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Substrate utilization during graded aerobic exercise in rainbow trout

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Summary

A biochemical approach was employed to examine the oxidative utilization of carbohydrate and lipid in red muscle of rainbow trout (*Oncorhynchus mykiss*) during sustained swimming at 30 and 60 % of their critical swimming speed (U_{crit}; for 2, 15 and 240 min) and during non-sustainable swimming at 90 % U_{crit} (for 2, 15 and 45 min). Measurements included pyruvate dehydrogenase (PDH) activity, creatine phosphate, ATP, glycogen, glycolytic intermediates, acetyl-CoA, acetyl-, total-, free-, short-chain fatty acyl- and long-chain fatty acyl-carnitine, intramuscular triacylglycerol and malonyl-CoA concentrations, and whole body oxygen consumption ($\dot{M}_{O_2}$). During the first 2 min at 30 and 60 % U_{crit}, oxidation of endogenous glycogen by PDH activation increased 4- and 8-fold, respectively, yielding 1.5- to 2.5-fold increases in acetyl-CoA and 2- to 6-fold increases in acetyl-carnitine concentrations. Within 15 min, PDH activity returned to control values (153.9±30.1 nmol g^{-1} wet tissue min^{-1}); after 240 min there were small 1.7- to 2.6-fold increases in long-chain fatty acyl-carnitine and approx. 50 % decreases in malonyl-CoA concentrations, indicating an overall enhancement of lipid oxidation. Sustainable swimming at 30 and 60 % U_{crit} was further characterized by 1.5- and 2.2-fold increases in $\dot{M}_{O_2}$, respectively. Non-sustainable swimming at 90 % U_{crit} was characterized by a sustained tenfold (approx.) elevation of red muscle PDH activity (approx. 1600 nmol g^{-1} wet tissue min^{-1}). Significant 67 % decreases in white muscle creatine phosphate and 73 % decreases in glycogen levels, without matching increases in lactate levels, point to significant recruitment of white muscle during high-speed swimming for power production, and the potential export of white muscle lactate to red muscle for oxidation. Overall, sustainable exercise at 30 and 60 % U_{crit} is supported by approximately equal contributions of carbohydrate (approx. 45 %) and lipid (approx. 35 %) oxidation, whereas non-sustainable swimming is supported primarily by carbohydrate oxidation with only moderate contributions from lipid oxidation.

Key words: Swimming, red muscle, white muscle, pyruvate dehydrogenase, lipid, carbohydrate, lactate shuttling, malonyl-CoA, rainbow trout, *Oncorhynchus mykiss*.

Introduction

Trunk musculature in trout is spatially divided into separate regions of red-oxidative and white-glycolytic fibers that are differentially recruited to propel the fish at different swimming speeds. Red muscle is almost solely recruited at swimming speeds up to 70–80 % of the critical swimming speed (U_{crit}), beyond which white muscle is also recruited (Hudson, 1973; Johnson, 1981; Wilson and Egginton, 1994; Burgetz et al., 1998). This spatial separation of red and white muscle in fish and their known recruitment patterns have long been recognized as advantages of fish in the study of muscle metabolism (Moyes and West, 1995). To this end, the rainbow trout has become the model organism for the study of white muscle metabolism during high-intensity exercise (Dobson et al., 1987; Richards et al., 2002b) and during post-exercise recovery (Milligan and Girard, 1993; Wang et al., 1994; Wood and Wang, 1999; Kieffer, 2000; Richards et al., 2002a). High-intensity exercise, as imposed in most studies, however, is probably an event rarely experienced by fish *in vivo*, except when human intervention is involved (e.g. angling). For the most part, fish probably swim naturally at speeds that can be sustained for prolonged periods (<80 % U_{crit}; Krohn and Boisclair, 1994).

In addition to the spatial separation and different recruitment patterns of red and white muscle, the two muscle types have different substrate preferences. Red muscle contraction relies heavily on ATP generated from mitochondrial oxidative phosphorylation (maximum ATP production of 29 μmol ATP g^{-1} wet tissue min^{-1}; Moyes and West, 1995). Mitochondria isolated from red muscle of carp (*Cyprinus carpio*) have a very high capacity to oxidize pyruvate, fatty acids and some amino acids (Moyes et al., 1989). Conversely, white muscle mitochondrial ATP production is very low (approx. 3.5 μmol ATP g^{-1} wet tissue min^{-1}; Moyes et al., 1992), therefore, white muscle relies primarily on creatine

phosphate (CrP) hydrolysis and glycolysis (i.e. substrate level phosphorylation) for ATP production. However, during recovery from high-intensity exercise, white muscle mitochondria oxidize lipids to fuel CrP and glycogen synthesis (Moyes et al., 1992; Richards et al., 2002a). Thus, it is generally thought that at swimming speeds ≤70–80 % U_{crit} red muscle contraction and swimming will be supported primarily by oxidative utilization of fuels, and as swimming speed increases there will be greater reliance on ATP production by substrate level phosphorylation in white muscle.

Despite the variety of substrates oxidized by red muscle mitochondria, considerable debate surrounds the pattern of substrate selection in fish swimming at speeds ≤U_{crit} (see Moyes and West, 1995). Classically, protein and lipids were considered to be the major fuels oxidized during sustained swimming and carbohydrate utilization was considered to be minimal (Dreidzic and Hochachka, 1978; Jobling, 1994). However, Lauff and Wood (1996), using respirometry, demonstrated that (i) juvenile rainbow trout primarily oxidize lipid during swimming at 55 % and 85 % U_{crit}, (ii) carbohydrate oxidation is the next most important, and (iii) protein oxidation is minimal. White muscle glycolytic utilization of carbohydrate contributes to ATP turnover during the swimming fast-start (Wokoma and Johnson, 1981) and at speeds ≥70 % U_{crit} (Burgetz et al., 1998). In addition, whole body oxidative utilization of carbohydrate increases as swimming speed approaches U_{crit} (Lauff and Wood, 1996).

The objective of the present study was to determine the biochemical pathways involved in carbohydrate and lipid utilization by red and white muscle of rainbow trout while swimming at speeds corresponding to 30, 60 and 90 % U_{crit}. Furthermore, insights into the regulation of lipid and carbohydrate oxidation were gained through measurements of pyruvate dehydrogenase (PDH) and malonyl-CoA. Previously, we demonstrated the integral role of PDH transformation in regulating carbohydrate and lipid metabolism in white muscle during a bout of high-intensity exercise (Richards et al., 2002b) and during recovery from exhaustive exercise (Richards et al., 2002a). In the present study we measured the activity of PDH in both red and white muscle, and changes in oxidative metabolites (e.g. acetyl-CoA and carnitine) and glycolytic intermediates in red muscle during graded swimming, in an attempt to determine whether lipid or carbohydrate was oxidized during graded swimming.

Materials and methods

Animal care

Rainbow trout *Oncorhynchus mykiss*, Walbaum (115.9±7.9 g; 22.3±0.4 cm; N=123) were obtained from Humber Springs Trout Hatchery, Orangeville, Ontario, Canada. Fish were held under flow-through conditions in 800 l tanks supplied with aerated, dechlorinated city of Hamilton tapwater (composition given in Alsop and Wood, 1997) at 15 °C for at least 2 months before experimentation. During holding, fish were fed daily with commercial trout pellets. On the day of an experiment, fish were removed from the tank before feeding. All experimental procedures comply with guidelines from the Canadian Council of Animal Care.

