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GLYCEROL AND FATTY ACID KINETICS IN RAINBOW TROUT: EFFECTS OF ENDURANCE SWIMMING

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Summary

Continuous infusions of 2-[^{3}H]glycerol and 1-[^{14}C]palmitate were performed *in vivo* in rainbow trout to measure the effects of prolonged swimming on (1) the rate of appearance of glycerol (R_a glycerol or lipolytic rate), (2) the rate of appearance of non-esterified fatty acids (R_a NEFA) and (3) the rate of triacylglycerol:fatty acid cycling (TAG:FA cycling or re-esterification). Our goals were to test the hypothesis that sustained exercise for up to 4 days causes the progressive mobilization of triacylglycerol reserves to supply fuel to contracting muscles, and to assess whether TAG:FA cycling plays a role in the regulation of NEFA availability in teleosts. Contrary to expectation, the rates of lipolysis and fatty acid release in resting trout are not affected by endurance exercise. Unlike mammals, which increase the rate of lipolysis by two- to fourfold during submaximal exercise, these active teleosts do not mobilize triacylglycerol reserves beyond resting levels to supply more NEFAs to working muscles. Furthermore, they maintain R_a glycerol and R_a NEFA well in excess of oxidative fuel requirements even at rest. More than two-thirds of the NEFAs produced are re-esterified, but the results show that TAG:FA cycling is not involved in the regulation of NEFA availability during or after swimming. We propose that the observed high rates of re-esterification represent an important feature of ectothermic metabolism that allows the restructuring of membrane phospholipids to be synchronized with frequent changes in body temperature.

Key words: lipolysis, non-esterified fatty acid kinetics, lipid mobilization, *in vivo* substrate flux, exercise, rainbow trout, *Oncorhynchus mykiss*.

Introduction

It is generally accepted that non-esterified fatty acids (NEFAs) derived from lipid stores in muscle, liver and adipose tissue represent an important metabolic fuel for endurance swimming in teleosts (Driedzic and Hochachka, 1978; Henderson and Tocher, 1987; Lauff and Wood, 1997; Mommsen et al., 1980; Walton and Cowey, 1982). However, the evidence showing that fish mobilize these reserves during exercise is only indirect, and little is known about the process of triacylglycerol (TAG) breakdown in ectotherms (Henderson and Tocher, 1987; Moyes and West, 1995). Early studies have shown that the size of lipid stores decreases during migration (Idler and Bitners, 1960) or after 24 h of swim-tunnel exercise in salmon (Krueger et al., 1968), and that fish tissues contain active lipolytic enzymes (Sheridan, 1988). It is also known that the adipose tissue lipases of teleosts and mammals respond differently to common lipolytic hormones such as catecholamines and glucagon (Michelsen et al., 1994; Migliorini et al., 1992) and that the distribution of TAG is far more variable in fish than in mammals (Weber and Zwingelstein, 1995). Overall, however, lipid mobilization is much better documented in mammals because the regulation of their NEFA and glycerol fluxes has been investigated thoroughly (Van der Vusse and Reneman, 1996). Results from mammalian experiments clearly show that prolonged exercise causes the stimulation of triacylglycerol hydrolysis and leads to a major increase in the rates of appearance (R_a) of NEFA and glycerol (Shaw et al., 1975; Weber et al., 1993; Wolfe et al., 1990). The effects of exercise on NEFA and glycerol kinetics in fish are unknown, and their glycerol fluxes have never been measured *in vivo*, even at rest.

Upon hydrolysis, each triacylglycerol yields 3 NEFA and 1 glycerol, but the ratios of R_a NEFA/R_a glycerol measured in mammals are always below 3. This is because a fraction of the fatty acids released through lipolysis is re-esterified *in situ*, whereas the glycerol cannot be metabolized directly because of the absence of glycerokinase in adipocytes (Weber et al., 1993; Wolfe et al., 1990). The simultaneous occurrence of lipolysis and re-esterification forms the triacylglycerol:fatty acid cycle (TAG:FA cycle), a substrate cycle known to play an important role in the regulation of NEFA availability in humans (Wolfe et al., 1990). When mammalian triacylglycerol reserves are mobilized during prolonged exercise, lipolysis is stimulated and the percentage of total fatty acids re-esterified decreases, thereby providing more NEFAs for oxidation in

working muscles (Campbell et al., 1992; Newsholme and Crabtree, 1976).

The goal of this study was to test the hypothesis that sustained swimming causes an increase in the R_a glycerol and R_a NEFA of fish, a response similar to that of mammals exercising at equivalent submaximal intensities. In addition, the flux through the TAG:FA cycle was quantified to determine what percentage of total NEFAs is re-esterified in fish and to assess whether this cycle is involved in the regulation of fatty acid availability in teleosts. Rainbow trout *Oncorhynchus mykiss* were exercised for 1 h at 1 body length s^{-1} (BL s^{-1}), but no significant increase in lipolytic rate was measured under these conditions. Consequently, additional experiments were carried out at a higher speed (1.5 BL s^{-1}) or during 4 days of continuous swimming.

Materials and methods

Animals

Rainbow trout, *Oncorhynchus mykiss* (Walbaum), of both sexes (body mass 451–823 g) were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and held in a 1300 l flow-through tank at 13 °C. Routine swimming speed in the holding tank was 0–0.3 BL s^{-1} depending on the distance from the centre of the tank. The fish were kept in dechloraminated, well-oxygenated water under a 12 h:12 h L:D photoperiod. Animals were acclimated to these conditions for at least 1 month before experiments, and they were fed Purina trout chow until satiation three times a week. The experiments were carried out between June and mid-October 1996 (1 h of swimming) and in June and July 1997 (4 days of swimming).

Catheterization

Animals were not fed for 24 h before catheterization or during the experiments. The surgery consisted of a double cannulation of the dorsal aorta under ethyl-*N*-aminobenzoate sulphonic acid (MS-222) anaesthesia, as described previously (Haman and Weber, 1996). For the first series of experiments (1 h of swimming at 1 or 1.5 BL s^{-1}), heparin (Organon, Teknica) was used as an anticoagulant at 10 i.u. ml^{-1} of Cortland saline (Wolf, 1963). However, heparin itself could potentially stimulate lipolysis and it was substituted with sodium citrate (12.9 mmol l^{-1}) for the second series of experiments (swimming for 4 days at 1 BL s^{-1}) to investigate this possibility. After surgery, the fish were allowed to recover for 36 h in a swim tunnel with a weak water current (11 cm s^{-1}), just strong enough to ensure adequate oxygenation. Under these conditions, the animals were not swimming, but were resting on the floor of the swim tunnel.

