2 electrode cuvette. Samples of arterial blood (300 µl, replaced immediately with an equal volume of saline) were collected from the DA cannula in normoxia, at 30 min exposure to each level of hypoxia, and following 1 h recovery to normoxia, to measure $C_{a O_2}$ and pHa.

The Hb–O_2 dissociation curve derived for rainbow trout at 14°C by Farrell and Clutterham (2003) was used to identify the percentage haemoglobin saturations that would prevail in blood at the P_{O_2} measured in the dorsal aorta and in the RM, in normoxia and at each level of hypoxia. The total O_2 content of blood in the RM ($C_{RM O_2}$, in mmol ml^{-1}) was then estimated as follows:

$$C_{RM O_2} = [\text{Hb sat RM} / \text{Hb sat DA}] \times C_{a O_2}\,, \qquad (2)$$

where 'Hb sat RM' and 'Hb sat DA' are the percentage saturations of haemoglobin in the red muscle and dorsal aorta, respectively. $C_{a O_2}$–$C_{RM O_2}$ is then an estimate of the amount of O_2 released between the DA and RM. It was assumed that, if the Root effect was causing $P_{RM O_2}$ to be high, then these estimates of apparent 'O_2 unloading' would decline drastically as $P_{a O_2}$ fell in hypoxia, in a manner that could not be accounted for by the simultaneous measurements of whole-animal $\dot{M}_{O_2}$.

Data analysis and statistics

One-way analysis of variance (ANOVA) for repeated measures was used to reveal effects of exercise or hypoxia on any single variable. A two-way repeated-measures ANOVA was used to assess the effects of progressive hypoxia on $P_{a O_2}$ *versus* $P_{RM O_2}$. Where changes in P_{O_2} were expressed as a percentage of the normoxic value, data were arc-sine transformed prior to analysis by ANOVA. In all cases, Bonferroni *post-hoc* tests were used to identify where significant differences lay. A probability of less than 5% (P<0.05) was taken as the fiducial level for statistical significance.

Results

Characteristics of red muscle P_{O_2} *in normoxia*

In all fish, $P_{RM O_2}$ was close to zero under anaesthesia, but gradually rose over a few hours during recovery (Fig. 1) and, at full recovery, was approximately 60 mmHg (Table 1). Under steady state normoxia, mean $P_{RM O_2}$ was significantly lower than both mean $P_{W O_2}$ and $P_{a O_2}$ (Table 1). There was variability in normoxic $P_{RM O_2}$ among fish, ranging from a low of 39 mmHg to a high of 101 mmHg, but no fish exhibited a higher P_{O_2} in their muscle than in either their inspired water or arterial blood. Therefore, there was no dramatic evidence that the Root effect influenced $P_{RM O_2}$. In contrast to the stable $P_{RM O_2}$ observed while fish were swimming steadily during normoxia, sharp reductions in $P_{RM O_2}$ occurred if the animal struggled in the respirometer, followed by a gradual return to the previous P_{O_2} (Fig. 1), but $P_{RM O_2}$ never decreased below ~40 mmHg.

Table 1. *Partial pressures of O_2 in the red muscle and arterial blood of rainbow trout under control normoxic conditions, and the effects of reducing water* P_{O_2} *in mild hypoxia*

	$P_{W O_2}$ (mmHg)		
	140 (normoxia)	100	75
$P_{RM O_2}$ (mmHg)	61±10[a]	51±6[b]	41±5[c]
$P_{a O_2}$ (mmHg)	119±5[d]	84±2[e]	61±3[a,b]
$P_{a O_2}$–$P_{RM O_2}$ (mmHg)	58±10[a]	33±6[b]	20±4[c]
Change in $P_{RM O_2}$ from normoxia (%)	–	–13±7[a]	–29±8[b]
Change in $P_{a O_2}$ from normoxia (%)	–	–29±3[b]	–48±3[c]

$P_{RM O_2}$, partial pressure of O_2 in the red muscle; $P_{a O_2}$, partial pressure of O_2 in arterial blood; $P_{W O_2}$, water P_{O_2}.

All values are means ± S.E.M., N=6.

