The Journal of Experimental Biology 213, 3802-3809

doi:10.1242/jeb.048512

Hypoxia stimulates lactate disposal in rainbow trout

Teye Omlin and Jean-Michel Weber*

Biology Department, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada

*Author for correspondence (jmweber@uottawa.ca)

Accepted 18 August 2010

SUMMARY

Current understanding of lactate metabolism in fish is based almost entirely on the interpretation of concentration measurements that cannot be used to infer changes in flux. The goals of this investigation were: (1) to quantify baseline lactate fluxes in rainbow trout (*Oncorhynchus mykiss*) under normoxic conditions; (2) to establish how changes in rates of lactate appearance (R_a) and disposal (R_d) account for the increase in blood lactate elicited by hypoxia; and (3) to identify the tissues responsible for lactate production. R_a and R_d lactate of rainbow trout were measured *in vivo* by continuous infusion of [U-^{14}C]lactate in trout exposed to 25% O_2 saturation or maintained in normoxia for 90 min. In normoxic fish, R_a lactate decreased from 18.2 to 13.1 μmol kg^{-1} min^{-1} and R_d lactate from 19.0 to 12.8. R_a and R_d were always matched, thereby maintaining a steady baseline blood lactate concentration of ~0.8 mmol l^{-1}. By contrast, the hypoxic fish increased blood lactate to 8.9 mmol l^{-1} and R_a lactate from 18.4 to 36.5 μmol kg^{-1} min^{-1}. This stimulation of anaerobic glycolysis was unexpectedly accompanied by a 52% increase in R_d lactate from 19.9 to 30.3 μmol kg^{-1} min^{-1}. White muscle was the main producer of lactate, which accumulated to 19.2 μmol g^{-1} in this tissue. This first study of non-steady-state lactate kinetics in fish shows that the increase in lactate disposal elicited by hypoxia plays a strategic role in reducing the lactate load on the circulation. Without this crucial response, blood lactate accumulation would double.

Key words: lactate kinetics, *in vivo* metabolite fluxes, anaerobic glycolysis, carbohydrate metabolism, continuous tracer infusion, low oxygen availability, *Oncorhynchus mykiss*.

INTRODUCTION

Aquatic environments are routinely exposed to hypoxia through natural eutrophication and thermal stratification, but modern human activities causing organic pollution and global warming have amplified this problem (Diaz and Rosenberg, 2008). Reports of mass mortality and of fisheries collapse are becoming more common (Wu, 2002), particularly for hypoxia-sensitive organisms like salmonids (Turner et al., 1983). The well-characterized response of rainbow trout to hypoxia combines physiological changes aimed at trying to maintain normal oxygen supply to tissues (hyperventilation, bradycardia, increased blood O_2-carrying capacity and redistribution of blood flow) with metabolic changes aimed at providing adequate amounts of ATP with less oxygen (stimulation of anaerobic glycolysis) (Richards et al., 2009). Carbohydrates become an essential source of energy because glycolysis can produce ATP in the absence of oxygen. Therefore, hypoxia results in the accumulation of lactate, and the metabolism of this anaerobic end product has been extensively studied in trout. Exposure to hypoxia for 1–3 h increases the blood lactate concentration from normoxic values of less than 1 mmol l^{-1} to 6–15 mmol l^{-1} (Dunn and Hochachka, 1986; Thomas et al., 1992; Van Raaij et al., 1996). Unfortunately, current understanding of lactate metabolism in fish is based almost entirely on the interpretation of concentration measurements that cannot be used to infer changes in glycolytic flux (Haman et al., 1997b; Stanley et al., 1985). This is because metabolite concentration is determined by dynamic changes in rates of appearance (R_a) and disposal (R_d), and widely different changes in these fluxes can elicit identical effects on concentration. For instance, the same increase in lactate concentration could be due to the stimulation of anaerobic glycolysis (increase in R_a) or to a reduction in the rate of lactate utilization (decrease in R_d). The only reliable clues about fluxes provided by monitoring concentration are: (1) that R_a and R_d always remain equal when concentration stays constant, but this is true when R_a and R_d undergo identical, parallel changes; and (2) that R_a and R_d are mismatched when concentration varies.

To date, the direct measurement of lactate fluxes has only been attempted in three fish studies, investigating endurance swimming (Weber, 1991), recovery from exhaustive exercise (Milligan and McDonald, 1988), and exposure to hypoxia (Dunn and Hochachka, 1987). They reveal that lactate is rapidly produced and utilized, even in a normoxic fish at rest, and that swimming and hypoxia stimulate the rate of lactate turnover. Regrettably, these early estimates of flux were all obtained using bolus injection, an obsolete method with significant limitations (Wolfe, 1992): (1) flux calculations are based on surface areas under specific activity-decay curves that are difficult to evaluate accurately; (2) each experiment yields only one value of flux, preventing the measurement of a time course; and (3) the bolus injection method assumes steady-state conditions and cannot be used to quantify R_a and R_d independently (Haman and Weber, 1996). By contrast, the more versatile continuous tracer infusion method is widely used in biology and medicine because it has none of the above limitations and because it provides more accurate values of flux (Allsop et al., 1978; Allsop et al., 1979). Adequate double catheterization techniques have been developed for continuous tracer infusion in fish (Haman et al., 1997a; Haman and Weber, 1996), and this more versatile method has been used successfully to investigate the kinetics of glucose (Haman et al., 1997b; Shanghavi and Weber, 1999; Weber and Shanghavi, 2000), glycerol (Bernard et al., 1999; Magnoni et al., 2008b), fatty acids (Weber et al., 2002) and triacylglycerol (Magnoni et al., 2008a). The lactate kinetics of fish have never been assessed by continuous tracer infusion.

A few studies provide measurements of lactate accumulation in trout tissues in an attempt to characterize the main sources of anaerobic end product during hypoxia (Bernier et al., 1996; Boutilier et al., 1987; Dunn and Hochachka, 1986). Their results support the notion that white muscle is the most important producer of lactate in hypoxic trout, but it is unclear whether this conclusion holds for a 90 min, acute exposure to 25% O_2 saturation at their preferred temperature of 13°C because a variety of conditions have been used in previous studies (exposures of 3–24 h, 13–20% O_2 saturation, and lower temperatures of 4–9°C).

The goals of our investigation were: (1) to measure the baseline lactate turnover rate of rainbow trout using the most reliable tracer method presently available; (2) to establish how changes in rates of R_a and R_d account for the increase in blood lactate concentration elicited by hypoxia; and (3) to identify the main tissues responsible for lactate production. We anticipated that environmental hypoxia would stimulate R_a lactate by activating anaerobic glycolysis in muscle and possibly liver, and that it would decrease R_d by reducing the capacity of tissues to oxidize lactate.

MATERIALS AND METHODS

Animals

Male and female rainbow trout *Oncorhynchus mykiss* (Walbaum) (482±17 g, *N*=24) were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and held in a 1300-liter flow-through tank in dechlorinated, well-oxygenated water at 13°C under a 12 h:12 h light–dark photoperiod. Fish were acclimated to these conditions for at least 2 weeks before experiments. They were fed floating fish pellets (Martin Mills, Elmira, Ontario, Canada) three times a week until satiation. They were randomly assigned to a control group (normoxia) or treatment group (hypoxia). All procedures were approved by the Animal Care Protocol Review Committee of the University of Ottawa and adhered to the guidelines established by the Canadian Council on Animal Care for the use of animals in research.

