LIPID MOBILIZATION IN EXERCISING SALMONIDS

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SUMMARY

Animals rely on lipids as a major fuel for endurance exercise because they pack more joules per gram than any other fuel. However, in contrast to mammals, information on how the mobilization of lipids from endogenous stores is managed to meet the needs of energy metabolism in swimming fish is sparse. Information on *in vivo* rates of lipid mobilization in swimming fish has been limited to relatively low exercise intensities and has only been investigated in a single species. Therefore, the goal of my thesis was to address this paucity of information by quantifying lipolytic rate in rainbow trout during graded exercise and fatty acid mobilization in Atlantic salmon during prolonged endurance exercise.

In the first part of my work, I hypothesized that like mammals, rainbow trout stimulate lipolysis above resting levels to a peak with increasing work intensity, but subsequently lower its rate at high intensities when ATP production from carbohydrates becomes dominant. To test this hypothesis, I measured the rate of appearance of glycerol (R_a glycerol) in the blood (resulting from the breakdown of triacylglycerol (TAG)) of trout at rest (control) and during graded exercise from rest to U_{crit}. Results showed that R_a glycerol in trout averaged 1.24 ± 0.10 μmol kg $^{-1}$ min^{-1} and that this rate was unaffected by exercise of any intensity. These experiments revealed that rainbow trout do not modulate lipolysis during exercise. Furthermore, I calculated that baseline lipolytic rate was much higher in trout than in mammals and that this rate is in constant excess of the requirements of energy metabolism.

My second investigation focused on measuring fatty acid mobilization in Atlantic salmon. To date, the majority of studies on energy metabolism in salmonids have used rainbow trout as the ubiquitous model for salmonids. I postulated that domesticated rainbow trout may be

far less impressive athletes than their wild anadromous form and other salmonids. In this regard, I proposed that studying energy metabolism in Atlantic salmon (even those from aquaculture) may help to deepen our understanding of the physiology of true long-distance migrant fish. To study the effects of prolonged endurance exercise on the mobilization of fatty acids from endogenous stores in these fish, I monitored the rate of appearance of fatty acids (R_a NEFA calculated from R_a Palmitate) in the blood during 72 hours of sustained swimming. I found that contrary to what has been previously described in rainbow trout, R_a Palmitate (and by proxy, R_a NEFA) is reduced by approximately 64% (from 0.75 ± 0.12 μmol $kg^{-1}min^{-1}$ to 0.27 ± 0.06 μmol $kg^{-1}min^{-1}$ and from 19.3 ± 7.8 μmol $kg^{-1}min^{-1}$ to 6.9 ± 2.0 μmol $kg^{-1}min^{-1}$ for R_a Palmitate and R_a NEFA, respectively) during prolonged endurance exercise in Atlantic salmon. However, like in trout, even this reduced rate of fatty acid mobilization exceeds the requirements of energy metabolism at rest and during swimming. While further experiments will be necessary, I speculated that this reduction in R_a NEFA may be caused by a partial inhibition of lipolysis to reduce the energetic cost of TAG:FA cycling and optimize fuel budgets during prolonged endurance exercise.

This thesis provides the first *in vivo* measurements of lipolysis during graded exercise in salmonids and the first *in vivo* measurements of fatty acid mobilization in Atlantic salmon. From the results mentioned above, I concluded that salmonids mobilize lipids in constant excess of the requirements for energy metabolism, possibly to allow for rapid reorganization of membrane phospholipids in response to changing environmental conditions. However, more anadromous and migratory phenotypes may rely on a tighter control of lipolysis to minimize the costs of substrate cycling and conserve energy on limited fuel stores.

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CHAPTER 1

GENERAL INTRODUCTION

Metabolic fuels

The various vital processes essential to supporting life require energy. Animals obtain this energy from ingested food or from body reserves of lipids, carbohydrates and proteins (Kaushik and Medale, 1994; Weber and Haman, 2004). These metabolic fuels are brought to tissues and are broken down to produce ATP, the universal energetic currency of working cells. All metabolic fuels are not created equal however; their relative contribution to ATP production varies based on nutritional state (Cherel et al., 1992; Weber and O'Connor, 2000; Kiens, 2006; Viollet et al., 2009), the level (Lauff and Wood, 1996; Brooks, 1997, 1998; Richards et al., 2002b; Schippers et al., 2014), type (Haman et al., 2002; Haman et al., 2004a; Weber and Haman, 2005) and duration of activity (Lauff and Wood, 1997; Weber and Haman, 2004; Weber, 2009, 2011), fuel availability (De Glisezinski et al., 1998; Haman et al., 2004b), as well as changes in physiological state (e.g. torpor; Heldmaier et al., 2004; Andrews et al., 2009) and hormonal balance (Reidy and Weber, 2000; Wortley et al., 2004; Isacco et al., 2012).