The $P_{RM O_2}$ value for each fish, used to derive the mean value shown here, was calculated as the mean of 30 measurements made every 1 min during either normoxia or either level of hypoxia, whereas the $P_{a O_2}$ for each fish was calculated as the average of two measurements, made at the beginning and end of the 30 min period (see text for further details).

The $P_{a O_2}$–$P_{RM O_2}$ is the partial pressure gradient between arterial blood and the RM.

For the $P_{a O_2}$ and $P_{RM O_2}$ data, a common superscript indicates no significant difference by two-way repeated-measures ANOVA with Bonferroni *post-hoc* comparisons amongst means. The same ANOVA was performed on the % change data, following their arc-sine transformation.

For the $P_{a O_2}$–$P_{RM O_2}$ data, a common superscript indicates no significant difference by one-way repeated-measures ANOVA with Bonferroni *post-hoc* comparisons amongst means.

In all cases, significance was attributed at P<0.05.

Effects of sustained exercise

As expected, exercise caused an exponential increase in O_2 uptake, and the maximum rate of $\dot{M}_{O_2}$ was observed at the maximum speed which the fish were able to sustain for a complete 30 min interval (Fig. 2). Exercise also caused a decline in pHa, particularly at U_{crit} (Fig. 2), which is evidence of a switch to glycolytic metabolism prior to exhaustion. Nevertheless, trout in the current study did not exercise exceptionally well, reaching a U_{crit} of 1.38±0.16 *BL* s^{-1} (N=5), which is lower than the U_{crit} of approximately 2.0 *BL* s^{-1} reported earlier for chronically instrumented rainbow trout at the same temperature (e.g. Shingles et al., 2001; Farrell and Clutterham, 2003). Arterial blood P_{O_2} also showed a significant reduction during sustained exercise and at exhaustion, but recovered rapidly, unlike both $\dot{M}_{O_2}$ and pHa, which slowly returned towards control values during 2 h of recovery. Despite the arterial acidosis, $C_{a O_2}$ was unchanged throughout exercise and at exhaustion (Fig. 2).