Catheterizations

Fish were fasted for at least 24 h before surgery. They were anesthetized with ethyl-*N*-aminobenzoate sulfonic acid (MS-222; 60 mg l^{-1}) in well-oxygenated water and their dorsal aorta was cannulated with two PE-50 catheters (Intramedic, Clay-Adams, Sparks, MD, USA), as described previously (Haman and Weber, 1996). After cannulation, the animals were allowed to recover for 24 h in opaque Plexiglas chambers (50×12×12 cm) supplied with the same quality water as the acclimation tank. Catheters were kept patent by flushing with 0.2 ml Cortland saline (Wolf, 1963) containing 50 u ml^{-1} heparin (Sigma-Aldrich, St Louis, MO, USA). Lactate kinetics were then measured by continuous tracer infusion in the same chambers. Only animals with a hematocrit >20% after recovery from surgery were used in experiments.

Lactate kinetics

The rates of lactate appearance (R_a) and lactate disposal (R_d) were measured by continuous infusion of [U-^{14}C]lactate (New England Nuclear, Boston, MA, USA; 4.84 GBq mmol $^{-1}$). Infusates were freshly prepared before each experiment by drying an aliquot of the solution obtained from the supplier under N_2 and resuspending in Cortland saline. Labeled lactate was infused for 4 h in resting animals (1500±25 Bq kg^{-1} min^{-1}, *N*=13), using a calibrated syringe pump (Harvard Apparatus, South Natick, MA, USA; at 1 ml h^{-1}). Lactate (labeled and unlabelled) was infused at rates accounting for <0.002% of the endogenous R_a lactate measured in resting, normoxic fish.

Oxygen levels in the water were monitored using a sympHony SP70D dissolved oxygen meter (VWR, West Chester, PA, USA). The water was kept normoxic throughout the control experiments (9.46±0.01 mg O_2 l^{-1}). For hypoxia experiments, the water was kept normoxic during the first hour (9.42±0.01 mg O_2 l^{-1}), before oxygen content was gradually reduced over 90 min by bubbling N_2 through a column containing glass marbles. After reaching 25% saturation (2.55±0.01 mg O_2 l^{-1}), measurements of lactate kinetics were continued for 90 min to quantify fluxes under hypoxic conditions. Blood samples (100 µl each) were drawn 50, 55 and 60 min after the start of infusion to ensure that isotopic steady state had been reached and to quantify baseline lactate fluxes. Additional samples were then taken every 15 min until the end of the experiments. The amount of blood sampled from each fish accounted for <10% of total blood volume. Blood samples were immediately deproteinized in 200 µl perchloric acid (PCA; 6% w/w) and centrifuged for 5 min at 16,000 ***g*** (Eppendorf 5415c, Brinkmann, Rexdale, Canada). Supernatants were frozen at –20°C and assayed within 24 h.

Tissue sampling

After the infusions, the fish were killed by injection of an overdose of sodium pentobarbital through one of the catheters (Euthanyl, Abraxis Pharmaceutical Products, Schaumburg, IL, USA). Heart, liver, red muscle, white muscle and brain were harvested in random order within 4 min after death (~2 g per tissue, except for heart and brain that were sampled entirely). Red and white muscle were always sampled below the dorsal fin. Tissues were immediately freeze-clamped in liquid N_2 and stored at –80°C until metabolite concentrations were measured.

Analyses of blood samples

Glucose and lactate concentrations were measured spectrophotometrically (Bergmeyer, 1985) using a Spectra Max plus 384 (Molecular Devices, Sunnyvale, CA, USA). Radioactivity was measured by scintillation counting (Beckman Coulter LS 6500, Fullerton, CA, USA) in Bio-Safe II scintillation fluid (RPI Corp, Mount Prospect, IL, USA). Lactate and glucose were separated using three ion exchange columns placed in series to determine specific activities [as described by Katz et al. (Katz et al., 1981), with modifications as follows]. The three columns contained 0.2 ml Dowex 50 (H^+ form; 100 mesh; to separate amino acids), 0.4 ml Dowex 1 (acetate form; 200 mesh; to separate lactate) and 0.3 ml Dowex 1 (borate form; 200 mesh; to separate glucose). Before passing through the columns, the deproteinized blood samples (150 µl each) were neutralized with 75 µl potassium bicarbonate (1 mol l^{-1}), and diluted with 5 ml deionized H_2O. Amino acids were eluted from column 1 with 4 ml ammonium hydroxide (2 mol l^{-1}), lactate from column 2 with 4 ml acetic acid (2 mol l^{-1}), and glucose from column 3 with 4 ml acetic acid (0.5 mol l^{-1}). Preliminary experiments with known amounts of labeled lactate and labeled glucose showed that this procedure separates and recovers >90% of total activity. After column separation, the sum of the activities recovered in the amino acid, lactate and glucose fractions accounted for 84±3% of total plasma activity (*N*=13 fish).

Tissue metabolites

Frozen tissues (0.5–1.0 g) were ground in liquid N_2 with a mortar and pestle, before homogenizing for 1 min in perchloric acid (6%; 4:1 v/w) with a Polytron (Kinematica, Luzern, Switzerland). Glucose and lactate concentrations were determined from supernatant as described for blood. Tissue glycogen concentration was measured on subsamples of the homogenates using amyloglucosidase (Fournier and Weber, 1994).

Calculations and statistics

Lactate and glucose specific activities ($Bq\,\mu mol^{-1}$) were calculated as the ratio between activity and concentration. The rates of appearance (R_a lactate) and disposal (R_d lactate) were calculated using the non-steady-state equations of Steele (Steele, 1959), with a volume of distribution of $100\,ml\,kg^{-1}$ (Stanley et al., 1985). To illustrate what would happen to blood lactate concentration if the rate of lactate disposal was not stimulated during hypoxia, hypothetical changes in concentration were computed (see Fig. 5). They were calculated for each time using R_a lactate measured during hypoxia and R_d lactate measured during control experiments under normoxic conditions. Statistical comparisons were performed using one- or two-way repeated measures analysis of variance (RM-ANOVA) with Bonferroni post-hoc test to determine which means were different from baseline. In cases in which the assumptions of normality or homoscedasticity were not met, Friedman repeated measures ANOVA on ranks was used with Dunn's test, or the data was normalized by logarithmic transformation before parametric analysis. All values presented are means ± s.e.m. and a level of significance of $P<0.05$ was used in all tests.

RESULTS

Water oxygen content

Changes in the oxygen concentration of the water during the measurement of lactate kinetics in control and hypoxic fish are presented in Fig. 1A. Control fish were kept in normoxic water averaging $9.5\,mg\,O_2\,l^{-1}$ and this concentration did not vary over time ($P>0.05$). For the hypoxic fish, oxygen content was progressively reduced from $9.4\,mg\,O_2\,l^{-1}$ (at time 0) to $2.6\,mg\,O_2\,l^{-1}$ over 1.5 h, and was maintained at that level for the remainder of the experiments. Oxygen concentrations of the hypoxia group were significantly lower than normoxic values starting 15 min after the onset of the transition phase ($P<0.001$).