Critical swimming speed (U_{crit}*)*

In order to establish the swimming capacity of the fish under study, critical swimming speed (U_{crit}) was determined on all fish at least 2 weeks before experimentation, according to the methodology outlined by Brett (1964). Briefly, groups of 4–6 fish were introduced into a Beamish-style swim tunnel (volume 156 l) and allowed to acclimatize for 1 h at a linear water velocity of 10 cm s^{-1} at 15 °C. This water velocity caused the fish to orient into the current, but all fish maintained station at the bottom of the tunnel with only periodic tail movements. After the acclimatization period, swimming speed was increased by 10 cm s^{-1} every 30 min until each fish fatigued. Fatigue was established when a trout fell against the back screen of the swim tunnel three consecutive times after being manually re-introduced into the current. U_{crit} was then calculated by

$$U_{crit} = V_{ls} + \left(\frac{t_s}{t_i}\right) V_i , \quad (1)$$

where V_{ls} is the velocity (cm s^{-1}) of the last completed swimming period, t_s is the time (min) spent swimming at the final swimming speed, t_i is the time increment (min), and V_i is the velocity increment (cm s^{-1}). Mean U_{crit} for all trout was 68.5±0.6 cm s^{-1} or 3.2±0.1 BL s^{-1} (where BL=body length; N=123).

Experimental protocol

On the day of an experiment, 2–3 fish were transferred into the swim tunnel and allowed to acclimatize for 1 h at a swimming speed of 10 cm s^{-1}. At the end of the acclimatization period, water velocity was increased over a 5 s period to one of three swimming speeds, corresponding to 30 % (20 cm s^{-1}), 60 % (41 cm s^{-1}) and 90 % (62 cm s^{-1}) U_{crit}. Fish swum at 30 % and 60 % U_{crit} for 2, 15 or 240 min (4 h), but at 90 % U_{crit} for only 2, 15 and 45 min, because at the high speed they fatigued between 50 and 80 min (N=9). During swimming at 90 % U_{crit}, periodic prodding was required to prevent fish from resting against the rear grid of the swim tunnel. Control fish were swum at 10 cm s^{-1} for either 2 min or 240 min after the initial acclimatization period. There were no differences between trout sampled at either control time, therefore in all figures and tables the controls are combined into a single point.

To sample fish while swimming, 25 ml clove oil (Sigma-Aldrich; Anderson et al., 1997) was introduced into the 156 l swim tunnel, resulting in anaesthesia after 2–4 min, when the fish fell against the back screen. Clove oil anaesthesia is an effective fish anaesthetic that has no detrimental effects on U_{crit} in juvenile or adult trout (Anderson et al., 1997). Fish continued to swim during the onset of anaesthesia and only fell against the back screen within the final 10–25 s. Any fish that struggled were discarded. At complete anaesthesia, the fish

were manually removed from the tunnel and a section of trunk extending from posterior to the dorsal fin to the anterior side of the anal fin was excised from each fish and immediately freeze-clamped between two metal plates pre-cooled in liquid N_2. Muscle sampling took less than 15 s. All samples were stored at –86 °C until analysis.

Analytical techniques

Frozen muscle was broken into small pieces (50–100 mg) under liquid N_2 in an insulated mortar and pestle. Care was taken to obtain only white muscle from the area dorsal to the lateral line and red muscle along the lateral line. Several pieces of red and white muscle were stored separately in liquid N_2 for determination of PDH activity by radiometric assay, as previously described (Richards et al., 2002a). The remaining broken muscle was lyophilized for 72 h, then red and white muscle fibers were separated by careful dissection, cleaned of blood, bone and connective tissue, and each stored dry at –86 °C for subsequent analysis.

For extraction of metabolites from red and white muscle, samples of lyophilized muscle (approx. 10 mg for red muscle, 20 mg for white muscle) were weighed into borosilicated tubes, 1 ml of ice-cold $HClO_4$ (1 mol l^{-1}) was added, and the suspension was homogenized at the highest speed of a Virtis hand-held homogenizer for 20 s at 0 °C. Red and white muscle homogenates were vortexed and 200 µl of homogenate slurry was removed, placed into a 1.5 ml centrifuge tube and frozen at –86 °C for determination of glycogen. The remaining homogenate was centrifuged for 5 min, 4,800 ***g*** at 4 °C and the supernatant was neutralized with 3 mol l^{-1} K_2CO_3. The neutralized extract was centrifuged for 5 min at 20 000 ***g*** at 4 °C and the supernatant was analyzed immediately for ATP, CrP and lactate. Red muscle extracts were further assayed fluorometrically for glucose, glucose 6-phosphate (Glu 6-P), fructose 6-phosphate (Fru 6-P), glucose 1-phosphate (Glu 1-P), glycerol 3-phosphate (Gly 3-P) and glycerol. All these assays followed methods described (Bergmeyer, 1983) that were modified for fluorometry. Red muscle extracts were also analyzed for acetyl-CoA, free CoA (CoA-SH), and acetyl-, free-, short-chain fatty acyl- (SCFA), long-chain fatty acyl- (LCFA) and total carnitine by radiometric methods previously described (Richards et al., 2002a). Red muscle intramuscular triacylglycerol (IMTG) was determined on lyophilized tissue as described (Keins and Richter, 1996). Malonyl-CoA was determined by high-performance liquid chromatography on a separate extraction as described (Richards et al., 2002a).

Respirometry

To determine oxygen consumption ($\dot{M}_{O_2}$) in control fish and fish swimming at 30 % and 60 % U_{crit}, we used an identical acclimatization and swimming procedure as outlined above. Oxygen consumption was not determined on fish swimming at 90 % U_{crit} because our metabolic data indicated that the fish were not in steady state at this swimming speed. In view of the high volume of the swim tunnel it was necessary to swim fish in groups to achieve measurable changes in water P_{O_2}. Briefly, four fish were introduced into the swim tunnel and allowed to acclimatize for 1 h at a flow speed of 10 cm s^{-1}. At the end of the acclimatization period, water speed was either increased to 30 % or 60 % U_{crit} or left at the control speed. After 2 h of swimming at the pre-determined speed, the swim tunnel was sealed from the atmosphere and an initial water sample was taken. Fish were swum for an additional 1.5–2 h, at which point another water sample was taken. Water samples (4 ml) were taken into gas-tight syringes and P_{O_2} was measured using a Radiometer E5046 oxygen electrode thermonstatted to 15 °C and attached to a Cameron Instruments OM-200 meter. Oxygen consumption, in µmol kg^{-1} wet mass min^{-1}, was calculated according to the Fick Principle:

$$\dot{M}_{O_2} = \frac{\Delta P_{O_2} \times \alpha O_2 \times V}{mt}, \quad (2)$$

where ΔP_{O_2} is the difference in P_{O_2} over the duration of the respirometry period, αO_2 is the solubility coefficient of O_2 in water at 15 °C (2.0111 µmol per mmHg l^{-1}) (1 mmHg=133.3 Pa) (Boutilier et al., 1984), V is the volume of water in the swim tunnel (156 l), m is the total mass of all four fish (kg), and t is the duration of the respirometry period (min). ATP turnover was calculated from $\dot{M}_{O_2}$, assuming a standard P:O_2 ratio of 6 (Hultman et al., 1967).