Swim tunnel

Measurements were started 48 h after surgery in a modified Blazka-type swim tunnel (Beamish, 1978) consisting of a polyvinylchloride tube (1.5 m in length and 0.2 m in diameter) immersed in a 550 l flow-through tank. Two 'honeycomb' grids were placed inside the tube to delimit a 28 l swimming chamber (0.9 m long) and to ensure laminar flow. The upper part of this chamber was closed by a transparent lid with a longitudinal slit allowing access to the catheters. Water flow was powered by an electrical trolling motor (Mini Kota, 117 kPa thrust) connected to an adjustable power supply (Harrison 6433B DC, Hewlett Packard) to control water velocity. This swim tunnel was adequate to exercise fish at speeds up to 80 cm s^{-1}. Velocity was calibrated by video-taping the repeated release of a dye suspension through the chamber and by counting film frames. For each experiment, water velocity was corrected according to Webb (1974) and Nelson et al. (1996) to take into account the water acceleration around individual animals of different sizes. A metal grid was placed at the downstream end of the swimming chamber and connected to a 12 V power supply. It was manually activated on very rare occasions when the fish was leaning against it during measurements. The upstream end of the swimming chamber was kept darker than the downstream section, and the animals typically swam in the upstream portion to avoid bright light.

Continuous infusions of isotopes

Labelled substrates were infused as described previously (Haman et al., 1997a,b; Haman and Weber, 1996). The infusate was prepared daily with 2-[^{3}H]glycerol (Amersham; 37.0 GBq $mmol^{-1}$) and 1-[^{14}C]palmitate (Amersham; 1.85–2.2 GBq $mmol^{-1}$). Trout plasma was collected from donor individuals from the same batch of fish and used as a source of lipid-binding proteins. The 1-[^{14}C]palmitate was supplied commercially in toluene, dried under nitrogen and immediately resuspended in ethanol to obtain a solution of 37,000 Bq μl^{-1}. A subsample of this solution was mixed with 400 µl of plasma and well agitated before adding 2-[^{3}H]glycerol dissolved in Cortland saline. While the fish was at rest, an infusion of the isotope mixture was started using a calibrated syringe pump (Harvard Apparatus, South Natick, MA, USA) at 1 ml h^{-1}. Infusion rates ranged from 469×10^3 to 1040×10^3 disints min^{-1} kg^{-1} min^{-1} for both isotopes, or less than 0.5 nmol glycerol kg^{-1} min^{-1} and less than 8 nmol palmitate kg^{-1} min^{-1}. This corresponded to trace amounts of less than 0.01 % of R_a glycerol and less than 0.8 % of R_a palmitate.

Exercise protocols and blood sampling

In the first series of experiments, glycerol and fatty acid kinetics were quantified before, during and after 1 h of swimming at 1 BL s^{-1} (N=6 animals, 38.2±0.5 cm body length) or at 1.5 BL s^{-1} (N=6, 39.2±0.5 cm) (means ± S.E.M.). The isotope infusion was started 1 h before swimming and it was continued for 30 min after the end of exercise to monitor recovery. For every fish, nine blood samples each of 0.5 ml were drawn throughout the infusion. In a second series of experiments, glycerol and fatty acid kinetics were measured before and during 4 days of continuous swimming at 1 BL s^{-1} (N=6, 40.2±0.9 cm body length). On day 1, the first isotope infusion was performed just before the start of exercise to quantify resting kinetics. Every 24 h after the beginning of exercise, an additional 1 h infusion was performed to monitor

the effects of endurance swimming. A 0.8 ml blood sample was taken at the end of each infusion. Before the start of the last infusion (day 4), an additional blood sample was taken to measure residual activity from the four previous infusions (one at rest and three during exercise). However, the residual specific activities of glycerol and palmitate were negligible in these samples, and no correction was made in the calculations. In both series of experiments, haematocrit was measured on each blood sample. It was always above 20 % and did not decrease significantly throughout the experiments. At the end of each experiment, the animals were killed by an overdose of sodium pentobarbital injected through one of the catheters.

Preparation of blood samples

Immediately after sampling, the blood was centrifuged and the plasma was separated. Twenty-five per cent of the plasma was stored at −20 °C and used later to measure total NEFA concentration with an analytical test kit (NEFA C, Wako Chemicals, Osaka, Japan). The remaining 75 % of the plasma was mixed with 25 ml of chloroform:methanol (Folch, 2:1) (Folch et al., 1957) immediately after separation to avoid the adsorption of glycerol onto the walls of the tubes. At this step, glass tubes prewashed with a solution of 0.2 mmol l^{-1} glycerol and rinsed with chloroform:methanol (Folch 2:1) were used to minimize glycerol adsorption. Preliminary experiments with known amounts of labelled glycerol showed that immediate extraction of plasma and transfer to pretreated glass tubes reduced the loss of glycerol to less than 2 %. The aqueous and organic phases were separated and re-extracted with chloroform and methanol:water (40:30), respectively. After centrifugation, each tube contained an aqueous and an organic phase, which were separated before pooling the two aqueous phases and the two organic phases. Each pooled phase was then dried using a rotating evaporator (Büchi RE 121 Rotavapor). The aqueous phase was resuspended in ethanol:water (1:1) and the organic phase in hexane:isopropanol (3:2). The extraction protocol used in these studies allowed the recovery of 95–98 % of total ^{3}H and ^{14}C activities.