Lactate concentration and specific activity

The time course of changes in blood lactate concentration is shown in Fig. 1B. Control fish maintained a low, baseline lactate concentration averaging $0.8\,mmol\,l^{-1}$ throughout the experiments ($P>0.05$). By contrast, the treatment group showed a progressive increase from 1.0 to $8.9\,mmol\,l^{-1}$ during hypoxia ($P<0.001$). Blood lactate specific activity during the measurement of lactate kinetics is presented in Fig. 2. Hypoxic fish showed a significant decrease ($P<0.001$; Fig. 1A), whereas a small increase was observed in control fish ($P<0.001$; Fig. 1B). Dunn's post-hoc test identified only the last two points of the control group as being different from baseline ($P<0.05$).

Effects of hypoxia on lactate fluxes

Changes in the rates of lactate appearance and disposal are shown in Fig. 3. Hypoxia caused a large increase in R_a and in R_d lactate ($P<0.001$; Fig. 3A). In fish exposed to hypoxia, R_a lactate increased from a baseline value of 18.4 to $36.5\,\mu mol\,kg^{-1}\,min^{-1}$, and R_d lactate increased from 19.9 to $30.3\,\mu mol\,kg^{-1}\,min^{-1}$ throughout the experiments. In control fish, R_a and R_d lactate were 18.2 and $19.0\,\mu mol\,kg^{-1}\,min^{-1}$ at time 0. After 3 h, these fluxes showed a slight decrease to 13.1 and $12.8\,\mu mol\,kg^{-1}\,min^{-1}$, respectively ($P<0.001$; Fig. 3B). Dunn's post-hoc test identified only the last three R_a and R_d values of the control group as being different from baseline ($P<0.05$). Initial (baseline) and final values (after exposure to normoxia or hypoxia) for blood lactate concentration, specific activity and lactate fluxes are summarized in Table 1.

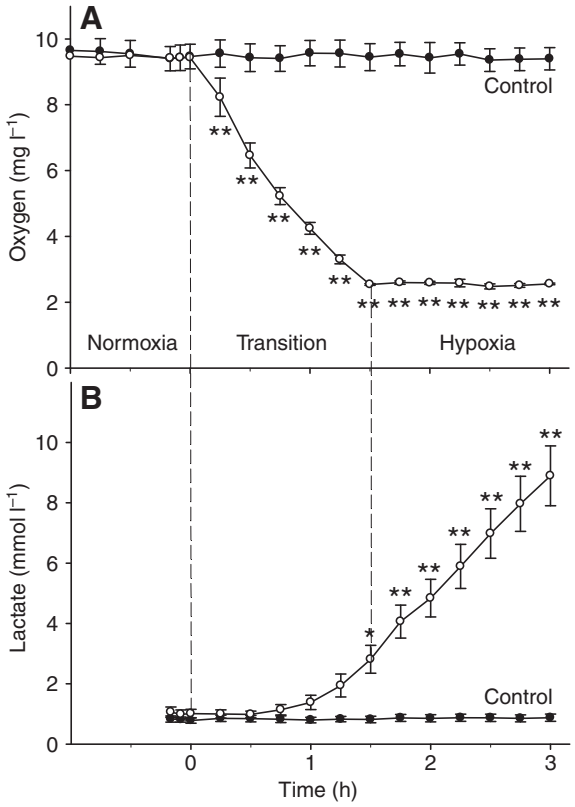

Fig. 1. Water oxygen content (A) and blood lactate concentration (B) in rainbow trout during the measurement of lactate kinetics. Filled circles show control fish kept at normoxia; open circles show treatment fish exposed to hypoxia. For treatment fish, normoxia, transition and hypoxia phases are separated by dotted lines. Values are means ± s.e.m. (N=8). Differences from baseline (time 0) are indicated by asterisks: *$P<0.05$ and **$P<0.001$.

Because changes in lactate concentration depend on the balance between R_a and R_d lactate, their difference is presented in Fig. 4. The R_a–R_d difference increased from –0.5±0.5 to a maximum of $8.3\pm1.7\,\mu mol\,kg^{-1}\,min^{-1}$ in fish exposed to hypoxia ($P<0.001$; Fig. 4A), but remained at baseline ($0.02\pm0.10\,\mu mol\,kg^{-1}\,min^{-1}$) in control fish ($P=0.12$; Fig. 4B). In the treatment group, all R_a–R_d values were significantly different from 0 after 70 min ($P<0.05$). Hypoxia caused an unexpected increase in the rate of lactate disposal (Fig. 3A) and, to evaluate its impact, we have calculated hypothetical changes in blood lactate concentration if this response did not happen. These calculated values are presented in Fig. 5 together with the concentrations observed in control and hypoxic fish for comparison. The closed triangles show that blood lactate concentration would increase to $18.0\,mmol\,l^{-1}$ if R_d lactate was not stimulated during hypoxia (instead of the maximum of $8.9\,mmol\,l^{-1}$ observed in real fish in which R_d lactate was strongly increased; Fig. 5).

Glucose metabolism

Changes in blood glucose concentration are presented in Fig. 6. Glycemia in treatment fish increased from 4.9 to $6.1\,mmol\,l^{-1}$ during exposure to hypoxia ($P<0.001$; Fig. 6A), whereas control fish maintained baseline values ($4.92\pm0.02\,mmol\,l^{-1}$) throughout the experiments ($P>0.05$; Fig. 6B). The Bonferroni post-hoc test reveals that all the values measured during hypoxia were hyperglycemic

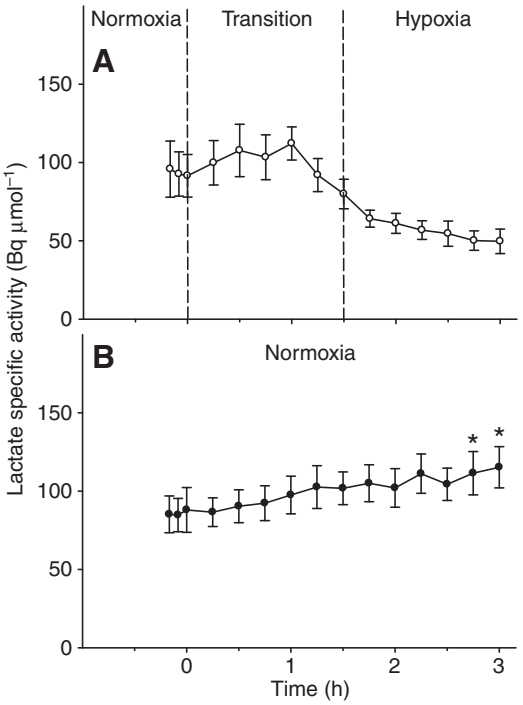

Fig. 2. Blood lactate specific activity in rainbow trout during continuous infusion of [U-^{14}C]lactate in hypoxic (A) and control (B) fish. Tracer infusions were started 1 h before time 0. In A, normoxia, transition and hypoxia phases are separated by dotted lines. Values are means ± s.e.m. (N=8 for hypoxia and 5 for control). Differences from baseline (time 0) are indicated by asterisks (*P<0.05).