Data presentation and statistical analysis

All data are presented as means ± S.E.M. (N, number of fish for all metabolite data or $\dot{M}_{O_2}$ measurements). All muscle metabolite concentrations determined on lypohilized tissue were converted back to wet masses by taking into account a wet:dry ratio of 4:1 (Wang et al., 1994). Statistical analysis was a one-way analysis of variance (ANOVA) followed by a least-significant difference method of pairwise multiple comparisons. Results were considered significant at $P<0.05$.

Results

Respirometry

Compared to control trout, $\dot{M}_{O_2}$ increased 1.5- and 2.2-fold in trout swimming at 30 % and 60 % U_{crit}, respectively (Fig. 1).

Red muscle

Creatine phosphate and adenylates

Red muscle [CrP] did not change in trout swum at 30 % U_{crit} for up to 240 min (Fig. 2A). In trout swimming at 60 % U_{crit}, there was a 26 % drop in [CrP] at 2 min, but by 15 min levels had returned to control values and remained unchanged for up to 240 min. At 90 % U_{crit}, there was a 54 % decrease in [CrP] at 2 min, which remained depressed compared to control values for up to 45 min. Swimming speed or duration had no effect on red muscle [ATP] (Fig. 2B).

Glycogen, glycolytic intermediates and intramuscular triacyl glycerol

Swimming at 30 % U_{crit} for 240 min or at 60 % U_{crit} for up to 15 min had no effect on red muscle [glycogen] (Fig. 3A).

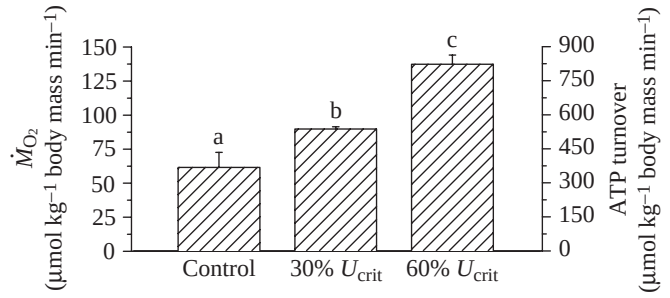

Fig. 1. Oxygen consumption (left axis) and theoretical ATP turnover (right axis) in control fish and in fish swum at 30 % and 60 % U_{crit}. See text for details. Values are means ± S.E.M.; N=5 for Control and 30 % U_{crit}; N=10 for 60 % U_{crit}. Bars with different letters are significantly different (P<0.05).

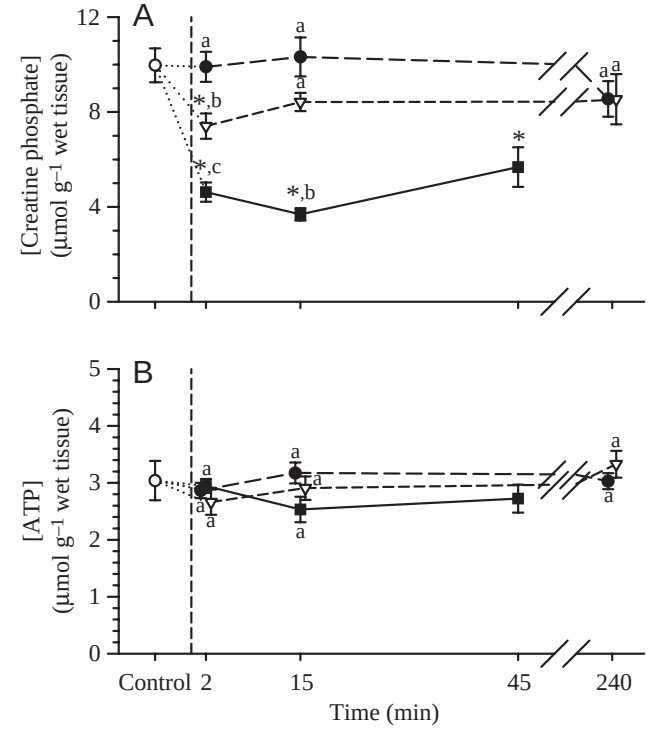

Fig. 2. Red muscle creatine phosphate (A) and ATP (B) concentrations in control fish (open circles) and in fish swum at 30 % U_{crit} (filled circles) and 60 % U_{crit} (open triangles) for up to 240 min and at 90 % U_{crit} (filled squares) for up to 45 min. The vertical dashed line represents the start of exercise. Overlapping data points are offset for clarity. Values are means ± S.E.M., N=8 for each point. Note the break in the horizontal axis. *Values significantly different (P<0.05) from control fish; different letters indicate values that are significantly different (P<0.05) within a time period.

However, after 240 min of swimming at 60 % U_{crit} there was a significant 34 % decrease in [glycogen] compared to levels in control trout. At 90 % U_{crit}, there was a significant 46 % decrease in [glycogen] after 15 min of swimming, which remained depressed by 57 % compared to control trout at 45 min. Red muscle [lactate] did not change in trout swum at 30 or 60 % U_{crit}

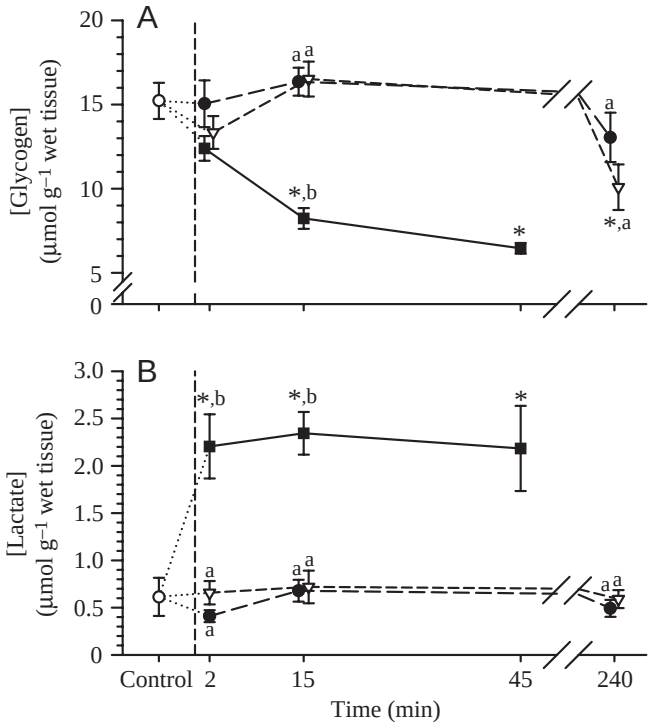

Fig. 3. Red muscle glycogen (A) and lactate (B) concentrations in control fish (open circles) and in fish swum at 30 % U_{crit} (filled circles) and 60 % U_{crit} (open triangles) for up to 240 min and at 90 % U_{crit} (filled squares) for up to 45 min. See Fig. 2 caption for other details.

for up to 240 min (Fig. 3B). However, after swimming for 2 min at 90 % U_{crit}, red muscle [lactate] had increased 3.6-fold, and remained elevated and stable compared to levels in control trout for up to 45 min. The rise in red muscle [lactate] (approx. 1.6 μmol g^{-1} wet tissue) was slight relative to the fall in [glycogen] (approx. 9 μmol glucosyl units g^{-1} wet tissue = 18 μmol lactate units g^{-1} wet tissue).