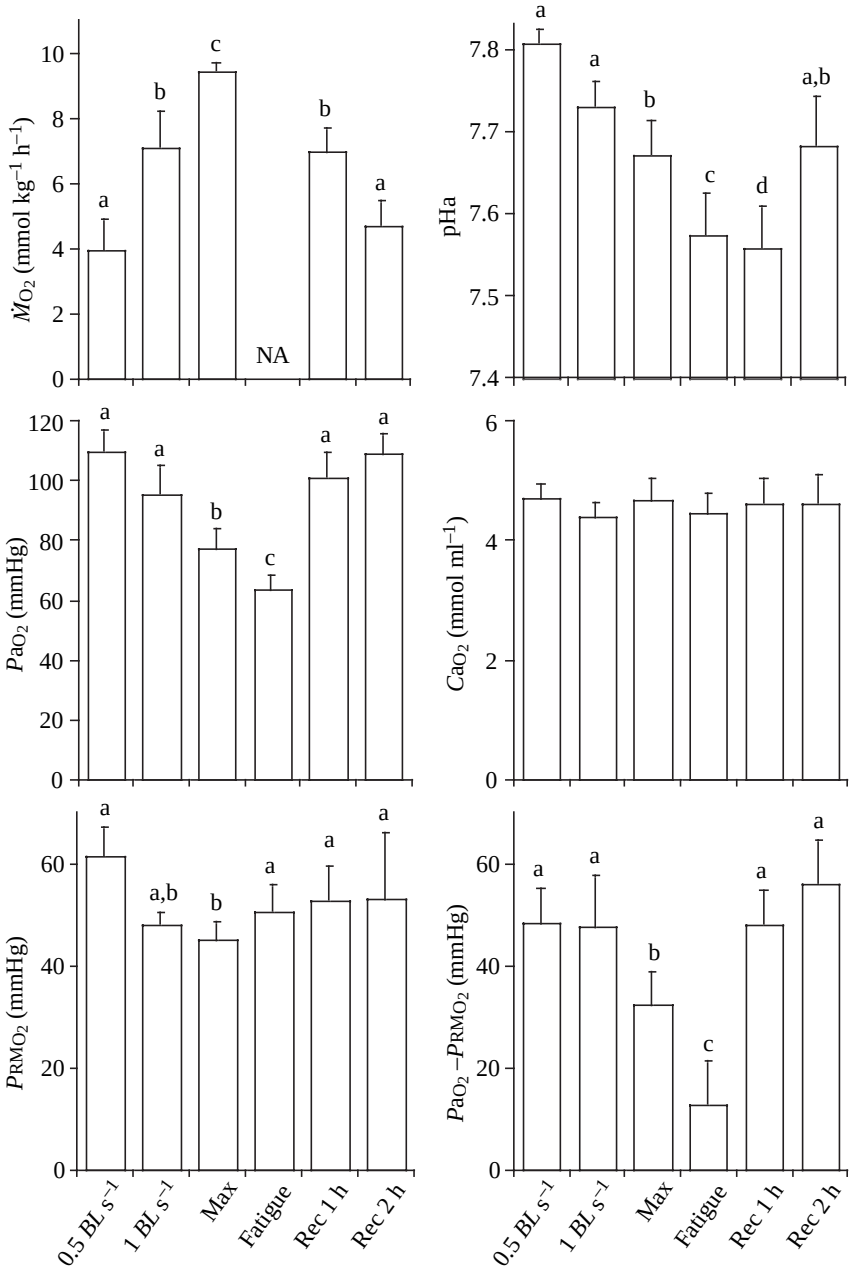

Fig. 2. Effects of sustained exercise, fatigue, and subsequent recovery, on rates of O_2 uptake ($\dot{M}_{O_2}$; mean ± S.E.M.); arterial blood pH (pHa), P_{O_2} (Pa_{O_2}) and total O_2 content (Ca_{O_2}); red muscle P_{O_2} (P_{RMO_2}), and the arterial to red muscle P_{O_2} gradient (Pa_{O_2}–P_{RMO_2}). Data are provided for rainbow trout swimming at two sustained speeds in body lengths s^{-1} (BL s^{-1}); at their maximum sustained swimming speed (Max); immediately upon fatigue (note that $\dot{M}_{O_2}$ data are not available for this instance), and at 1 h and 2 h of recovery (Rec). NA, data not available. N=5 in all cases, for those variables where exercise elicited statistically significant effects by one-way ANOVA for repeated variables, a common superscript indicates no significant difference between means by Bonferroni *post-hoc* test (P<0.05).

During sustained exercise, P_{RMO_2} showed a significant drop and although the mean value remained above 40 mmHg, P_{RMO_2} never exceeded Pa_{O_2}. Moreover, P_{RMO_2} rose significantly at the moment of exhaustion to a level that was not statistically different from the control, and remained thus for the ensuing 2 h recovery period. The partial pressure gradient between arterial blood and the RM dropped as exercise intensity increased, to a low at fatigue, but then returned rapidly to control values during recovery (Fig. 2). Given that the partial pressure gradient dropped as $\dot{M}_{O_2}$ increased during exercise, resolution of the Fick equation revealed that O_2 delivery, hence blood flow, to the red muscle would have to increase by a factor of 4.6±1.1 times (mean ± S.E.M., N=5) between swimming speeds of 0.5 *BL* s^{-1} and 1.38 *BL* s^{-1}.

Effects of exposure to hypoxia

Mild hypoxia had no significant effects on $\dot{M}_{O_2}$ and Ca_{O_2} (Table 2), so it could be assumed that rates of tissue O_2 demand and blood O_2 transport capacity did not change significantly. Furthermore, the absence of any changes in pHa indicates that there was no major increase in the release of lactic acid and CO_2 from the tissues (Table 2). Thus, the most significant effect of mild hypoxia was a decrease in the P_{O_2} of arterial blood as it left the gills (Table 1). Correspondingly, the P_{RMO_2} showed close temporal sensitivity to changes in Pw_{O_2} and Pa_{O_2} during exposure to hypoxia and the return to normoxia (Fig. 3). Red muscle P_{O_2} was significantly reduced from normoxic values at both levels of hypoxia (Pw_{O_2}=100 mmHg and 75 mmHg), but changes in P_{RMO_2} were significantly less than those in Pa_{O_2}, and so the arterial to RM P_{O_2} gradient declined as hypoxia deepened (Fig. 3, Table 1). Proportional (%) changes in P_{O_2}, relative to normoxic values, were much more pronounced than the net changes in the RM but, nonetheless, were significantly smaller in the RM than in the arterial blood (Fig. 3, Table 1). At no time during either hypoxia or recovery did any fish exhibit a higher P_{RMO_2} than their Pa_{O_2}. In fact, the estimates of apparent O_2 unloading did not decrease as hypoxia deepened but, rather, increased slightly (Table 2).