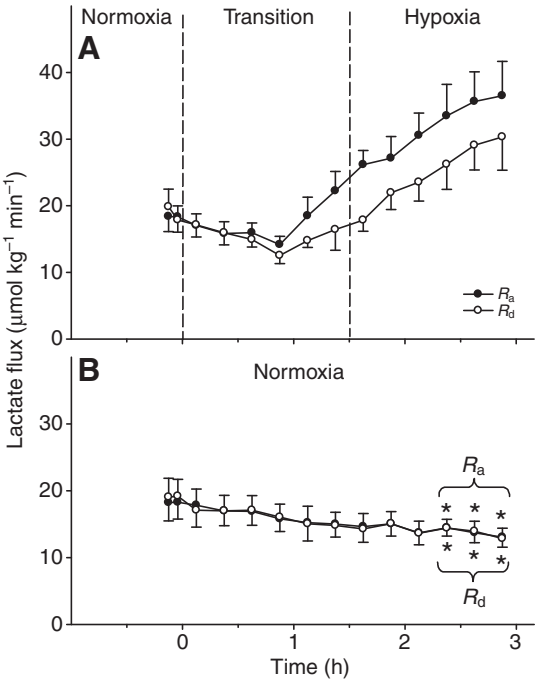

Fig. 3. Lactate fluxes of rainbow trout exposed to hypoxia (A) or kept at normoxia (B). Filled circles show the rate of appearance of lactate (R_a); open circles show the rate of lactate disposal (R_d). Tracer infusions were started 1 h before time 0. In A, normoxia, transition and hypoxia phases are separated by dotted lines. For the control fish (B), most R_d values overlap with R_a. Values presented are means and s.e.m. (N=8 for hypoxia and 5 for control). Differences from baseline (time 0) are indicated by asterisks (*P<0.05).

(Fig. 6A). Changes in blood glucose specific activity during continuous infusion of labeled lactate are shown in Fig. 7. Some lactate was used as a precursor for glucose synthesis. Therefore, the specific activity of glucose increased in fish exposed to hypoxia (P<0.001; Fig. 7A), as well as in control fish kept at normoxia (P<0.001; Fig. 7B).

Tissue carbohydrates

The effects of hypoxia on the concentrations of the main carbohydrates in rainbow trout tissues are shown in Fig. 8. Changes in lactate, glucose and glycogen concentrations were observed. All of the tissues accumulated lactate during hypoxia (control *vs* hypoxia: P<0.001), with white muscle showing the highest increase from 3.6 to 19.2 μmol g^{-1} (Fig. 8A). Comparisons among hypoxia values reveal that lactate concentration was higher in white muscle than in all other tissues (P<0.01; statistics not shown on graph). Increases in glucose concentration were observed in liver, red muscle and white muscle, but no change was observed in the other tissues (Fig. 8B). Hypoxia caused significant glycogen depletion in the liver from 299 to 158 μmol glucosyl units g^{-1} (P<0.001), but had no effect in other tissues (P>0.05; Fig. 8C).

DISCUSSION

Our study shows that rainbow trout acutely exposed to hypoxia do not only increase lactate production (R_a), but also strongly stimulate lactate disposal (R_d). We report the first measurements of non-steady-state lactate kinetics in an ectotherm. The classic increase in blood lactate concentration observed during hypoxia results from a

Table 1. Blood metabolite concentrations and specific activities, and rates of lactate appearance and disposal, before and after normoxia (control) or hypoxia in rainbow trout

	Normoxia		Hypoxia	
	Initial	Final	Initial	Final
Lactate (mmol l^{-1})	0.8±0.1 (8)	0.9±0.1 (8)	1.0±0.1 (8)	8.9±1.0 (8)**
Glucose (mmol l^{-1})	5.0±0.4 (8)	4.8±0.4 (8)	4.9±0.5 (8)	6.1±0.6 (8)*
Lactate S.A. (Bq μmol^{-1})	88.0±14.3 (5)	115.2±13.1 (5)*	91.5±13.6 (8)	49.7±7.8 (8)
Glucose S.A. (Bq μmol^{-1})	2.7±0.6 (4)	14.8±3.7 (4)*	3.7±1.0 (8)	6.9±1.2 (8)*
R_a lactate (μmol kg^{-1} min^{-1})	18.2±2.7 (5)	13.1±1.3 (5)*	18.4±2.3 (8)	36.5±5.2 (8)
R_d lactate (μmol kg^{-1} min^{-1})	19.0±2.8 (5)	12.8±1.3 (5)*	19.9±2.6 (8)	30.3±5.0 (8)*

All measurements were taken during continuous infusion of [U-^{14}C]lactate. Values are means ± s.e.m. with sample size in parentheses. Initial values were measured at time 0 and final values at 3 h. S.A., specific activity; R_a, rate of appearance; R_d, rate of disposal. Statistical differences are indicated by * (P<0.05) or ** (P<0.001).

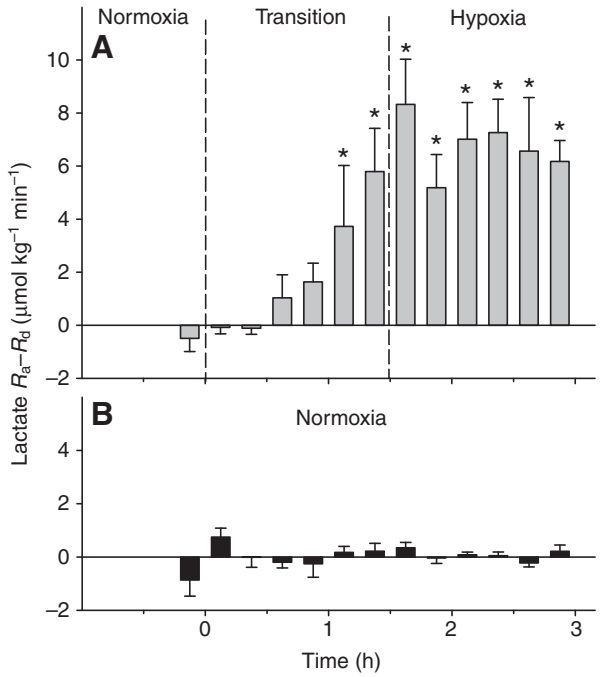

Fig. 4. Changes in the difference between the rates of lactate appearance and lactate disposal (R_a–R_d) in rainbow trout exposed to hypoxia (A) or kept at normoxia (B). Tracer infusions were started 1 h before time 0. In A, normoxia, transition and hypoxia phases are separated by dotted lines. Values are means and s.e.m. (N=8 for hypoxia and 5 for control). Differences from baseline are indicated by asterisks (*P<0.05).