Red muscle [glucose] remained constant compared to controls in trout swimming at 30 and 60 % U_{crit} for up to 240 min (Fig. 4A). At 90 % U_{crit}, muscle [glucose] remained at control values for the first 2 min, then increased by 1.9-fold by 15 min and remained elevated for up to 45 min. There was no effect of swimming at 30 % U_{crit} on red muscle [Glu 6-P] (Fig. 4B). At 60 % U_{crit}, muscle [Glu 6-P] increased 2.2-fold within 2 min, but returned to control values by 15 min and remained low until 240 min. In trout swimming at 90 % U_{crit}, [Glu 6-P] increased 6.2-fold and gradually returned to control values by 45 min. Swimming at 30 and 60 % U_{crit} for up to 240 min did not affect red muscle [Fru 6-P] except for a small, but significant, decrease after 240 min of swimming at 30 % U_{crit} (Table 1). Swimming at 90 % U_{crit} caused an initial 2.3-fold increase in [Fru 6-P], which returned to control values by 15 min and remained low at 45 min. Red muscle [Glu 1-P] was below detection in all fish regardless of swimming speed or duration (data not shown). Red muscle [Gly 3-P] did not change in trout swimming at 30 % U_{crit} for up to 240 min

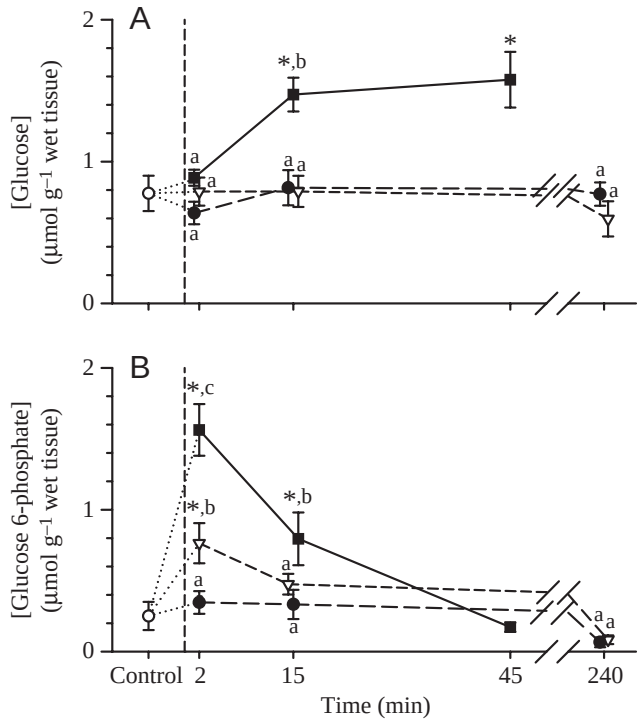

Fig. 4. Red muscle glucose (A) and glucose 6-phosphate (B) concentrations in control fish (open circles) and in fish swum at 30 % U_{crit} (filled circles) and 60 % U_{crit} (open triangles) for up to 240 min and at 90 % U_{crit} (filled squares) for up to 45 min. See Fig. 2 caption for other details.

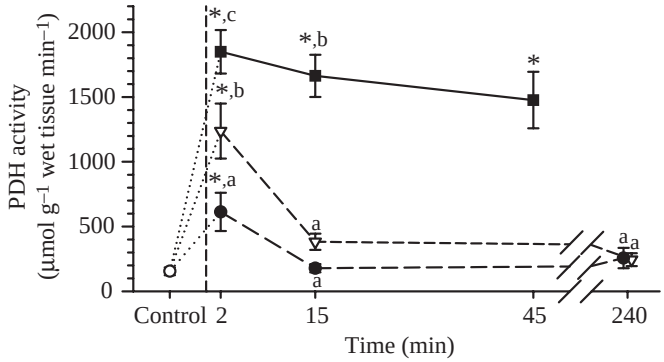

Fig. 5. Red muscle pyruvate dehydrogenase (PDH) activity in control fish (open circles) and in fish swum at 30 % U_{crit} (filled circles) and 60 % U_{crit} (open triangles) for up to 240 min and at 90 % U_{crit} (filled squares) for up to 45 min. See Fig. 2 caption for other details.

(Table 1). However, at 60 % U_{crit} there was a significant 27-fold increase in [Gly 3-P] after 15 min of swimming, which decreased to control values by 240 min. In trout swimming at 90 % U_{crit} there was a 29-fold increase in [Gly 3-P], which further increased to 55-fold at 45 min. Swimming speed or duration had no effect on red muscle [glycerol] except in fish swimming at 90 % U_{crit} for 45 min, where [glycerol] was 2.6-fold higher than in control fish. Swimming speed or duration had no effect on red muscle IMTG levels (Table 1).

Pyruvate dehydrogenase activity

During the first 2 min of swimming, red muscle PDH activity increased by 4, 8 and 12-fold in trout swimming at 30, 60 and 90 % U_{crit}, respectively (Fig. 5). In trout that continued swimming at 30 and 60 % U_{crit}, PDH activity returned to values that were not significantly different from those in control fish, and remained low for the full 240 min of swimming. However, in trout that continued swimming at 90 % U_{crit}, PDH activity remained approximately 10- to 12-fold higher than in control trout throughout the swimming period.

Acetyl group accumulation and carnitine

In general, swimming speed and duration had no effect on red muscle CoA-SH levels except at 15 min, where trout swimming at 60 and 90 % U_{crit} exhibited significantly lower (approx. 30 %) CoA-SH levels compared to control trout and

Table 1. *Red muscle glycolytic intermediates, glycerol, intramuscular triacylglycerol, free CoA and short chain fatty acyl-carnitine concentrations in control fish and fish swum at 30, 60 and 90 %* U_{crit} *for up to 240 min*

		Swimming speed								
		30 % U_{crit}			60 % U_{crit}			90 % U_{crit}		
Metabolite	Control	2 min	15 min	240 min	2 min	15 min	240 min	2 min	15 min	45 min
Fru 6-P	0.11±0.02	0.06±0.02^{a}	0.12±0.03^{a}	0.01±0.0^{*,a}	0.12±0.05^{a}	0.09±0.02^{a}	0.09±0.05^{a}	0.25±0.04^{*,b}	0.15±0.03^{a}	0.08±0.01
Gly 3-P	0.04±0.03	0.03±0.03^{a}	0.12±0.07^{a}	0.09±0.09^{a}	0.32±0.09^{a,b}	1.07±0.39^{*,b}	0.13±0.07^{a}	1.17±0.12^{*,b,c}	2.82±0.29^{*,c}	2.19±0.58*
Glycerol	2.03±0.37	0.93±0.19^{a}	1.58±0.25^{a}	1.94±0.41^{a}	1.27±0.45^{a}	1.71±0.37^{a}	2.22±0.50^{a}	1.19±0.55^{a}	2.57±0.53^{a}	5.20±0.96*
IMTG	207±10 (8)	198±12^{a} (8)	200±11^{a} (6)	181±12^{a} (8)	212±16^{a} (4)	197±22^{a} (5)	203±24^{a} (7)	201±8^{a} (7)	197±8^{a} (8)	216±24 (7)
CoA-SH	53.6±5.4	48.5±7.3^{a}	56.8±6.0^{a}	50.0±3.8^{a}	57.6±6.2^{a}	37.4±4.6^{*,b}	49.1±4.9^{a}	46.7±3.8^{a}	38.4±5.1^{*,b}	46.8±4.4
SCFA-Car	13±25	49±12^{a}	61±9^{a}	33±10^{a}	49±4^{a}	10±8^{a}	31±19^{a}	1±1^{a}	1±1^{b}	30±19

Values are means ± S.E.M., N=8 except where indicated in parentheses.