Discussion

Characteristics of red muscle P_{O_2} *in normoxia*

Farrell and Clutterham (2003) used the same micro-optodes to measure mixed venous P_{O_2} (Pv_{O_2}) in the ductus Cuvier of rainbow trout at a similar temperature. They found that Pv_{O_2} declined to 20 mmHg immediately after surgery, and recovered to a steady-state value of approximately 35 mmHg

Table 2. *Trout oxygen uptake, arterial blood O_2 content, arterial pH and apparent rates of blood O_2 unloading between the dorsal aorta and the red muscle, as a function of water* P_{O_2}

	P_{wO_2} (mmHg)		
	140 (normoxia)	100	75
$\dot{M}_{O_2}$ (mmol kg^{-1} h^{-1})	4.76±0.79	5.11±0.90	4.63±0.64
Ca_{O_2} (mmol ml^{-1})	4.76±0.45	4.41±0.36	4.20±0.35
pHa	7.82±0.02	7.81±0.03	7.84±0.03
Apparent O_2 unloading (mmol ml^{-1})	1.46±0.37	1.80±0.29	2.26±0.37

$\dot{M}_{O_2}$, rate of oxygen uptake; Ca_{O_2}, arterial blood O_2 content; pHa, arterial pH; PwO_2, water P_{O_2}.

All values are mean ± S.E.M., N=6.

Apparent O_2 unloading refers to the decline in total O_2 content between blood in the dorsal aorta and in the RM, calculated as described in the text. There was no significant effect of hypoxia upon any variable.

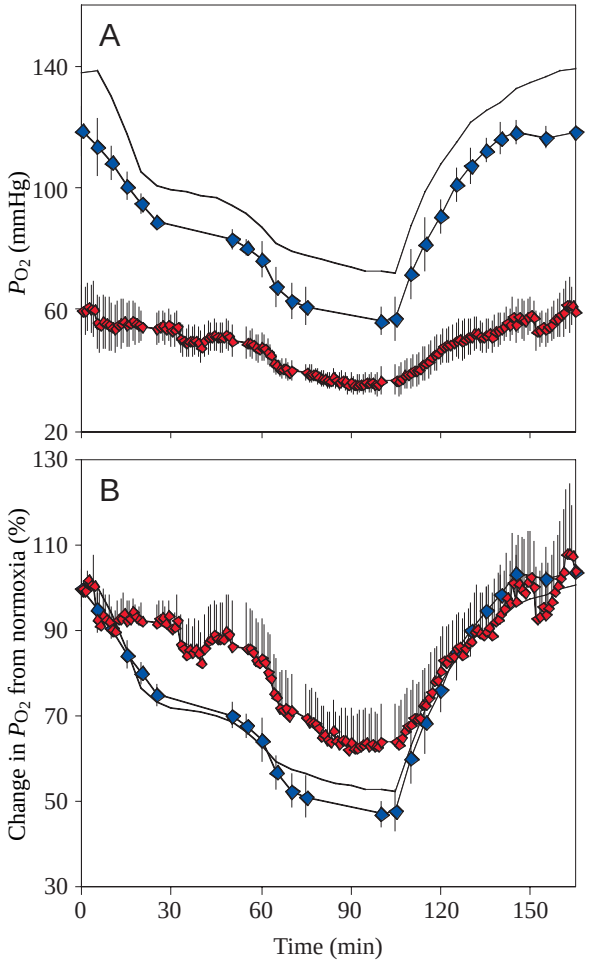

Fig. 3. (A) Temporal changes (mean ± S.E.M.) in red muscle P_{O_2} (P_{RMO_2}, red diamonds) and arterial blood P_{O_2} (Pa_{O_2}, blue diamonds) in rainbow trout during exposure to mild hypoxia and recovery to normoxia (water P_{O_2}, P_{wO_2}, shown as the simple line). (B) Percentage changes (mean ± S.E.M.) in P_{RMO_2} (blue diamonds), Pa_{O_2} (red diamonds) and P_{wO_2} (simple line), from their respective normoxic values, over the same period. N=6 in all cases.