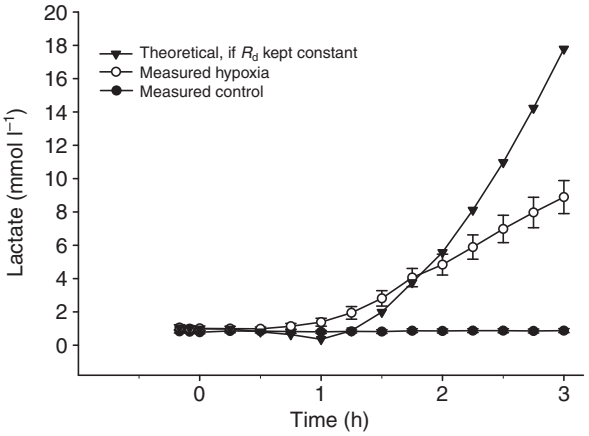

Fig. 5. Theoretical increase in blood lactate concentration (triangles) calculated for rainbow trout exposed to hypoxia if lactate disposal stayed at baseline, normoxic values. Real blood lactate concentrations measured in trout exposed to hypoxia (open circles) or kept at normoxia (filled circles) are also indicated for comparison.

mismatch caused by the more rapid increase in R_a than in R_d (+98% *vs* +52%). The increase in lactate production comes as no surprise, but the stimulation of R_d lactate is unexpected in an animal experiencing oxygen deprivation. This metabolic response is essential because it greatly reduces the lactate load imposed on the circulation of hypoxic fish. Simple measurements of changes in concentration could not have shown how fish cope with hypoxic stress and their response could only be characterized through *in vivo* flux measurements. Differential accumulation of lactate in various tissues reveals that white muscle is the principal source of the anaerobic end product.

Stimulation of lactate disposal

The most striking result from this study is the stimulation of R_d lactate elicited by hypoxia (Fig. 3, Table 1). The only two pathways available to clear lactate are oxidation and gluconeogenesis. Intuitively, it would make sense if hypoxia actually decreased R_d lactate because lowering oxidation would spare oxygen and reducing gluconeogenesis would spare energy. So, how can lactate oxidation and glucose production from lactate be activated during hypoxia? The overall need for oxidative fuel is the same in normoxic and hypoxic fish because the oxygen deprivation regime used in our experiments does not cause metabolic depression (Haman et al., 1997b). The observed increase in R_d lactate could therefore be caused by a change in fuel selection, favoring lactate and reducing the use of alternative fuels such as glucose and fatty acids. During hypoxia, oxidative tissues probably increase lactate use by mass action effect when its availability is greatly elevated in the circulation (Fig. 1B). This scenario is consistent with numerous studies on fish and mammals demonstrating that oxidative tissues, such as heart, brain and red muscle, prefer lactate over other fuels (Bilinski and Jonas, 1972; Drake et al., 1980; Lanctin et al., 1980; Smith et al., 2003). Studies by Polakof and Soengas have also recently quantified the use of lactate by fish liver, brain and Brockmann bodies (Polakof and Soengas, 2008a; Polakof and Soengas, 2008b), and the mammalian heart has been shown to increase its rate of lactate oxidation during hypoxia (Mazer et al., 1990). However, no direct measurement of lactate oxidation in heart, brain, liver, gut and erythrocytes of hypoxic fish are yet available, and all of these tissues could play a significant role in stimulating R_d lactate when oxygen supply is limited.

Even though glucose synthesis from lactate costs some ATP, the balance of evidence presently available from the literature suggests that gluconeogenesis is stimulated during hypoxia. The increases in glucose concentration observed here in the blood (Fig. 6A) and in the liver (Fig. 8B) are consistent with this idea, but our measurements of glucose specific activity (Fig. 7) cannot be used to quantify gluconeogenic flux from lactate. They only provide a qualitative demonstration that trout synthesize glucose from lactate, without revealing whether gluconeogenesis is stimulated during hypoxia. In a previous study on frogs kept in a low oxygen environment for several weeks, the authors concluded that lactate was partly recycled via increased hepatic gluconeogenesis (Donohoe and Boutilier, 1999). Another study on rainbow trout exposed to acute hypoxia also suggests that gluconeogenesis is activated (Wright et al., 1989). Direct measurements of gluconeogenic enzymes in Gulf killifish reveal that fructose 1,6-bisphosphatase activity is stimulated by hypoxia, although phosphoenolpyruvate carboxykinase (PEPCK) was not affected in these experiments (Martinez et al., 2006). Perhaps the most compelling evidence comes from a study investigating how gluconeogenesis is activated by hypoxia in isolated rat hepatocytes. The authors demonstrate that the expression of gluconeogenic enzymes is stimulated when oxygen is lacking, and they characterize the mechanism of PEPCK activation by hypoxia-inducible factor 1 (HIF-1) (Jeong et al., 2005). More research will be needed to show whether this same mechanism also activates gluconeogenesis in fish. Finally, it has been demonstrated that high blood lactate concentration (maintained via a lactate clamp) stimulates gluconeogenesis in exercising humans

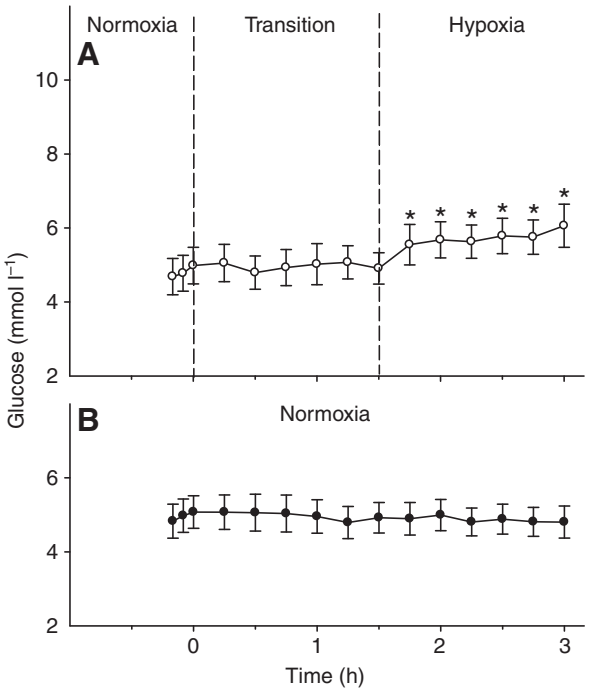

Fig. 6. Blood glucose concentration in rainbow trout exposed to hypoxia (A) or kept at normoxia (B). In A, normoxia, transition and hypoxia phases are separated by dotted lines. Values are means ± s.e.m. (*N*=8). Differences from baseline (time 0) are indicated by asterisks (**P*<0.05).

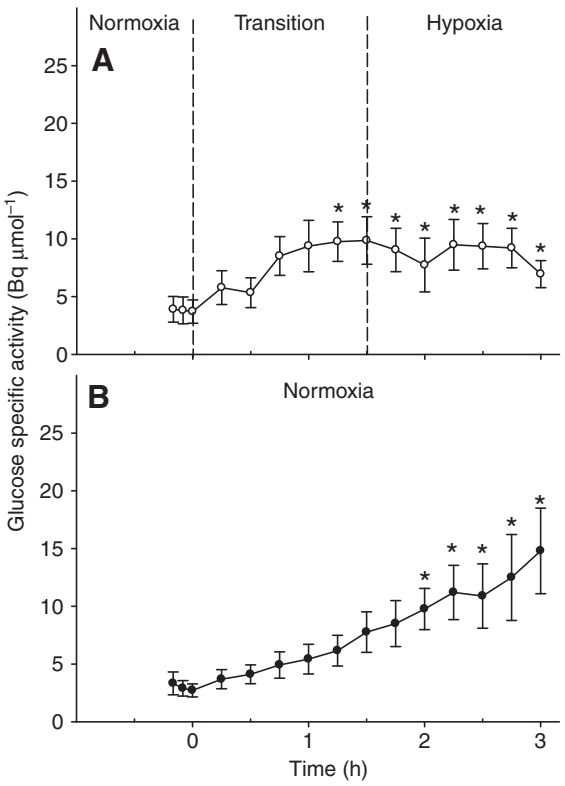

Fig. 7. Glucose specific activity in the blood of rainbow trout exposed to hypoxia (A) or kept at normoxia (B). Tracer infusions were started 1 h before time 0. In A, normoxia, transition and hypoxia phases are separated by dotted lines. Values are means ± s.e.m. (*N*=8 for hypoxia and 4 for control). Differences from baseline (time 0) are indicated by asterisks (**P*<0.05).