Fru 6-P, fructose 6-phosphate; Gly 3-P, glycerol 3-phosphate; IMTG, intramuscular triacylglycerol; CoA-SH, free CoA; SCFA-carnitine, short chain fatty acyl-carnitine.

Fru 6-P, Gly 3-P and glycerol, values are μmol g^{-1} wet tissue; IMTG is expressed in glycerol units (1 glycerol=3 fatty acids from IMTG); CoA-SH and SCFA-Car values are nmol g^{-1} wet tissue.

*Value significantly different from control (P<0.05); within a time period, values with different letters are significantly different.

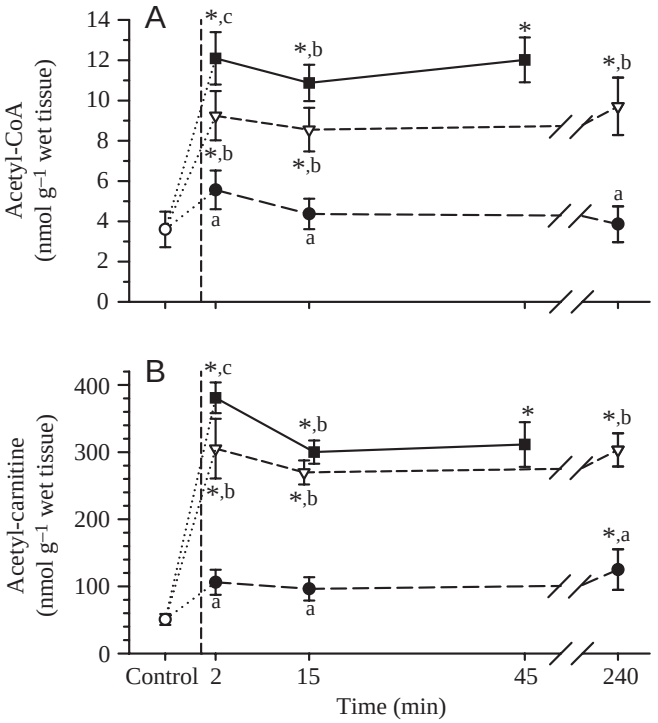

Fig. 6. Red muscle acetyl-CoA (A) and acetyl-carnitine (B) concentrations in control fish (open circles) and in fish swum at 30 % U_{crit} (filled circles) and 60 % U_{crit} (open triangles) for up to 240 min and at 90 % U_{crit} (filled squares) for up to 45 min. See Fig. 2 caption for other details.

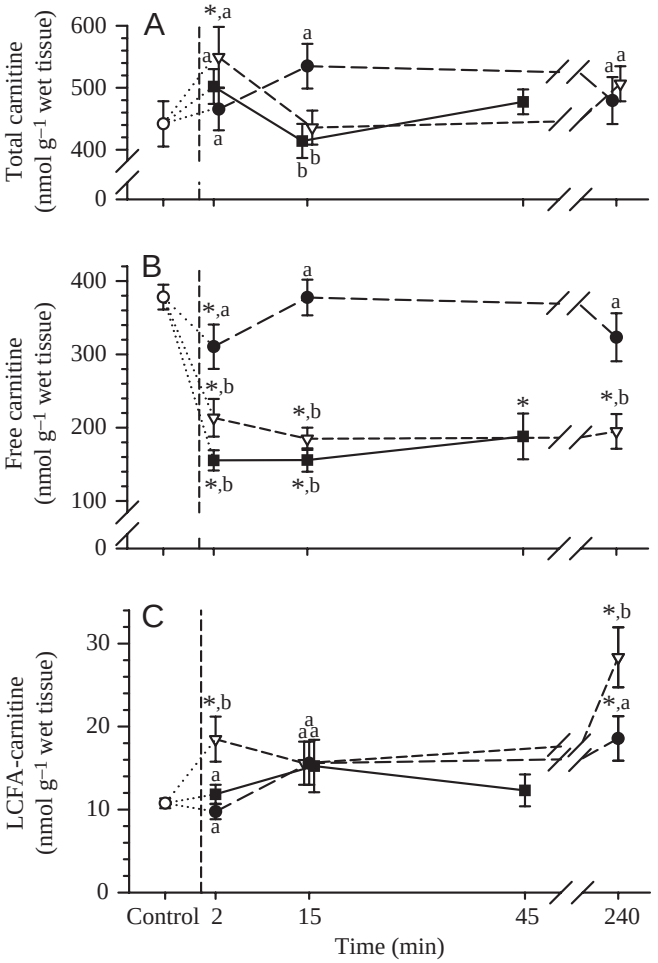

Fig. 7. Red muscle total carnitine (A), free carnitine (B) and long-chain fatty acyl (LCFA)-carnitine (C) concentrations in control fish (open circles) and in fish swum at 30 % U_{crit} (filled circles) and 60 % U_{crit} (open triangles) for up to 240 min and at 90 % U_{crit} (filled squares) for up to 45 min. See Fig. 2 caption for other details.

trout swimming at 30 % U_{crit} (Table 1). During the first 2 min of swimming, red muscle [acetyl-CoA] increased by 1.5-, 2.6- and 3.6-fold in trout swimming at 30, 60 and 90 % U_{crit}, respectively, and remained at these elevated levels throughout the swimming period (Fig. 6A). Similarly, during the first 2 min of swimming, there were 2.1-, 6.0- and 7.5-fold increases in [acetyl-carnitine] in trout swimming at 30, 60 and 90 % U_{crit}, respectively (Fig. 6B). These elevations in [acetyl-carnitine] remained constant for the duration of the swimming.

Swimming speed and duration had no effect on red muscle total carnitine concentration, except for a minor, but significant elevation after 2 min of swimming at 60 % U_{crit} (Fig. 7A). Red muscle free-carnitine concentration decreased by 18 %, 56 % and 59 % in trout swimming for 2 min at 30, 60 and 90 % U_{crit}, respectively, and remained close to these values throughout the swimming period (Fig. 7B). Swimming speed or duration had no effect on red muscle SCFA-carnitine concentration, except that trout swimming at 90 % U_{crit} for 15 min had significantly lower SCFA-carnitine levels than trout swimming at 30 % U_{crit} (Table 1). [LCFA-carnitine] remained constant compared to the levels in control trout for the first 15 min of swimming at 30 % U_{crit}, then increased 1.7-fold by 240 min (Fig. 7C). At 60 % U_{crit}, there was an initial 1.6-fold increase in [LCFA-carnitine] at 2 min, and a 2.5-fold increase by 240 min. Swimming at 90 % U_{crit} for 45 min did not affect [LCFA-carnitine].

Red muscle [malonyl-CoA] did not change within the first 2 min of swimming at any of the three swimming speeds (Fig. 8). However, by 15 min, there were significant 55 % decreases in [malonyl-CoA] in fish swimming at 30 and 60 % U_{crit}. [Malonyl-CoA] remained at these levels for up to 240 min of swimming at these two speeds. In contrast, in trout swimming at 90 % U_{crit}, there was a gradual decrease in muscle [malonyl-CoA] to values that were 36 % lower than in control fish at 45 min.