Glycerol

Glycerol concentration was determined on a volume of aqueous phase corresponding to 100 µl of plasma. This volume was dried under nitrogen and resuspended in hydrazine buffer before measuring the concentration of glycerol using a Beckman DU 640 spectrophotometer at 340 nm (Weber et al., 1993). At this step, the total tritium activity of the aqueous phase was only found in glycerol and glucose, and it was counted on the equivalent of 5 µl of plasma. Percentage activity in glycerol was obtained by separating glucose from glycerol using thin-layer chromatography (TLC); a subsample of the aqueous phase was evaporated and concentrated in ethanol:water before spotting it onto a silica gel plate (60 F_{254}, Merk, Germany). The plate was developed using chloroform:methanol (8:3), and the glucose and glycerol fractions were scraped into separate scintillation vials. They were resuspended in 3 ml of ethanol:water (1:1) and counted in ACS-II scintillation fluid (Amersham, Canada) using a Tri-Carb 2500 counter (Packard, Canada). No significant difference in absolute ^{3}H activity was found in glucose for fish swimming at 1 or 1.5 BL s^{-1}.

Fatty acids

The neutral lipids, NEFAs and phospholipids present in the organic phase were separated by filtration on Supelclean solid-phase extraction tubes (LC-NH2, Sigma, St Louis, MO, USA). The neutral lipids were eluted with chloroform:isopropanol (2:1), the NEFAs with isopropyl ether:acetic acid (98:2) and the phospholipids with methanol. A subsample of each fraction was counted to determine the distribution of ^{14}C and ^{3}H activity in plasma lipids. Because no ^{14}C is incorporated into fatty acids other than palmitate, the activity found in the NEFA fraction was equal to palmitate activity. The remainder of the NEFA fraction was used to determine the relative distribution of individual fatty acids using gas chromatography, as described previously (McClelland et al., 1995; Tserng et al., 1981).

Calculations and statistics

Palmitate concentration was calculated by multiplying NEFA concentration by the fractional contribution of palmitate to total NEFA. Glycerol R_a and palmitate R_a were calculated using the steady-state equation of Steele (1959). R_a NEFA was determined by dividing R_a palmitate by the fractional contribution of palmitate to total NEFAs. TAG:FA cycling (=fatty acid re-esterification) is known to occur both intracellularly (where fatty acids are re-esterified *in situ* without entering the circulation) and extracellularly (where they travel to their site of re-esterification through the circulation) (Newsholme and Crabtree, 1976; Wolfe et al., 1990). In the present study, only the absolute and relative rates of intracellular cycling were assessed, and they were calculated according to Wolfe et al. (1990):

$$\text{TAG:FA cycling} = (3 \times R_a \text{ glycerol}) - R_a \text{ NEFA},$$

$$\%\ \text{TAG:FA cycling} = (\text{TAG:FA cycling})/(3 \times R_a \text{ glycerol}).$$

A *t*-test was used to evaluate the effects of swimming speed (1.0 *versus* 1.5 BL s^{-1}) and anticoagulant (heparin *versus* sodium citrate). In all other cases, statistical differences were assessed using two-way analysis of variance (ANOVA) or Friedman's analysis of variance on ranks (when the assumption of normality or homoscedasticity was not met) with time and fish as the main factors. When significant changes were detected by ANOVA, Dunnett's test was used to determine which means were different from the resting value. Percentages were transformed to the arcsine of their square root before statistical analysis, and all values are given as means ± S.E.M. A level of significance of $P<0.05$ was used in all tests.

Results

Effects of short-term exercise

Swimming for 1 h at 1 BL s^{-1} had no significant effect on the concentrations and fluxes of glycerol, palmitate and total fatty acids ($P>0.05$). Therefore, values for all these variables

Table 1. *Mean plasma concentrations and rates of appearance of glycerol, palmitate and total non-esterified fatty acids throughout experiments where these variables were measured before, during and after swimming for 1 h at 1 BL s^{-1} in rainbow trout*

Variable	Mean value
[Glycerol] (mmol l^{-1})	0.38±0.03
Glycerol SA (disints min^{-1} μmol^{-1})	$1.26\times10^5\pm0.06\times10^5$
R_a glycerol (μmol kg^{-1} min^{-1})	8.1±0.7
[Palmitate] (μmol l^{-1})	124±5
Palmitate SA (disints min^{-1} μmol^{-1})	$8.18\times10^5\pm0.3\times10^5$
R_a palmitate (μmol kg^{-1} min^{-1})	1.09±0.07
[NEFA] (mmol l^{-1})	0.57±0.03
R_a NEFA (μmol kg^{-1} min^{-1})	4.85±0.24
TAG:FA cycling (μmol kg^{-1} min^{-1})	9.49±2.08
Percentage re-esterification	76.7±1.2

Specific activities (SA), calculated rates of TAG:FA cycling and percentage re-esterification are also presented.

Values are means ± S.E.M. (N=6).

R_a, rate of appearance; NEFA, non-esterified fatty acid; TAG, triacylglycerol; FA, fatty acid; BL, body length

of lipid metabolism were averaged throughout the experiments (Table 1). At this swimming speed, the rate of TAG:FA cycling stayed constant over time (P>0.05), averaging 9.49±2.08 μmol kg^{-1} min^{-1}, and 76.7±1.2 % of the fatty acids released by lipolysis were re-esterified (Table 1). Similarly, animals swimming for 1 h at the higher intensity of 1.5 BL s^{-1} showed no significant changes in these same variables of lipid metabolism (P<0.05) (Figs 1–3). Mean plasma glycerol and NEFA concentrations in animals swimming at 1.5 BL s^{-1} were 0.20±0.01 and 0.63±0.03 mmol l^{-1}, respectively. Glycerol and palmitate specific activities remained constant (P>0.05), but they differed among animals (P<0.001) because infusion rates varied among experiments (Figs 1B, 2B). Mean R_a glycerol and R_a NEFA in animals swimming at 1.5 BL s^{-1} were 3.47±0.24 and 3.73±0.28 μmol kg^{-1} min^{-1}, respectively. Swimming for 1 h at 1.5 BL s^{-1} had no effect on the rate of TAG:FA cycling (P>0.05). It averaged 6.73±0.59 μmol kg^{-1} min^{-1}, and 61.6±2.0 % of all the NEFAs released by lipolysis were re-esterified. Glycerol concentration, R_a glycerol, R_a palmitate and R_a NEFA were significantly lower at 1.5 than at 1 BL s^{-1} (P<0.001). This