within 30 min. These measurements of P_{vO_2} are very different from our measurements for RM, where P_{RMO_2} declined almost to zero during surgery and, although it then rose rapidly during the first few hours of recovery, it did not achieve a steady-state value for approximately 20 h. This result clearly shows that the RM muscle can become severely hypoxic during deep anaesthesia and the slow recovery of P_{RMO_2} may reflect reduced distribution of blood to the RM, as a consequence of post-surgical cardiac depression and decreased total peripheral resistance, and/or increased muscle O_2 demand, perhaps to metabolise the anaesthetic or repay an O_2 debt.

Farrell and Clutterham (2003) also found that P_{vO_2} dropped precipitously to around 20 mmHg whenever the fish struggled, and attributed this to sudden increases in muscle O_2 extraction. The sharp reductions in P_{RMO_2} that were observed when fish struggled could be due either to increased O_2 demand and extraction, or to a decrease in local blood flow associated with struggling behaviours. The latter may be the main contributing factor, as struggling behaviours are associated with bradycardia and reduced cardiac output (Stevens et al., 1972; Farrell, 1982; Farrell and Jones, 1992), and would also result in hypoperfusion if increased intramuscular pressure compresses the supplying segmental arteries. Even so, the fact that mean P_{RMO_2} did not decrease below ~40 mmHg is a novel finding indicating that RM remained well supplied with oxygen during spontaneous struggling behaviours in rainbow trout. These observations suggest that the previously observed precipitous decrease in P_{vO_2} (Farrell and Clutterham, 2003) during struggling is driven by tissues in addition to RM, the most likely candidate being the WM.

Another novel finding of the present study is that the P_{RMO_2} of approximately 60 mmHg measured in the free-swimming normoxic trout is appreciably higher than the P_{O_2} of 2–4 mmHg measured with microelectrodes in the WM of eels (Jankowsky, 1966). Unfortunately, this earlier study does not detail exactly how the probes were implanted and whether the eels were conscious during subsequent measurements (Jankowsky, 1966). Therefore, given our observation of a low P_{RMO_2} during anaesthesia, further studies with WM are warranted to confirm this difference between RM and WM. In contrast, it is very clear that the normoxic P_{RMO_2} in rainbow trout is significantly higher than in the skeletal muscle of mammals, where P_{O_2} values measured with implanted microelectrodes range from 25 mmHg to 35 mmHg in conscious humans *Homo sapiens* (Jung et al., 1999; Suttner et al., 2002) and dogs *Canis canis* (Hutter et al., 1999). Similar values were obtained in anaesthetised rats *Rattus norvegicus*, using phosphorescence quenching techniques (Behnke et al., 2001). In view of this difference, we provide the first direct evidence to support the earlier suggestions by Egginton (2002) that the anatomy and

physiology of RM in teleost fish could lead to an elevated P_{O_2} compared with mammals.