White muscle

White muscle [CrP] did not change in trout swimming at 30 % U_{crit} for up to 240 min or in trout swimming at 60 % U_{crit} for up to 15 min (Fig. 9A). At 240 min of swimming at 60 % U_{crit}, there was a 43 % decrease in [CrP]. At 90 % U_{crit}, muscle [CrP] decreased by 33 % at 2 min and 67 % at 45 min. White muscle [ATP] did not change in trout swimming at 30 or 60 % U_{crit} for up to 240 min (Fig. 9B). At 90 % U_{crit}, white muscle

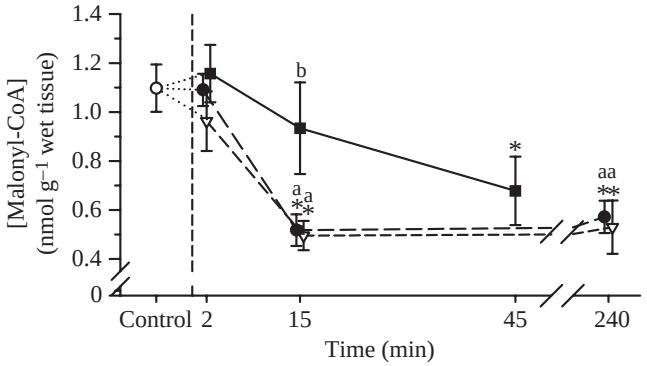

Fig. 8. Red muscle malonyl-CoA concentrations in control fish (open circles) and in fish swum at 30 % U_{crit} (filled circles) and 60 % U_{crit} (open triangles) for up to 240 min and at 90 % U_{crit} (filled squares) for up to 45 min. See Fig. 2 caption for other details.

[ATP] remained at control values for the first 15 min of swimming, but decreased by 46 % at 45 min.

White muscle glycogen levels remained relatively stable in trout swum at 30 % U_{crit}, except for a small, but significant decrease at 15 min, which recovered by 240 min (Fig. 10A). There was an initial 18 % decrease in white muscle [glycogen] at 2 min of swimming at 60 % U_{crit}, which decreased by a further 34 % by 240 min. At 90 % U_{crit}, there was a 33 % decrease in [glycogen] at 2 min, which continued to decrease (by 73 %) at 45 min. There were no changes in white muscle [lactate] in trout swum at 30 and 60 % U_{crit} for 240 min (Fig. 10B). Trout swum at 90 % U_{crit} for 2 min showed a 3.8-fold increase in white muscle [lactate], which further increased at 15 min to 7.3-fold the control values, then remained constant until 45 min. This rise in white muscle [lactate] at 90 % U_{crit} (approx. 8 μmol g^{-1} wet tissue) only accounts for approx. 30 % of the relative fall in white muscle [glycogen] (approx. 13 μmol glycosyl units g^{-1} wet tissue =approx. 26 μmol lactate units g^{-1} wet tissue).

Swimming speed or duration had no effect on white muscle PDH activity: mean PDH activity was 39.8±4.4 nmol g^{-1} wet tissue min^{-1} (N=35; data not shown).

Discussion

Overview

The present study is the first to examine the time course of substrate utilization by red and white muscle in trout during sustainable (at 30 % and 60 % U_{crit}; 240 min) and non-sustainable (at 90 % U_{crit}; 45 min) swimming. Sustainable swimming is powered predominantly by red muscle, with only minor changes in cellular energy status and small depletions of intramuscular substrates. Furthermore, there is a shift from the initial oxidation of carbohydrate observed during the first 2 min of swimming to oxidation of lipid during sustained swimming (>15 min). Non-sustainable swimming at 90 % U_{crit} is characterized by large depletions of intramuscular fuels, with oxidative phosphorylation of carbohydrate predominating in red muscle, and substrate level phosphorylation predominating in white muscle.

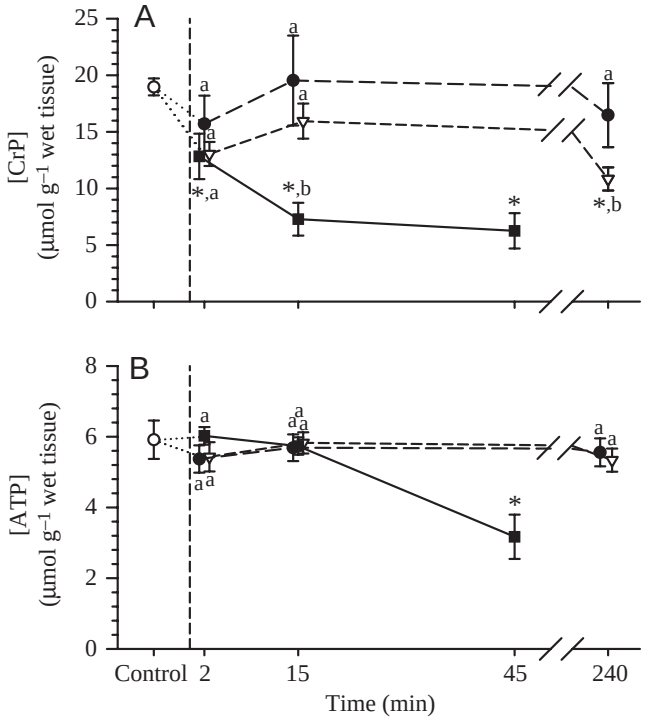

Fig. 9. White muscle CrP (A) and ATP (B) concentrations in control fish (open circles) and in fish swum at 30 % U_{crit} (filled circles) and 60 % U_{crit} (open triangles) for up to 240 min and at 90 % U_{crit} (filled squares) for up to 45 min. See Fig. 2 caption for other details.

Our data generally agree with previous studies suggesting that mainly red muscle is used to power swimming at speeds up to 70 % or 80 % U_{crit}, beyond which both red and white muscle contribute (Hudson, 1973; Johnson, 1981; Wilson and Egginton, 1994; Burgetz et al., 1998). At all swimming speeds, there were some changes in red muscle metabolites (e.g. Figs 2A, 6A,B) and PDH activity (Fig. 5), indicating that red muscle contributes to power production during swimming. During sustained swimming, there were moderate decreases in white muscle [CrP] after 240 min at 60 % U_{crit} (Fig. 9A), and small decreases in white muscle [glycogen] at 2 min and 240 min at 60 % U_{crit}, and at 15 min at 30 % U_{crit} (Fig. 10A). These minor decreases in [CrP] and [glycogen] suggest that there is an early but minor recruitment of white muscle during sustainable swimming, possibly to power bursts. The larger depletions of white muscle [CrP] observed over 45 min at 90 % U_{crit} (Fig. 9A), along with decreases in white muscle [ATP] (Fig. 9B) and [glycogen] (Fig. 10A), point to a substantial recruitment of white muscle during non-sustainable swimming. These results are in general agreement with those of Burgetz et al. (1998) who demonstrated a significant contribution of white muscle ‘anaerobic’ metabolism in trout at ≥70 % U_{crit}. The recruitment of white muscle probably acts to enhance swimming performance by providing greater power output, but may also play a role in supplying lactate to the red muscle for oxidation, particularly at high swimming speeds (Moyes and West, 1995). Note that lactate buildup was less