(Roef et al., 2003). Overall, therefore, the increase in R_d lactate observed in trout is probably jointly supported by an increase in lactate oxidation caused by a switch in fuel preference and by the activation of gluconeogenesis that might be required to sustain glucose supply to the nervous system.

Minimizing the lactate load on the circulation

Several previous studies have reported large increases in blood lactate levels for trout acutely exposed to hypoxia. Using the same conditions as in this study (90 min at 25% O_2 saturation), Van Raaij et al. reported final blood lactate concentrations of 6 mmol l^{-1} (Van Raaij et al., 1996). In other studies where different hypoxic stresses were applied, circulating lactate levels reached 7 mmol l^{-1} (180 min at 13% O_2 saturation and 4°C) (Dunn and Hochachka, 1986) and 15 mmol l^{-1} (60 min at 30% O_2 saturation and 10°C) (Thomas et al., 1992). Even though the activation of anaerobic glycolysis is clearly responsible for the observed accumulation of lactate, the true extent of this activation could not be established without information on fluxes. Here, we show that R_a lactate is stimulated during hypoxia, but that the accumulation of lactate resulting from this stimulation is strongly attenuated by the simultaneous increase in lactate disposal. The underlying changes in kinetics causing lactate accumulation in the circulation are presented in Fig. 4A, which shows the difference between the rates of production and disposal. To illustrate the real physiological impact of the hypoxia-induced increase in R_d lactate, we have calculated theoretical values for blood lactate levels if this response was abolished. If the rate of lactate disposal was artificially maintained at baseline levels throughout hypoxia, blood lactate concentration would reach 18 mmol l^{-1} or twice the value observed at the end of our experiments (see Fig. 5 for comparison). Therefore, the large increase in lactate disposal taking place during hypoxia plays a crucial role in minimizing the lactate load placed on the circulation.

Lactate production and hypoxia

The erroneous notion that anaerobic glycolysis is activated only when O_2 supply fails to meet demand is further put to rest by the fact that normoxic, resting rainbow trout produce lactate at the remarkable rate of 13–18 μmol kg^{-1} min^{-1} (Fig. 3, Table 1). These values obtained by continuous tracer infusion are higher than previous estimates measured by bolus injection (1–4 μmol kg^{-1} min^{-1}) (Dunn and Hochachka, 1987; Milligan and McDonald, 1988; Weber, 1991). Potential causes for this difference include large errors in estimating surface areas under specific activity decay curves (the main reason why bolus injection is now rarely used), label recycling, and, for one of the studies (Dunn and Hochachka, 1987), failure to subtract glucose activity from total activity when estimating lactate specific activity.

In normoxic fish, high rates of lactate production are exactly matched by equally high rates of lactate disposal (Fig. 3B, Fig. 4B) that act in tandem to maintain a low and steady lactate concentration in the circulation (<1 mmol l^{-1}; Fig. 1B). Work by Richards et al. has shown that the pyruvate dehydrogenase complex probably plays a key regulatory role in sustaining lactate production in normoxic fish (Richards et al., 2002). Rainbow trout support higher baseline turnover rates for lactate (~15 μmol kg^{-1} min^{-1}; this study) than for glucose (~8 μmol kg^{-1} min^{-1}) (Haman et al., 1997b; Shanghavi and Weber, 1999; Weber and Shanghavi, 2000), and the same pattern

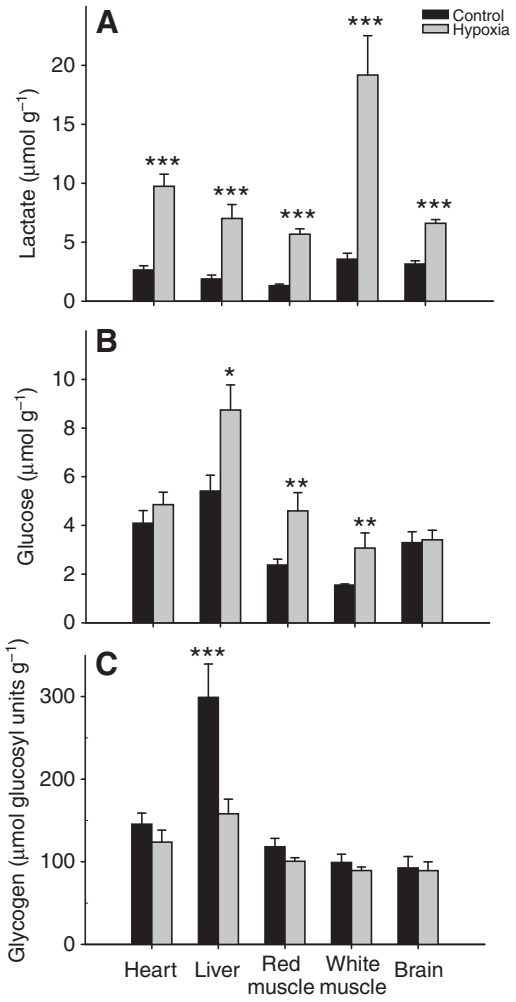

Fig. 8. Tissue concentrations of lactate (A), glucose (B) and glycogen (C) in rainbow trout kept at normoxia (filled bars) or exposed to hypoxia (gray bars). Values are means + s.e.m. (N=6) expressed per gram of wet tissue. Significant effects of hypoxia within each tissue are indicated by asterisks: *P<0.05), **P<0.01 and ***P<0.001. Comparison of hypoxia values for lactate concentration (A) shows that white muscle is higher than all other tissues (P<0.01; statistics not shown on graph).

has been observed in humans (Stanley et al., 1985; Weber et al., 1990). Animals appear to maintain high rates of lactate turnover, even under normoxic conditions at rest, because lactate is a particularly mobile intermediate of carbohydrate metabolism involved in numerous fuel shuttles (Gladden, 2004).