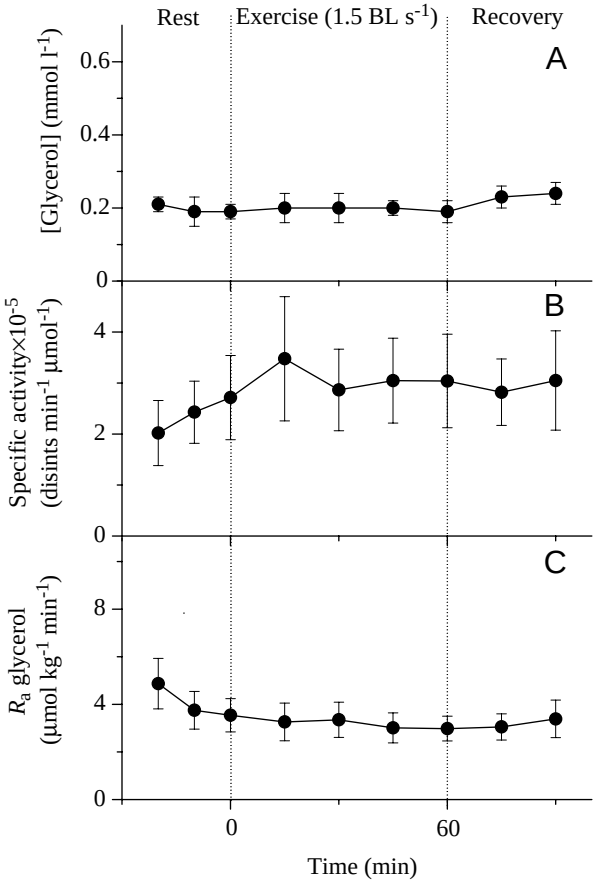

Fig. 1. Glycerol concentration (A), specific activity (B) and rate of appearance R_a (C) in rainbow trout during rest, exercise at 1.5 BL s^{-1} and recovery. Values are means ± S.E.M. (N=6). BL, body length.

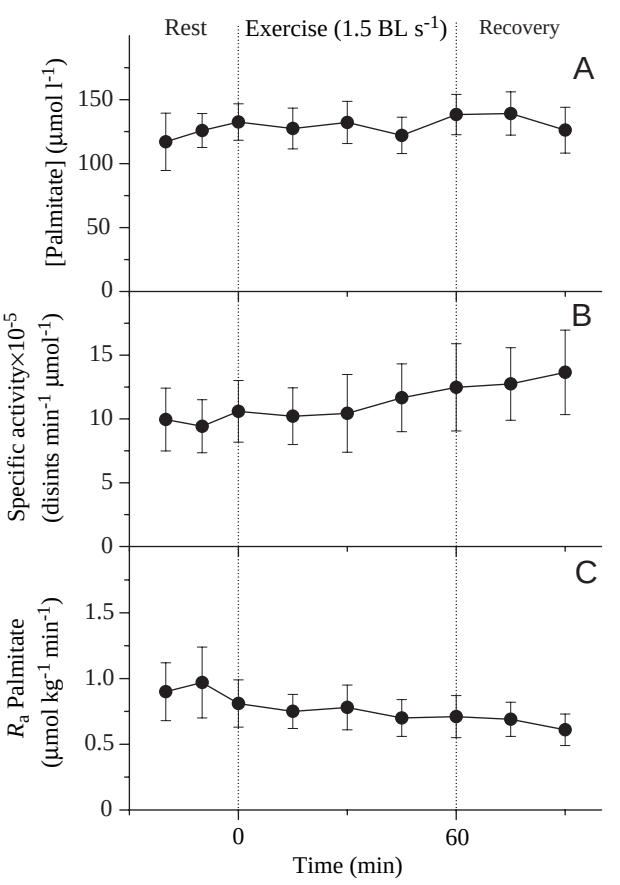

Fig. 2. Palmitate concentration (A), specific activity (B) and rate of appearance R_a (C) in rainbow trout during rest, exercise at 1.5 BL s^{-1} and recovery. Values are means ± S.E.M. (N=6). BL, body length.

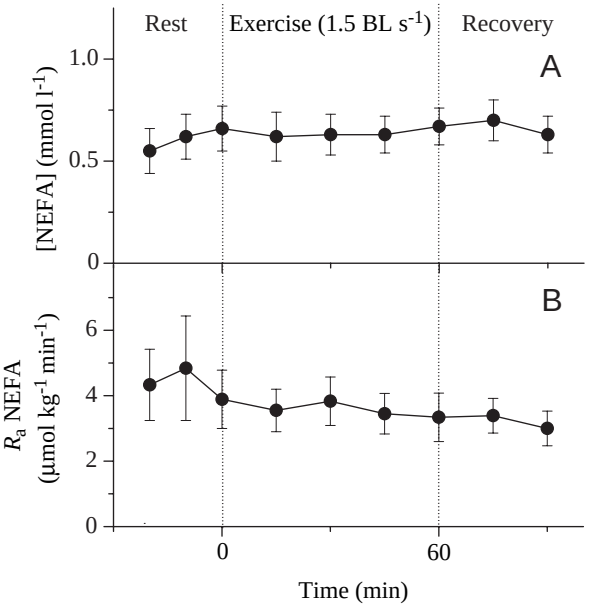

Fig. 3. Non-esterifed fatty acid (NEFA) concentration (A) and rate of appearance R_a (B) in rainbow trout during rest, exercise at 1.5 BL s^{-1} and recovery. Values are means ± S.E.M. (N=6). BL, body length.

difference was already present at rest for glycerol concentration and R_a, but this was not the case for R_a palmitate and R_a NEFA. The absolute and relative rates of re-esterification were also significantly different between the two speeds, even at rest (P<0.001).

Effects of prolonged exercise

Four days of swimming at 1 BL s^{-1} had no significant effects on the concentrations and kinetics of glycerol, palmitate and NEFA (P>0.05, Figs 4–6). Specific activities were intentionally omitted from these figures because the use of different infusion rates on different days for repetitive experiments on each fish prevented these values from providing meaningful information on isotopic steady state. The mean rate of TAG:FA cycling was 7.66±0.92 μmol kg^{-1} min^{-1} and did not change between rest, exercise and recovery (P>0.05). Throughout the experiments, 63.8±2.8 % of all the NEFAs released by lipolysis were re-esterified. Table 2 presents the NEFA composition of trout plasma before and during exercise. Four days of swimming had no effect on the relative contributions of individual fatty acids to total NEFAs (P>0.05).