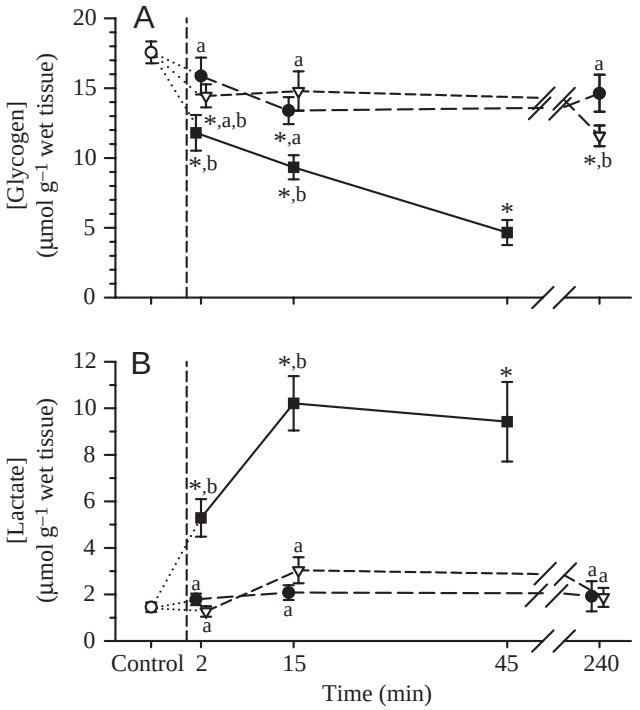

Fig. 10. White muscle glycogen (A) and lactate (B) concentrations in control fish (open circles) and in fish swum at 30 % U_{crit} (filled circles) and 60 % U_{crit} (open triangles) for up to 240 min and at 90 % U_{crit} (filled squares) for up to 45 min. See Fig. 2 caption for other details.

than glycogen depletion in white muscle at 60 and 90 % U_{crit} (Fig. 10A,B), suggesting that lactate was exported for oxidation elsewhere.

Sustainable swimming

During sustainable swimming at 30 % and 60 % U_{crit} there were increases in $\dot{M}_{O_2}$ and thus the oxidative generation of ATP (Fig. 1). Our $\dot{M}_{O_2}$ data agree well with those previously reported for trout at rest (90 μmol kg^{-1} body mass min^{-1}) and during swimming at 45 % and 75 % U_{crit} (107 and 158 μmol kg^{-1} body mass min^{-1}, respectively; Kieffer et al., 1998). Similarly, Lauff and Wood (1996) found resting rainbow trout had an $\dot{M}_{O_2}$ of 92 μmol kg^{-1} body mass min^{-1}, which increased to 150 μmol kg^{-1} body mass min^{-1} at 55 % U_{crit}. This increase in $\dot{M}_{O_2}$ with increasing swimming speeds probably reflects an increase in red muscle ATP turnover from oxidative phosphorylation, which occurs in the absence of changes in red muscle [ATP] (Fig. 2B) and with only very minor changes in red muscle [CrP] (Fig. 1A) and [glycogen] (Fig. 3A).

Substrate utilization at 30 % and 60 % U_{crit} can be divided into two phases: an initial acclimatization period that relies on carbohydrate oxidation, followed by a prolonged period that is characterized by enhanced lipid oxidation. During the first 2 min at 60 % U_{crit} there is an increase in glycolytic flux, indicated by the accumulation of Glu 6-P (Fig. 4B), and a small, non-significant decrease in [glycogen] (Fig. 3A). By analogy with the data of Richards et al. (2002b), this increase in glycolysis was probably mediated through the transformation of the rate-limiting enzyme glycogen phosphorylase into its active form. Enhanced glycolysis, without an increase in lactate production (Fig. 2B), yields sufficient substrate for the speed-dependent increase in PDH activity (Fig. 5). This increase in PDH activity observed at 30 and 60 % U_{crit} indicates an increase in the catalytic rate of PDH and tricarboxylic acid (TCA) cycle flux and oxidative phosphorylation.

Pyruvate dehydogenase is the rate-limiting enzyme for entry of glycolytically derived pyruvate into the TCA cycle for ATP production *via* oxidative phosphorylation. In mammals, PDH activity is regulated by reversible covalent modification and by product inhibition (Weiland, 1983). At the onset of exercise, Ca^{2+} release from the sarcoplasmic reticulum probably acts as the initial cue to activate red muscle PDH *via* a stimulation of PDH phosphatase, which dephosphorylates and activates PDH. The increase in PDH activity at 2 min of swimming (Fig. 5) resulted in product accumulation as acetyl-CoA (Fig. 6A). However, [acetyl-CoA] was kept relatively low within the mitochondria through the formation of acetyl-carnitine (Fig. 6B), which can be transported from the mitochondrial matrix to the cytoplasm. Total acetyl group accumulation accounted for only 7 and 19 % of the total PDH activity during the first 2 min at 30 and 60 % U_{crit}, respectively, indicating a large increase in acetyl-CoA oxidation.

During sustained swimming (15–240 min) at 30 and 60 % U_{crit}, PDH activity (Fig. 5) returned to control values, indicating a relative reduction in carbohydrate oxidation and a shift toward lipid oxidation. Inactivation of PDH after 15 min was probably *via* a mechanism similar to that proposed by the glucose–fatty acid cycle in mammals (van der Vusse and Reneman, 1996; Randle, 1998). We have previously demonstrated that the regulation of PDH transformation in fish white muscle during high-intensity exercise (Richards et al., 2002b) and recovery (Richards et al., 2002a) is similar to that described in mammals. During submaximal exercise, increase in β-oxidation causes a sustained elevation in acetyl-CoA/CoA-SH ratio (cf. Fig. 6A, Table 1) and an increase in [NADH]/[NAD^+]. These increases in acetyl-CoA and NADH levels may override the stimulatory effects of Ca^{2+} on PDH phosphatase. They may also reduce the transformation of PDH by stimulating PDH kinase, resulting in the phosphorylation and deactivation of PDH. Increased PDH kinase activity has been proposed to explain reduced carbohydrate oxidation in rat red quadriceps muscle during sustained exercise (Denyer et al., 1991). Sustained elevations of muscle [acetyl-CoA] would also inhibit PDH activity *in vivo* through product inhibition.

During sustained exercise at 60 % U_{crit} (240 min) there was a small, but significant, decrease in red muscle [glycogen] (Fig. 3A), suggesting that some carbohydrate utilization persisted. Based on measured red muscle PDH activity observed during sustained swimming (15–240 min), and taking into account the fact that red muscle constitutes 7 % of the body mass (Moyes and West, 1995), carbohydrate oxidation at 30 and 60 % U_{crit}

could account for 47 and 44 % of the $\dot{M}_{O_2}$, respectively. If we assume in the present study that 20 % of the $\dot{M}_{O_2}$ is due to protein oxidation (from Lauff and Wood, 1996), then the remaining increase in $\dot{M}_{O_2}$ observed at 30 and 60 % U_{crit} (33 and 36 % of $\dot{M}_{O_2}$, respectively) must be due to increased lipid oxidation. Thus, sustained swimming is supported by the oxidation of approx. 45 % carbohydrate, 35 % lipid and 20 % protein. These relative increases in $\dot{M}_{O_2}$ due to lipid oxidation at 30 and 60 % U_{crit} can be supported by the oxidation of only 4.4 and 7.3 μmol g^{-1} wet mass palmitate over the swimming period (15–240 min). Oxidation of these low concentrations of palmitate would yield changes in red muscle IMTG of only 1.5–2.4 μmol glycerol units g^{-1} red muscle from 15 to 240 min, concentrations that are well within the error of IMTG measurement (Table 1). Therefore, in the present study, no changes in IMTG were expected or observed.