If the P_{O_2} in respiring tissues is determined by the rate at which O_2 is supplied in the blood, the distance and speed it diffuses, and the rate at which the tissue consumes it (Egginton, 2002), then a comparison of these variables between teleost RM and skeletal muscles of mammals might provide insight into why P_{RMO_2} is so high. Mass-specific blood flow rates to trout RM may be up to twice the level reported for mammalian skeletal muscles at rest, although they are similar during exercise (Egginton, 1987, 2002; Taylor et al., 1996). Rainbow trout haemoglobin has a similar affinity for O_2 to that of, for example, humans and rats (Wilmer et al., 2000). Egginton (2002) calculated and compared the mean geometric supply area (domain of influence) as well as the mean Krogh's diffusion distance for capillaries in the tibialis anterior (TA) of both rats and Syrian hamsters *Mesocritus auratus versus* those in the RM of both rainbow trout and striped bass *Morone saxatilis*. In these two teleosts, domains and diffusion distances were approximately 20% smaller than in the hamster, whereas rat domains were approximately eightfold larger and diffusion distances approximately twofold larger than in the other three species (Egginton, 2002). Thus, the higher blood flow and smaller capillary domains clearly favour a higher P_{O_2} in the red muscle of the rainbow trout relative to the skeletal muscles of the rat (Behnke et al., 2001). The lower body temperature of the fish, however, will lead to a significant reduction in O_2 diffusivity, an effect that is only partially offset by a concurrent reduction in tissue O_2 consumption (Taylor et al., 1997; Egginton, 2002).

Insights into red muscle O_2 supply during graded exercise

Our measurements of P_{RMO_2} in fish during graded exercise, at exhaustion and during recovery are also novel. Furthermore, it is evident that they contrast with results in exercising mammals. In both conscious humans and the anaesthetised rat, sustained exercise reduces intramuscular P_{O_2} from around 30 mmHg to below 20 mmHg, a change that is attributed to increased rates of O_2 extraction by the working muscle (Jung et al., 1999; Behnke et al., 2001). The significant decrease in P_{RMO_2} during sustained exercise in the current study presumably occurred for the same reason. However, P_{RMO_2} declined to only 45 mmHg at the maximum rates of exercise performance and $\dot{M}_{O_2}$, which is considerably higher than the P_{O_2} values observed in mammals (Jung et al., 1999; Behnke et al., 2001). Egginton et al. (2000), using morphological data and analysis of the resulting physico-chemical conditions for O_2 diffusion, estimated that the P_{O_2} gradient between capillaries and the centre of a red muscle fibre may be less than 4 mmHg in trout at maximum sustained exercise. Thus, the high P_{RMO_2} suggests that rainbow trout RM may not become hypoxic at high levels of sustained exercise, a suggestion that is supported by two other lines of evidence. First, resolution of the Fick equation revealed that the reduction in the arterial to RM P_{O_2} gradient that occurred between a swimming speed of 0.5 *BL* s^{-1} and maximal exercise would have required an approximately fivefold increase in blood supply to meet the measured increase in $\dot{M}_{O_2}$. This increase compares favourably with the eightfold increase in blood supply to RM measured with microspheres during maximum sustained exercise in rainbow trout (Taylor et al., 1996). Second, at exhaustion P_{RMO_2} increased rather than decreased. This contrasts with tetrapod skeletal muscles, where fatigue is associated with a profound decline in P_{O_2} to below 50% of resting values (Molé et al., 1999; Howlett and Hogan, 2001). This suggests that when WM is recruited to power swimming speeds above 70% of U_{crit} (Burgetz et al., 1998; Lee et al., 2003) subsequent exhaustion is not linked to major reductions in RM O_2 supply. Consequently, convective O_2 supply to the RM seems not to be a limiting factor for maximum aerobic performance in rainbow trout. Prolonged exercise at 90% of U_{crit} leads to depletion of oxidative substrates in trout RM (Richards et al., 2002), so this may be the cause of fatigue. Alternatively, RM may simply reduce its activity when WM is recruited during incremental exercise, a gait transition representing an orderly and necessary transition to a muscle that can generate the required increase in tailbeat frequency and muscular power output (see Jones and Randall, 1978). One consequence of this gait transition is that P_{RMO_2} remains high, higher than P_{vO_2} at fatigue (Farrell and Clutterham, 2003). Further research into this area is clearly required, not least to determine the validity of an incremental graded exercise protocol in investigating factors limiting maximum rates of aerobic metabolism and performance in fish.