The stimulation of glycolytic flux by hypoxia was measured here as a two-fold increase in R_a lactate from 18 to 36 μmol kg^{-1} min^{-1} (Fig. 3A, Table 1). The only other study dealing with the effects of acute oxygen deprivation on trout lactate kinetics reported an increase from 3 to 20 μmol kg^{-1} min^{-1} (Dunn and Hochachka, 1987), but these previous values might not reflect true rates because they were estimated by bolus injection (see limitations above). Nevertheless, hypoxia-induced changes in lactate production in trout are consistent with the response observed in mammals. Humans acutely exposed to hypobaric hypoxia show an initial 4-fold increase in lactate production before the response is somewhat attenuated after 3 weeks of acclimation to high altitude (Brooks et al., 1991). Several earlier studies of lactate metabolism in fish had compared proton and lactate movements after exhaustive exercise (e.g. Turner and Wood, 1983; Turner et al., 1983). Rates of proton and lactate flux were estimated from changes in pH and in lactate concentration, either in white muscle or in the circulation. In future investigations of acid-base balance in exercising fish, it could be useful to use measurements of R_a lactate as a tool to estimate rates of proton production indirectly. This approach could be helpful because the stoichiometry of lactate and proton production rarely deviates from a 1:1 ratio (Marcinek et al., 2010) and, to our knowledge, direct measurements of proton flux have not been attempted.

What are the main sites of lactate production during hypoxia? The much higher accumulation of lactate in white muscle (19 μmol g^{-1}) than in other tissues (7–10 μmol g^{-1}) shows that white muscle is probably the principal source of lactate in hypoxic trout (Fig. 8A). This observation is consistent with previous studies reporting changes in tissue lactate concentrations in rainbow trout (Bernier et al., 1996; Boutilier et al., 1987; Dunn and Hochachka, 1986) and in Amazonian cichlids (Richards et al., 2007). It is unclear to what extent the lower accumulation of lactate in other tissues than in white muscle is due to endogenous production or to the influx of white muscle-derived lactate via the blood. White muscle lactate is produced from local glycogen, but no significant decline in glycogen concentration could be demonstrated in this tissue (Fig. 8C). However, we can calculate that only 8% of the large and variable white muscle glycogen stores would be sufficient to explain the increase in lactate concentration, making the demonstration of a statistically significant decrease very difficult. For this reason, only one previous study of acute hypoxia exposure shows a decrease in white muscle glycogen (Boutilier et al., 1987), whereas two others, like ours, report only non-significant trends (Bernier et al., 1996; Dunn and Hochachka, 1986). During hypoxia, it has been suggested that high rates of lactate production in white muscle might be necessary to maintain the cytoplasmic redox state in this tissue (Richards et al., 2007).

Conclusions

The accumulation of anaerobic end product in hypoxic trout is caused by the unequal stimulation of lactate production and disposal. This study demonstrates that the doubling of R_a lactate is unexpectedly accompanied by a 52% increase in R_d lactate. This hypoxia-induced increase in lactate disposal plays a strategic role in reducing the lactate load on the circulation, and is probably mutually supported by increasing lactate oxidation through a change in metabolic fuel preference and by stimulating the use of lactate as a gluconeogenic substrate. Resting trout kept in normoxic water produce lactate at higher baseline rates than were previously estimated by bolus injection, and these rates are exactly matched by high rates of lactate disposal to maintain low and steady blood lactate concentrations (<1 mmol l^{-1}). During hypoxia, measurements of lactate accumulation in tissues shows that white muscle is the major producer of anaerobic end product. This first *in vivo* study of non-steady-state lactate kinetics in fish characterizes the fundamental changes in flux that underlie their physiological response to hypoxia.

ACKNOWLEDGEMENTS

We thank Bill Fletcher for his invaluable help in animal care. This work was supported by an NSERC discovery grant to J.-M.W.

REFERENCES

Allsop, J. R., Wolfe, R. R. and Burke, J. F. (1978). The reliability of rates of glucose appearance in vivo calculated from constant tracer infusions. *Biochem. J.* **172**, 407-416.

Allsop, J. R., Wolfe, R. R., DiStephano, J. J., III and Burke, J. F. (1979). The reliability of rates of glucose appearance in vivo calculated from single tracer injections. *Can. J. Physiol. Pharmacol.* **57**, 1267-1274.