During sustained swimming, however, there were small, but significant increases in LCFA-carnitine levels at 240 min of swimming at 30 and 60 % U_{crit} (Fig. 7C). In muscle, carnitine plays two major roles (Brass, 2000). First, carnitine acts to transport long-chain fatty acids into the mitochondria for β-oxidation. Second, excess acetyl groups from acetyl-CoA are bound to carnitine, forming acetyl-carnitine, which keeps [acetyl-CoA] relatively low within the mitochondria in order to sustain flux through β-oxidation. In trout red muscle, there is a clear reliance on carnitine to bind acetyl groups from acetyl-CoA production (Figs 6, 7). However, the significant increase in [LCFA-carnitine] observed during sustained swimming (Fig. 7C) implicates long-chain fatty acid oxidation as the source of acetyl groups. Short-chain fatty acyl-carnitines are also oxidized at high rates in mitochondria isolated from carp red muscle (Moyes et al., 1989), but we observed no increase in SCFA-carnitine levels during exercise (Table 1). This does not preclude the oxidation of short-chain fatty acids, however, because their movement across the inner mitochondrial membrane is not necessarily dependent on carnitine (van der Vusse and Reneman, 1996).

The binding of long-chain fatty acids to carnitine is catalyzed by carnitine palmitoyltransferase-1 (CPT-1), which is thought to be the rate-limiting step in fatty acid oxidation (van der Vusse and Reneman, 1996). Based on mammalian research, CPT-1 is thought to be regulated *in vivo* by malonyl-CoA production (Ruderman et al., 1999). Malonyl-CoA is the first committed step in fatty acid synthesis and is formed by the acetyl-CoA carboxylase carboxylation of acetyl-CoA. During sustained exercise at 30 and 60 % U_{crit} (≥15 min), malonyl-CoA concentrations decreased in trout red muscle (Fig. 8) despite a sustained elevation in acetyl-CoA (Fig. 6A). These decreases in [malonyl-CoA] would relieve the resting inhibition of CPT-1 during sustained exercise and enhance fatty acid oxidation through an increase in carnitine-dependent transport of fatty acids into the mitochondria. In rat muscle, submaximal exercise reduces the concentration of malonyl-CoA, as seen in trout red muscle (Fig. 8), and relieves inhibition of CPT-1. However, the response of CPT-1 to malonyl-CoA is not consistent between species. In the human, submaximal exercise at 60 % V_{O_2max} does not cause a decrease in muscle [malonyl-CoA], despite the fact that this exercise intensity is characterized by enhanced lipid oxidation (Romijn et al., 1993). In trout, malonyl-CoA may play different roles in red *versus* white muscle. We have previously demonstrated that during recovery from high-intensity exercise, a period characterized by enhanced white muscle lipid oxidation (low PDH activity, elevated acetyl-CoA, decreased IMTG and increased LCFA-carnitine levels), white muscle [malonyl-CoA] increases rather than decreases (Richards et al., 2002a). This increase in [malonyl-CoA] observed in white muscle during recovery from high-intensity exercise may act to elongate fatty acids for oxidation, as proposed by Richards et al. (2002a). These apparent differences in the regulation of CPT-1 by malonyl-CoA in red and white muscle of trout merit further research.

In the present study, we provide indirect biochemical evidence for lipid oxidation during sustained exercise. Our results agree well with those of Lauff and Wood (1996), who assessed substrate use in juvenile trout by respirometry. However, direct evidence for enhanced lipid oxidation during sustained exercise remains to be demonstrated. Bernard et al. (1999) demonstrated that triacylglycerol:fatty acid cycling is not enhanced during endurance exercise in trout. However, basal rates of triacylglycerol:fatty acid cycle are high in trout so could not limit fatty acid utilization during exercise. Quantification of the absolute rates and relative contributions of endogenous and exogenous lipids in fuelling sustained swimming needs to be addressed in isolated muscles using pulse-chase techniques (Dyke et al., 1997).

Non-sustainable swimming

During swimming at 90 % U_{crit}, there was a sustained elevation of red muscle PDH activity (Fig. 5) and a large depletion of red muscle glycogen and accumulation of lactate (Fig. 3A,B), indicating that trout rely heavily on carbohydrate as the substrate for high-speed swimming. In particular, the high PDH activity indicates that oxidative phosphorylation of carbohydrate is a major source of ATP at 90 % U_{crit}. In addition, there was a significant contribution of white muscle CrP (Fig. 9A) hydrolysis and glycolysis (Fig. 10A,B) to total power output and a potential role of the white muscle supplying substrate, as lactate, to the red muscle.

The contributions of lipid oxidation during swimming at 90 % U_{crit} are likely to be smaller than at 30 and 60 % U_{crit}. In the present study, we did not observe changes in [LCFA-carnitine] in red muscle of trout swimming at 90 % U_{crit} for 45 min and only demonstrated decreased red muscle malonyl-CoA concentrations at 45 min of swimming. However, given the large ATP turnover generated by a small amount of lipid oxidation, we cannot rule out a contribution of lipid oxidation to non-sustainable exercise. These data agree with those of Lauff and Wood (1996), indicating a greater reliance on carbohydrate as the carbon source during high-speed swimming at 80 % U_{crit} and a lower, but sustained utilization of lipid fuels.

In trout swimming at 90 % U_{crit}, approx. 25 % of the substrate

required to support the measured red muscle PDH activity could have been supplied by endogenous glycogen, the remaining substrate being supplied by hepatic glucose release or by white muscle lactate production (Moyes and West, 1995). However, Shanghavi and Weber (1999) have recently demonstrated that hepatic glucose release does not contribute significantly to red muscle ATP turnover in trout swimming at approx. 70 % U_{crit}. Numerous studies have proposed that the large store of white muscle glycogen could contribute substrate to red muscle during steady-state exercise (e.g. Wokoma and Johnson, 1981). In the present study, the observed decrease in white muscle glycogen at 60 and 90 % U_{crit} could not be accounted for solely by white muscle lactate accumulation. Taking into account the fact that white muscle PDH activity did not change with swimming speed or duration (PDH activity was approx. 40 nmol g^{-1} wet mass min^{-1}), the difference between glycogen depletion and combined lactate accumulation and oxidation reveals a discrepancy of 2.0 and 15.6 μmol g^{-1} white muscle lactate at 60 and 90 % U_{crit}, respectively. Based on whole body calculations, if this lactate were transported from the white muscle to the red muscle for oxidation, the discrepancy could account for an additional 37 % (0.06 μmol g^{-1} red muscle min^{-1}) and 211.5 % (2.96 μmol g^{-1} red muscle min^{-1}) of the total substrate needed for the measured red muscle PDH activity at 60 and 90 % U_{crit}, respectively. However, based on estimates of lactate turnover in trout plasma during swimming at 85 % U_{crit} (9.7 μmol kg^{-1} min^{-1}; Weber, 1991), the primary circulation has the capacity to shuttle 10 % of the substrate required. Clearly, more work is needed to resolve this apparent discrepancy.

In conclusion, the present biochemical evidence indicates that sustained swimming in trout (30 and 60 % U_{crit}) is primarily supported by red muscle contraction where carbohydrate serves as the initial substrate for ATP production. During extended periods of sustained swimming there is a partial shift in substrate utilization away from carbohydrate to an activation of long-chain fatty acid oxidation. Non-sustainable swimming at 90 % U_{crit} relies extensively on carbohydrate as the substrate for ATP production, through both red muscle oxidative phosphorylation and white muscle glycolysis. The likely contributions of lipid oxidation to ATP production during swimming at 90 % U_{crit} are lower than at 30 and 60 % U_{crit}, but may still be important.

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