Insights into the impact of the Root effect upon red muscle O_2 tensions

There was no evidence that the Root effect influenced tissue O_2 tension enough to raise P_{RMO_2} above P_{aO_2} in the rainbow trout. In fact, the opposite was always true, both in normoxia and in mild hypoxia, when the P_{aO_2} to P_{RMO_2} gradient was reduced. Indeed, P_{RMO_2} was sensitive to changes in P_{aO_2} and although the proportional changes in P_{RMO_2} during hypoxia were significantly less than the changes in P_{aO_2}, this could be attributed to the sigmoid shape of the trout Hb–O_2 dissociation curve. Thus, as P_{aO_2} declined, the arterial to RM P_{O_2} difference shifted left towards the steep portion of the dissociation curve, such that a smaller drop in P_{O_2} was required to elicit the same degree of O_2 unloading.

In addition to P_{RMO_2} being elevated compared with measurements in mammalian muscles, it is interesting that P_{RMO_2} was also consistently higher than published values for mixed P_{vO_2} in the trout, both in normoxia and at comparable degrees of hypoxia (Holeton and Randall, 1967; Farrell and Clutterham, 2003). Indeed, the measured values for P_{RMO_2} lie almost exactly midway between published values for P_{aO_2} and P_{vO_2} at the appropriate water P_{O_2} (Holeton and Randall, 1967). In contrast, the reported range for mammalian intramuscular P_{O_2} (Hutter et al., 1999; Jung et al., 1999; Behnke et al., 2001; Suttner et al., 2002) is consistently lower than that of mixed P_{vO_2}, which is typically around 40 mmHg (Hutter et al., 1999; Wilmer et al., 2000). The higher P_{RMO_2} relative to P_{vO_2} in the trout can be interpreted in one of two ways. One possibility is

that venous return from RM is a relatively small contribution to mixed venous blood. The other possibility is that the high P_{RMO_2} of rainbow trout relative to mammals could be, at least in part, a consequence of a Root effect in blood perfusing the RM. The Root effect would be engendered by transient changes in erythrocyte pH caused by the faster rates of CO_2 diffusion than O_2 diffusion, and the strong coupling of O_2 and CO_2 movements that are known to exist in trout blood (Brauner and Randall, 1998; Brauner et al., 2000). That is, when arterial blood enters the RM of trout, rapid diffusion of metabolic CO_2 into the erythrocyte would cause a transient drop in pH and cause a Root 'off-shift', driving O_2 off the haemoglobin and raising P_{O_2}. The deoxygenated haemoglobin would, however, then bind protons (the Haldane effect) and cause blood pH to rise again, eliciting a Root 'on-shift' that binds O_2 back onto the haemoglobin and lowers P_{O_2} in the venous blood leaving the tissue.

While such a role of the Root effect is conjecture at this time, we can eliminate the possibility that we measured an artefact of mixed arterial, tissue and venous P_{O_2} values rather than intramuscular P_{O_2}. If this had been the case, P_{RMO_2} should have varied directly with P_{aO_2} during hypoxia and exercise, which it did not. Furthermore, similar concerns would, presumably, exist for mammalian studies of intramuscular P_{O_2} that involved the implantation of microelectrodes (Hutter et al., 1999; Jung et al., 1999; Suttner et al., 2002). Thus, in addition to the anatomical reasons for elevated P_{RMO_2} that have been raised by Egginton (2002), the current study has not eliminated the possibility that O_2 tensions are also influenced by the action of the Root effect within the muscle vasculature. Future investigations should perhaps be aimed at experimental manipulation of the Root effect to investigate how this change influences P_{RMO_2} relative to P_{aO_2} and mixed P_{vO_2}.

Conclusions

The results show that the P_{O_2} prevailing in the RM of rainbow trout is higher than that reported for skeletal muscles of rats and humans. While there was a significant decrease in P_{RMO_2} during sustained exercise, it did not decline below 40 mmHg and increased slightly at exhaustion. These observations are taken as a strong indication that O_2 supply to the RM does not become limiting either at the moment of recruitment of WM or at exhaustion. We found no dramatic evidence that the Root effect raises O_2 tensions in red muscle because P_{RMO_2} remained almost exactly midway between previously published values of P_{aO_2} and P_{vO_2} for rainbow trout and was sensitive to reductions in P_{aO_2} during mild hypoxia. Further work is needed to explain the higher P_{O_2} in the RM relative to mixed venous blood because, while this may reflect a limited contribution from RM to mixed venous return, the phenomenon might also be a consequence of a transient Root effect in the RM vasculature.

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