Distribution of radioactivity in plasma metabolites

The pattern of incorporation of radioactivity observed during the short-term experiments at 1 BL s^{-1} did not differ from that at 1.5 BL s^{-1}, so only the data obtained at 1.5 BL s^{-1} are presented in Fig. 7. The percentage of ^3H activity in glycerol decreased as tritiated glycerol was progressively incorporated into neutral lipids (P<0.05). In contrast, the percentage of total tritium activity in plasma glucose and phospholipids did not change over time (P>0.05) (Fig. 7A). A

Table 2. *Fatty acid composition as a percentage of total non-esterified fatty acids for rainbow trout plasma before and during four days of continuous swimming at 1 BL s^{-1}*

		Swimming period (days)			
NEFA	Rest	1	2	3	4
16:0	25.1±0.9	25.3±0.9	24.5±0.5	25.7±1.0	24.8±0.4
16:1	5.4±0.3	6.1±0.4	6.0±0.3	6.2±0.3	6.3±0.4
18:0	22.7±1.2	19.5±1.4	19.4±1.1	18.3±1.2	20.1±1.2
18:1	14.6±1.3	16.3±1.4	16.8±1.0	16.7±1.3	16.1±1.4
18:2	4.2±0.4	4.8±0.4	5.5±0.4	5.7±0.1	5.7±0.3
18:3	7.3±0.7	8.4±0.9	8.2±0.7	8.2±0.7	7.8±0.6
20:0	3.7±0.6	3.5±1.1	4.0±1.0	3.7±0.9	3.9±1.1
20:4	1.5±0.1	1.4±0.2	1.5±0.2	1.4±0.2	1.6±0.3
22:1	10.3±2.3	9.9±1.8	9.3±1.2	8.9±1.1	9.0±0.9
22:6	4.3±1.0	4.8±1.0	5.0±0.8	5.3±1.1	4.8±0.7

Values are means ± S.E.M. (N=6).
Exercise did not cause any significant change in NEFA composition (P>0.05).
NEFA, non-esterified fatty acid.

similar pattern was observed for ^{14}C. The percentage of total ^{14}C activity decreased in NEFAs as palmitate was progressively incorporated into neutral lipids (P<0.05), whereas ^{14}C activity in phospholipids stayed constant and very low (Fig. 7B).

During the long-term experiments, multiple isotope infusions were performed over 4 days and, therefore, the distribution of ^3H and ^{14}C was very different from that observed during the short-term experiments. Tritiated glycerol and [^{14}C]palmitate were both incorporated in small amounts into neutral lipids (P<0.01) and in large amounts into phospholipids (P<0.01)

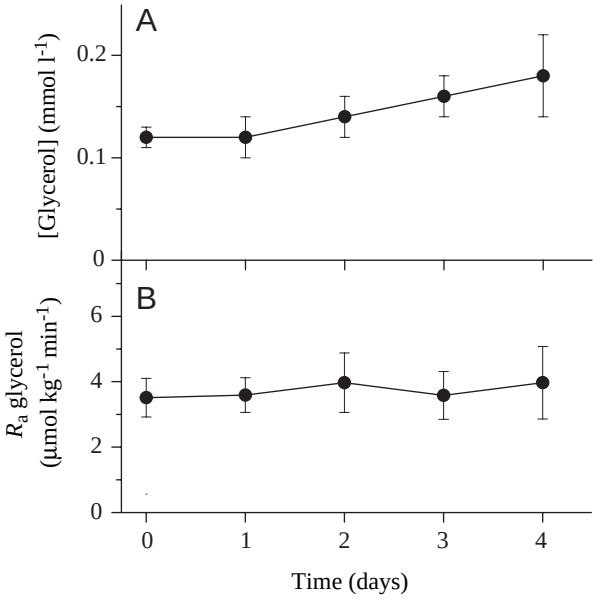

Fig. 4. Glycerol concentration (A) and rate of appearance R_a (B) in rainbow trout at rest (day 0) and during 4 days of exercise at 1 BL s^{-1}. Values are means ± S.E.M. (N=6). BL, body length.

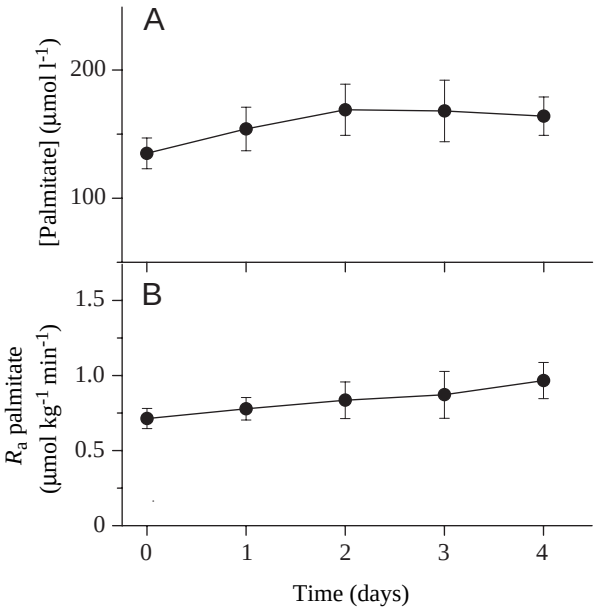

Fig. 5. Palmitate concentration (A) and rate of appearance R_a (B) in rainbow trout at rest (day 0) and during 4 days of exercise at 1 BL s^{-1}. Values are means ± S.E.M. (N=6). BL, body length.

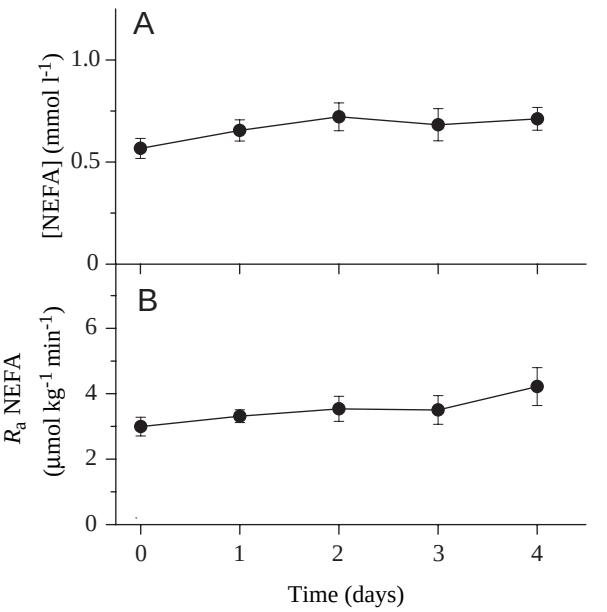

Fig. 6. Non-esterifed fatty acid (NEFA) concentration (A) and rate of appearance R_a (B) in rainbow trout at rest (day 0) and during 4 days of exercise at 1 BL s^{-1}. Values are means ± S.E.M. (N=6). BL, body length.