Bergmeyer, H. U. (1985). *Methods of Enzymatic Analysis*, 701 pp. Weinheim: VCH.
Bernard, S. F., Reidy, S. P., Zwingelstein, G. and Weber, J.-M. (1999). Glycerol and fatty acid kinetics in rainbow trout: effects of endurance swimming. *J. Exp. Biol.* **202**, 279-288.
Bernier, N., Harris, J., Lessard, J. and Randall, D. (1996). Adenosine receptor blockade and hypoxia-tolerance in rainbow trout and Pacific hagfish. I. Effects on anaerobic metabolism. *J. Exp. Biol.* **199**, 485-495.
Bilinski, E. and Jonas, R. E. E. (1972). Oxidation of lactate to carbon dioxide by rainbow trout *Salmo gairdneri* tissues. *J. Fish Res. Bd Can.* **29**, 1467-1471.
Boutilier, R. G., Dobson, G., Hoeger, U. and Randall, D. J. (1987). Acute exposure to graded levels of hypoxia in rainbow trout (*Salmo gairdneri*): Metabolic and respiratory adaptations. *Respir. Physiol.* **71**, 69-82.
Brooks, G. A., Butterfield, G. E., Wolfe, R. R., Groves, B. M., Mazzeo, R. S., Sutton, J. R., Wolfel, E. E. and Reeves, J. T. (1991). Decreased reliance on lactate during exercise after acclimatization to 4,300 m. *J. Appl. Physiol.* **71**, 333-341.
Donohoe, P. H. and Boutilier, R. G. (1999). The use of extracellular lactate as an oxidative substrate in the oxygen-limited frog. *Respir. Physiol.* **116**, 171-179.
Drake, A. J., Haines, J. R. and Noble, M. I. M. (1980). Preferential uptake of lactate by the normal myocardium in dogs. *Cardiovasc. Res.* **14**, 65-72.
Dunn, J. F. and Hochachka, P. W. (1986). Metabolic responses of trout (*Salmo Gairdneri*) to acute environmental hypoxia. *J. Exp. Biol.* **123**, 229-242.
Dunn, J. F. and Hochachka, P. W. (1987). Turnover rates of glucose and lactate in rainbow trout during acute hypoxia. *Can. J. Zool.* **65**, 1144-1148.
Fournier, R. A. and Weber, J.-M. (1994). Locomotory energetics and metabolic fuel reserves of the Virginia opossum. *J. Exp. Biol.* **197**, 1-16.
Gladden, L. B. (2004). Lactate metabolism: a new paradigm for the third millennium. *J. Physiol.* **558**, 5-30.
Haman, F. and Weber, J.-M. (1996). Continuous tracer infusion to measure in vivo metabolite turnover rates in trout. *J. Exp. Biol.* **199**, 1157-1162.
Haman, F., Powell, M. and Weber, J.-M. (1997a). Reliability of continuous tracer infusion for measuring glucose turnover rate in rainbow trout. *J. Exp. Biol.* **200**, 2557-2563.
Haman, F., Zwingelstein, G. and Weber, J.-M. (1997b). Effects of hypoxia and low temperature on substrate fluxes in fish: plasma metabolite concentrations are misleading. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **42**, R2046-R2054.
Jeong, H. C., Min, J. P., Kook, W. K., Yoon, H. C., Sun, H. P., Won, G. A., Ung, S. Y. and Cheong, J. (2005). Molecular mechanism of hypoxia-mediated hepatic gluconeogenesis by transcriptional regulation. *FEBS Lett.* **579**, 2795-2801.
Katz, J., Okajima, F., Chenoweth, M. and Dunn, A. (1981). The determination of lactate turnover in vivo with ^{3}H- and ^{14}C-labelled lactate. The significance of sites of tracer administration and sampling. *Biochem. J.* **194**, 513-524.
Lanctin, H. P., McMorran, L. E. and Driedzic, W. R. (1980). Rates of glucose and lactate oxidation by the perfused isolated trout (*Salvelinus frontinalis*) heart. *Can. J. Zool.* **58**, 1708-1711.
Magnoni, L., Vaillancourt, E. and Weber, J.-M. (2008a). High resting triacylglycerol turnover of rainbow trout exceeds the energy requirements of endurance swimming. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295**, R309-R315.
Magnoni, L., Vaillancourt, E. and Weber, J.-M. (2008b). In vivo regulation of rainbow trout lipolysis by catecholamines. *J. Exp. Biol.* **211**, 2460-2466.
Marcinek, D. J., Kushmerick, M. J. and Conley, K. E. (2010). Lactic acidosis in vivo: testing the link between lactate generation and H^{+} accumulation in ischemic mouse muscle. *J. Appl. Physiol.* **108**, 1479-1486.
Martinez, M. L., Landry, C., Boehm, R., Manning, S., Cheek, A. O. and Rees, B. B. (2006). Effects of long-term hypoxia on enzymes of carbohydrate metabolism in the Gulf killifish, *Fundulus grandis*. *J. Exp. Biol.* **209**, 3851-3861.
Mazer, C. D., Stanley, W. C., Hickey, R. F., Neese, R. A., Cason, B. A., Demas, K. A., Wisneski, J. A. and Gertz, E. W. (1990). Myocardial metabolism during hypoxia: maintained lactate oxidation during increased glycolysis. *Metabolism* **39**, 913-918.
Milligan, C. L. and McDonald, D. G. (1988). In vivo lactate kinetics at rest and during recovery from exhaustive exercise in coho salmon (*Oncorhynchus kisutch*) and starry flounder (*Platichthys stellatus*). *J. Exp. Biol.* **135**, 119-131.
Polakof, S. and Soengas, J. (2008a). Differential effects of in vivo and in vitro lactate treatment on liver carbohydrate metabolism of rainbow trout. *Comp. Biochem. Physiol. A Physiol.* **151**, 205-210.
Polakof, S. and Soengas, J. (2008b). Involvement of lactate in glucose metabolism and glucosensing function in selected tissues of rainbow trout. *J. Exp. Biol.* **211**, 1075-1086.
Richards, J. G., Heigenhauser, G. J. F. and Wood, C. M. (2002). Glycogen phosphorylase and pyruvate dehydrogenase transformation in white muscle of trout during high-intensity exercise. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **282**, R828-R836.
Richards, J. G., Wang, Y. S., Brauner, C. J., Gonzalez, R. J., Patrick, M. L., Schulte, P. M., Choppari-Gomes, A. R., Almeida-Val, V. M. and Val, A. L. (2007). Metabolic and ionoregulatory responses of the Amazonian cichlid, *Astronotus ocellatus*, to severe hypoxia. *J. Comp. Physiol. B. Biochem. Syst. Environ. Physiol.* **177**, 361-374.
Richards, J. G., Farrell, A. P. and Brauner, C. J. (2009). Hypoxia. In *Fish Physiology*, Vol. 27 (ed. A. P. Farrell and C. J. Brauner), 528 pp. London: Elsevier, Academic Press.
Roef, M. J., De Meer, K., Kalhan, S. C., Straver, H., Berger, R. and Reijngoud, D. J. (2003). Gluconeogenesis in humans with induced hyperlactatemia during low-intensity exercise. *Am. J. Physiol. Endocrinol. Metab.* **284**, E1162-E1171.
Shanghavi, D. S. and Weber, J.-M. (1999). Effects of sustained swimming on hepatic glucose production of rainbow trout. *J. Exp. Biol.* **202**, 2161-2166.
Smith, D., Pernet, A., Hallett, W. A., Bingham, E., Marsden, P. K. and Amiel, S. A. (2003). Lactate: a preferred fuel for human brain metabolism in vivo. *J. Cereb. Blood Flow Metab.* **23**, 658-664.
Stanley, W. C., Gertz, E. W., Wisneski, J. A., Morris, D. L., Neese, R. A. and Brooks, G. A. (1985). Systemic lactate kinetics during graded exercise in man. *Am. J. Physiol. Endocrinol. Metab.* **249**, E595-E602.
Steele, R. (1959). Influence of glucose loading and of injected insulin on hepatic glucose output. *Ann. NY Acad. Sci.* **82**, 420-430.
Thomas, S., Perry, S. F., Pennec, Y. and Maxime, V. (1992). Metabolic alkalosis and the response of the trout, *Salmo fario*, to acute severe hypoxia. *Respir. Physiol.* **87**, 91-104.
Turner, J. D. and Wood, C. M. (1983). Factors affecting lactate and proton efflux from pre-exercised, isolated-perfused rainbow trout trunks. *J. Exp. Biol.* **105**, 395-401.
Turner, J. D., Wood, C. M. and Clark, D. (1983). Lactate and proton dynamics in the rainbow trout (*Salmo Gairdneri*). *J. Exp. Biol.* **104**, 247-268.
Van Raaij, M. T. M., Van Den Thillart, G. E. E. J. M., Vianen, G. J., Pit, D. S. S., Balm, P. H. M. and Steffens, A. B. (1996). Substrate mobilization and hormonal changes in rainbow trout (*Oncorhynchus mykiss*, L.) and common carp (*Cyprinus carpio*, L.) during deep hypoxia and subsequent recovery. *J. Comp. Physiol. B. Biochem. Syst. Environ. Physiol.* **166**, 443-452.
Weber, J.-M. (1991). Effect of endurance swimming on the lactate kinetics of rainbow trout. *J. Exp. Biol.* **158**, 463-476.
Weber, J.-M. and Shanghavi, D. S. (2000). Regulation of glucose production in rainbow trout: role of epinephrine *in vivo* and in isolated hepatocytes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **278**, R956-R963.
Weber, J.-M., Klein, S. and Wolfe, R. R. (1990). Role of the glucose cycle in control of net glucose flux in exercising humans. *Jl Appl. Physiol.* **68**, 1815-1819.
Weber, J.-M., Brichon, G., Bodennec, J. and Zwingelstein, G. (2002). Palmitate and oleate metabolism of rainbow trout in vivo. *Comp. Biochem. Physiol. A Physiol.* **131**, 409-416.
Wolf, K. (1963). Physiological saline for fresh water teleosts. *Prog. Fish-Cult.* **25**, 135-140.
Wolfe, R. R. (1992). *Radioactive and Stable Isotope Tracers in Biomedicine. Principles and Practice of Kinetic Analysis.* 471 pp. New York: Wiley-Liss.
Wright, P. A., Perry, S. F. and Moon, T. W. (1989). Regulation of hepatic gluconeogenesis and glycogenolysis by catecholamines in rainbow trout during environmental hypoxia. *J. Exp. Biol.* **147**, 169-188.