(Fig. 8). This incorporation was accompanied by concomitant decreases in the percentage of ^3H activity in glucose and in the percentage of ^{14}C activity in NEFAs (P<0.01).

Choice of anticoagulant

Mean resting values for concentrations and R_a of glycerol and NEFA were calculated for all experiments where heparin was used as an anticoagulant, and this was repeated for the experiments where sodium citrate was used (Table 3). Plasma glycerol concentration was higher with heparin (P<0.05), but the choice of anticoagulant had no significant effect on NEFA concentration, R_a NEFA or R_a glycerol (P>0.05).

Discussion

Contrary to expectation, the present study shows that rainbow trout do not mobilize their triacylglycerol reserves beyond resting levels, even when they swim continuously for 4 days, covering a mean distance of 140 km. It provides the first *in vivo* measurements of lipid kinetics and re-esterification during prolonged exercise in an ectothermic animal. Here, metabolic rates could not be quantified by respirometry because the swim tunnel available for our experiments was not a closed system. However, using data from other investigations of exercising salmonids (Beamish, 1978; Kiceniuk and Jones, 1977; Webb, 1971), we can estimate that swimming speeds of 1 and 1.5 BL s^{-1} correspond to exercise intensities of 30 and 50 % $\dot{M}_{O_2max}$, or to 50 and 70 % of maximum sustainable swimming speed (U_{crit}) for animals of this size. In mammals, aerobic exercise causes a very strong stimulation of lipolysis with a maximal response occurring between 40 and 60 % $\dot{M}_{O_2max}$. The lipolytic responses of exercising vertebrates that are currently known are summarized in Fig. 9. Most mammalian species measured to date increase R_a glycerol and R_a NEFA by two- to fourfold (Weber et al., 1996; Wolfe et al., 1990), whereas rainbow trout maintain these fluxes at resting levels (Figs 1–3), even when they swim for several days (Figs 4–6) at a mean speed normally used by migrating salmonids in the wild (Beamish, 1978; Quinn, 1988).

Table 3. *Resting concentrations and rates of appearance of glycerol and non-esterified fatty acids in rainbow trout in tracer infusion experiments where heparin or sodium citrate was used as an anticoagulant*

Anticoagulant	N	[Glycerol] (mmol l^{-1})	R_a glycerol (μmol kg^{-1} min^{-1})	[NEFA] (mmol l^{-1})	R_a NEFA (μmol kg^{-1} min^{-1})
Heparin	12	0.25±0.13	5.90±0.48	0.62±0.42	4.39±0.39
Sodium citrate	6	0.12±0.01*	3.51±0.59	0.58±0.05	2.99±0.29

Values are means ± S.E.M.
* indicates a significant difference between anticoagulants.
R_a, rate of appearance; NEFA, non-esterified fatty acid.

Potential limitations of this study

What are the possible reasons why no changes in lipid kinetics were observed? First, it could be argued that exercise did not cause an increase in R_a glycerol and R_a NEFA because the animals were not truly at rest at the beginning of the experiments. Therefore, lipolysis may already have been stimulated before swimming started. Previous results show that such a scenario is very unlikely. In experiments where metabolic rate and NEFA kinetics were measured simultaneously in animals resting in opaque acrylic boxes (Haman et al., 1997b), true resting metabolic rates were observed ($41\,\mu mol\,O_2\,kg^{-1}\,min^{-1}$) together with mean R_a NEFA values that were similar to or greater than those measured here ($4.8–5.9\,\mu mol\,kg^{-1}\,min^{-1}$). Alternatively, no change in R_a NEFA would have been observed if trout had relied mainly on the mobilization of intramuscular triacylglycerol (TAG) reserves rather than on circulating NEFAs because the fatty acids oxidized in contracting muscles would not appear in the blood (Moyes and West, 1995; Wolfe et al., 1990). In fact, early *in vitro* experiments suggest that red muscle palmitate may not enter the circulation before being oxidized (Bilinski and Lau, 1969). However, the fact that R_a glycerol does not increase during exercise in our experiments argues strongly against this possibility because trout muscles lack glycerokinase activity (Newsholme and Taylor, 1969) and, therefore, cannot metabolize the glycerol generated from muscle TAG *in situ*. The stimulation of TAG hydrolysis in trout muscle would have to be accompanied by an increase in R_a glycerol.

Role of the triacylglycerol:fatty acid substrate cycle in fish

The rates of appearance of glycerol and NEFA have allowed us to determine that, on average, 68 % of all the fatty acids released by lipolysis are re-esterified intracellularly in rainbow trout. Similar relative rates of re-esterification have been observed in mammals, where more than 60 % of the fatty acids

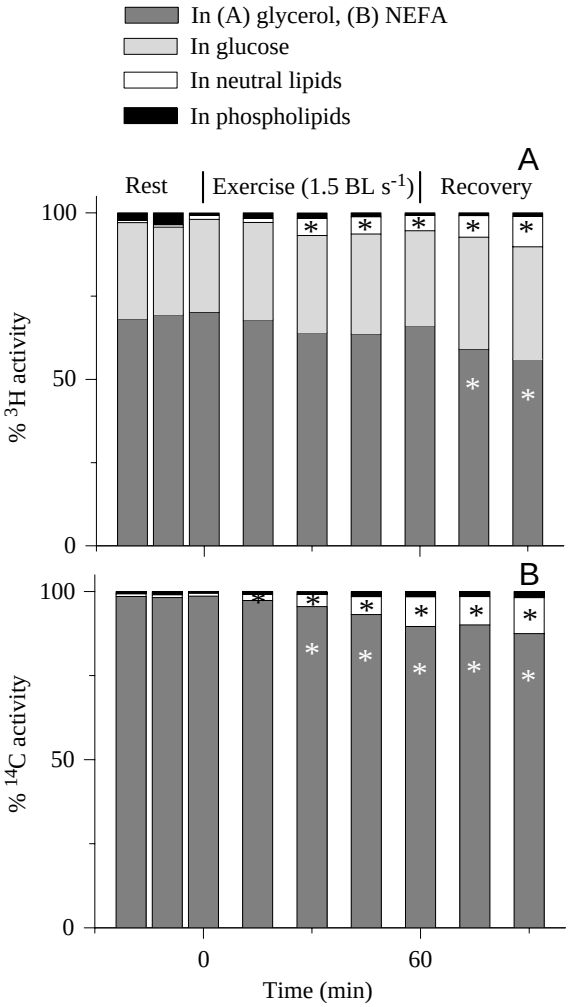

Fig. 7. Relative distribution of ^3H (A) and ^{14}C activity (B) in plasma metabolites during rest, exercise at $1.5\,BL\,s^{-1}$ and recovery. Values are mean percentages of total activity (N=6). Asterisks indicate values significantly different from rest (P<0.05). BL, body length. NEFA, non-esterifed fatty acid.

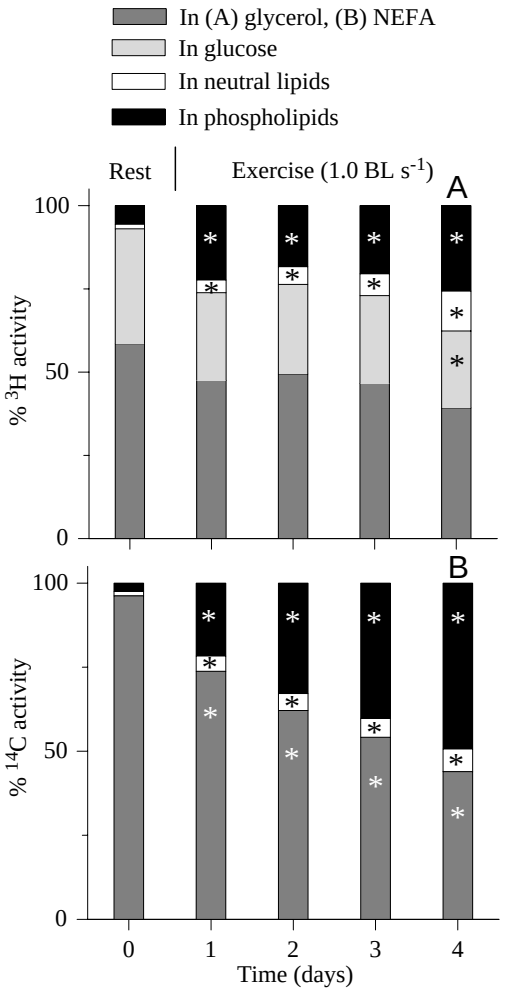

Fig. 8. Relative distribution of ^3H (A) and ^{14}C activity (B) in plasma metabolites during rest and 4 days of exercise at $1\,BL\,s^{-1}$. Values are mean percentages of total activity (N=6). Asterisks indicate values significantly different from rest (P<0.05). BL, body length. NEFA, non-esterifed fatty acid.

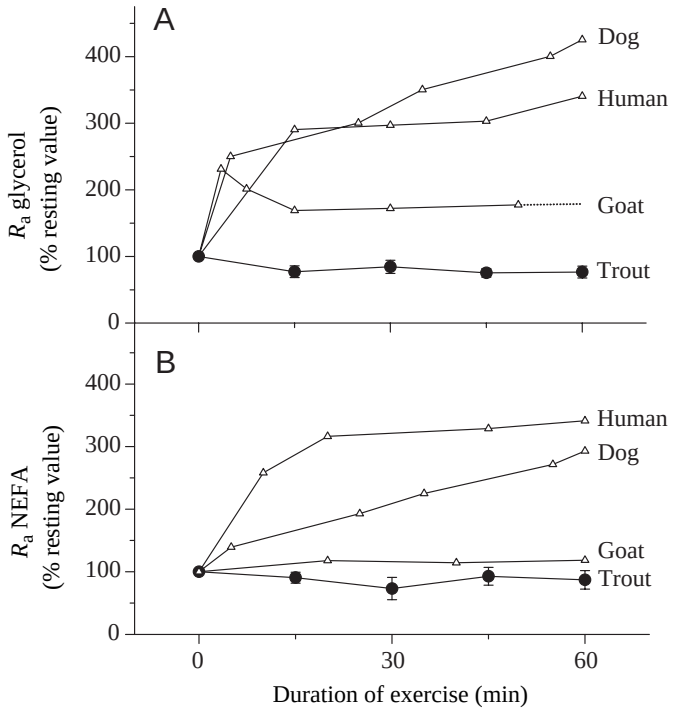

Fig. 9. Comparative effects of submaximal exercise on the lipid kinetics of trout and mammals. Relative changes in the rate of appearance (R_a) of glycerol (A) and in R_a NEFA (non-esterifed fatty acid) (B) are given as percentages of resting values at equivalent exercise intensities (1.5 BL s^{-1} for trout and 40 % $\dot{M}_{O_2max}$ for mammals). The trout values are means ± S.E.M. (N=6). The mammalian values were calculated from other published studies on dogs (Shaw et al., 1975), goats (Weber et al., 1993, 1996), and humans (Wolfe et al., 1990). BL, body length.

enter the TAG:FA cycle (Wolfe et al., 1990). Our method only allowed us to measure intracellular re-esterification (i.e. when the fatty acids do not enter the circulation between hydrolysis and esterification). High NEFA concentrations may be present in the cytosol of fish adipocytes, a situation known to stimulate intracellular re-esterification (Wolfe et al., 1990). In humans, simultaneous measurements of total lipid oxidation by indirect calorimetry and of R_a NEFA/R_a glycerol have shown that most of the re-esterification occurs extracellularly. In the present study, extracellular re-esterification was not measured, but minimal estimates of this variable can be calculated because NEFAs have only two possible fates: oxidation and re-esterification. If we assume (1) that the resting metabolic rate of our fish was 40 μmol O_2 kg^{-1} min^{-1} (Haman et al., 1997b), (2) that an average trout NEFA has 18 carbons requiring 26 O_2 for oxidation, and (3) that trout energy metabolism is entirely supported by fatty acid oxidation, then at least 20 % of all the NEFAs must undergo extracellular re-esterification. Overall, our results clearly show that rainbow trout sustain NEFA fluxes well in excess of their metabolic needs for oxidation and that the large majority of the fatty acids produced are re-esterified. In ectotherms, high rates of TAG:FA cycling may be essential to cope with fluctuations in body temperature. The high NEFA fluxes observed here may represent a fundamental component of the thermocompensatory mechanisms necessary to carry out a rapid reorganization of membrane phospholipids to maintain membrane fluidity (Bell et al., 1986) and normal diffusion processes (Desaulniers et al., 1996; Sidell and Hazel, 1987).

Because prolonged exercise had no effect on flux through the TAG:FA cycle, our results suggest that this cycle is not involved in the regulation of NEFA availability in fish. This contrasts with the known regulatory role played by the mammalian TAG:FA cycle. In both goats (Weber et al., 1993) and humans (Wolfe et al., 1990), exercise causes a strong increase in lipolytic rate and a simultaneous decrease in the relative rate of re-esterification, thereby amplifying NEFA availability for oxidation. In addition, relative re-esterification rises above 90 % immediately after the end of mammalian exercise to avoid reaching lethal plasma NEFA concentrations, while lipolysis is slowly downregulated. No such regulatory function for the TAG:FA cycle was observed during or after swimming in rainbow trout. However, the possibility remains that this cycle plays a regulatory role in fish, but under different circumstances than during exercise, when significant changes in R_a NEFA may occur.

Cost of TAG:FA cycling

The process of re-esterification requires the energy of 7–8 phosphate bonds per TAG formed (six to activate the NEFA and one to produce glycerol phosphate from free glycerol or glucose). One additional phosphate bond is used if the TAG backbone is generated through the conversion of glycerol to glucose and back to glycerol phosphate (Elia et al., 1987). In trout cells, we can estimate that the hydrolysis of 1 mol of ATP releases approximately 59 kJ (Golding et al., 1995), and that the production of 1 mol of TAG costs 470 kJ. The mean rate of NEFA re-esterification for all experiments was approximately 8 μmol NEFA kg^{-1} min^{-1} or 2.7 μmol TAG kg^{-1} min^{-1}. Therefore, the rate of intracellular TAG:FA cycling observed here in trout costs 1.3 J kg^{-1} min^{-1} (approximately 2.8 μmol O_2 kg^{-1} min^{-1}) or 7 % of resting oxygen consumption (assuming a resting $\dot{M}_{O_2}$ of 40 μmol O_2 kg^{-1} min^{-1}). The cost of the high TAG:FA cycling rate of trout drops well below 7 % of $\dot{M}_{O_2}$ when the animals are swimming, a relatively small expenditure allowing a rapid homeostatic response by membrane phospholipids as water temperature varies.

NEFA oxidation during swimming

During exercise, the rate of fatty acid oxidation of rainbow trout could be increased in the absence of a change in R_a NEFA. However, the stimulation of NEFA oxidation would have to occur in parallel with a decrease in re-esterification. Such a decrease was not observed, supporting the idea that NEFA oxidation is not stimulated during exercise. Using indirect calorimetry, Lauff and Wood (1996) showed that total lipid oxidation increased by 24 and 75 % over resting values when juvenile rainbow trout underwent prolonged swimming at 55 and 80 % U_{crit}, respectively. The following scenarios may explain these different findings: (1) other lipids, such as circulating TAG

and phospholipids, may be used preferentially to provide energy for aerobic swimming (Weber and Zwingelstein, 1995); (2) the rate of NEFA oxidation may actually increase during exercise, but at the expense of extracellular re-esterification, a variable that was not measured in the present study; or (3) the lipolytic responses of adult and juvenile trout are different. Further experiments designed to measure NEFA oxidation, extracellular re-esterification and the fluxes of circulating TAG (especially of very low-density lipoproteins) and phospholipids will be needed to resolve these issues.

Incorporation of labelled glycerol and palmitate

The liver can use circulating glycerol and NEFAs to synthesize other lipids or carbohydrates before releasing them back into the circulation. The results show that glycerol and palmitate are readily used by rainbow trout for the synthesis of neutral plasma lipids (mono-, di- and triacylglycerol) as indicated by the progressive incorporation of label observed in the short-term (Fig. 7) and the long-term (Fig. 8) experiments. These same precursors are also clearly used for the synthesis of circulating phospholipids, but this process was only observed after repeated tracer infusions over several days (Fig. 8). Unfortunately, these incorporation data can only be interpreted in a qualitative sense because the specific activities of neutral lipids and phospholipids were not quantified in this study. Therefore, actual rates of synthesis and turnover cannot be calculated for these metabolites.

Use of heparin in lipid metabolism studies

Heparin was used as an anticoagulant in some of the experiments (short-term swimming), but was substituted with sodium citrate in others (4 day swimming) to investigate a possible stimulatory effect of heparin on lipolysis. When the resting values of fish treated with the two anticoagulants were compared, no significant differences in R_a glycerol and R_a NEFA were observed (Table 3). Consequently, heparin can be safely used in future studies of lipid kinetics in fish at the doses necessary to maintain catheter patency. In our experience, sodium citrate was not a practical alternative because it is much less potent and it substantially decreased our ability to avoid catheter clotting compared with heparin.

In conclusion, the rates of triacylglycerol hydrolysis and fatty acid release that occur in resting rainbow trout are not affected by prolonged endurance exercise. Unlike mammals, these active teleosts do not upregulate lipolysis to supply more NEFAs to their working muscles. This study provides the first measurements of lipid kinetics in fish and shows that, at rest as well as during exercise, trout maintain glycerol and NEFA fluxes well in excess of their need for oxidative fuel. More than two-thirds of all the NEFAs produced are re-esterified, but the TAG:FA substrate cycle is not involved in the regulation of NEFA availability during or after swimming. We propose that these high rates of re-esterification represent an important feature of ectothermic metabolism that allows the restructuring of membrane phospholipids to be sychronized with frequent changes in body temperature.

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