Fuel selection during exercise

The effects of exercise intensity on fuel selection have been studied extensively in mammals. Lipids are the preferred fuel for endurance exercise because they are more energy dense and do not require water to be stored, therefore allowing superior storage capacity over proteins and carbohydrates (Weber, 2011). Because of this, animals can sustain exercise for longer and on smaller reserves (by unit mass; Weber, 2011) by preferentially oxidizing lipids to produce ATP. Mammals utilize lipids as their primary fuel for ATP production while at rest and to support locomotion up to roughly half of maximal effort (Hultman, 1995; Roberts et al., 1996; van Loon et al., 2001; McClelland, 2004), with exercise intensity often standardized as $\%VO_2$

$_{MAX}$ (Brooks and Mercier, 1994; Brooks, 1998; Weber, 2011). In other words, ATP production through β-oxidation of fatty acids drives energy metabolism up to ~ 50% $VO_{2\ MAX}$. At higher exercise intensities, the maximal rates of ATP production through β-oxidation are too slow to meet total energetic demand (Roberts et al., 1996; Weber, 2011) and a shift towards a carbohydrate-dominated energy metabolism occurs (Hultman, 1995; Roberts et al., 1996; Brooks, 1998; van Loon et al., 2001; McClelland, 2004; Weber, 2011). Carbohydrates have much higher maximal rates of oxidation when compared to lipids and are also the only metabolic fuel that can be used to produce ATP in the absence of oxygen (Weber, 2011). This makes carbohydrates particularly well-suited to support intense exercise when energy demand is high and oxygen supply to tissues is limited by its diffusion and transport capacity (Weibel et al., 1996). Exercise intensity also influences the source of the fuels used for ATP production. In general, lower intensity exercise is supported by circulating lipids and carbohydrates, whereas high intensity exercise increases reliance on intramuscular fuel stores (Romijn et al., 1993b; Weber et al., 1996b; Weber et al., 1996a).

The effects of exercise duration on fuel preference are also known. Generally, as duration increases, carbohydrate utilization decreases while lipid utilization increases (Jeukendrup, 2003). Like intensity, exercise duration also influences the provenance of fuels. Short bouts of exertion are supported primarily by intramuscular fuel stores while longer efforts rely more on circulating lipids and carbohydrates (Coggan, 1991; Romijn et al., 1993b).

Barring minor differences in the point of crossover from predominantly lipid to predominantly carbohydrate use associated with differences in aerobic scope (Schippers et al., 2014), this relationship between exercise intensity, duration and fuel preference is generally conserved across mammals (Roberts et al., 1996; McClelland, 2004; Weber, 2011).

Finally, protein catabolism for ATP production during exercise in most animals is limited because it usually comes at a detriment to proper cellular structure and function (Weber, 2011). Certain insects have been shown to use amino acids such as proline as a substrate for ATP production (Scaraffia and Wells, 2003). Migrating salmonids have also been shown to rely on protein to fuel energy metabolism (Hendry and Berg, 1999), but significant increase in protein utilization is not observed during routine exercise (Alsop and Wood, 1997; Kieffer et al., 1998). It only occurs at the very end of long migrations when all other fuel reserves are depleted and the animal is near death (Hendry and Berg, 1999; Weber, 2011). Typically, the contribution of protein catabolism to ATP production in most exercising vertebrates is low (Weber, 2011).

Energy metabolism in exercising fish

Fish, like all other vertebrates, rely on a mix of lipids, carbohydrates and proteins as essential fuels for energy metabolism. However, the breadth of current knowledge and understanding of fish energy metabolism is not as complete as in mammals. Early investigations into energy metabolism in fish dating back to the 1950-60s revolved around the regulation of carbohydrate metabolism in response to exercise and hypoxia (Black, 1957; Heath and Pritchard, 1965). These studies provided evidence that during vigorous exercise (Black, 1957) and severe hypoxia (Heath and Pritchard, 1965), energy metabolism in fish is supported principally by carbohydrate use. Indeed, vigorous exercise in fish is powered primarily by the white muscle (Johnston, 1980; Richards et al., 2002a) and relies heavily on carbohydrates for ATP production (Lauff and Wood, 1996) as demonstrated by the depletion of white muscle glycogen reserves (Wang et al., 1994; Richards et al., 2002b; Choi and Weber, 2016) and the accumulation of lactate in muscle and to a lesser extent in the blood (Wood, 1991; Richards et al., 2002b; Van

Ginneken et al., 2004). These findings have been further corroborated by measurements of lactate flux during graded swimming in rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) which show that increasing exercise intensity causes an accumulation of lactate in the circulation due to a greater reliance on glycolysis and a mismatch between the rate of appearance (R_a) and disposal (R_d) of lactate (Teulier et al., 2013). In contrast to mammals however, muscle and blood lactate concentrations in most teleosts remain elevated for several hours following exercise due to white muscle lactate retention (Schulte et al., 1992; Gleeson, 1996; Milligan, 1996; Wang et al., 1997; Weber et al., 2016). Recently, Omlin and Weber (2013) showed that this lactate retention by white muscle could be explained by the generally low expression of monocarboxylate transporters (MCTs) in this tissue and in particular the very poor expression of MCT4; the primary exporter of lactate found in mammalian muscles. This retention of lactate by fish muscle is thought to play a role in supplementing *in situ* glycogen synthesis during recovery from exhaustive exercise (Wood, 1991; Gleeson, 1996; Van Ginneken et al., 2004) rather than relying on the Cori cycle to clear lactate and replenish glycogen stores (Weber et al., 1986; Wood, 1991; Buck et al., 1992; Weber et al., 2016).

In addition to their reliance on carbohydrates as the primary fuel for ATP production during high-intensity exercise and similarly to mammals, fish also use carbohydrates (to an extent) to fuel sustainable, aerobic exercise. Measurements of respiratory quotient (RQ; the ratio of expired CO_2 to inspired O_2) in swimming trout have shown that carbohydrates may account for around 20% of oxidative fuel use at rest and their relative contribution to ATP production increases with exercise intensity (Lauff and Wood, 1996). Increased pyruvate dehydrogenase (PDH) activity, glucose, glucose-6-phosphate and malonyl-CoA concentrations in red muscle are also indicative of greater reliance on carbohydrates for oxidative phosphorylation as exercise

intensity increases (Richards et al., 2002b). However, during sustained swimming, reliance on carbohydrates to fuel ATP production is limited by exercise duration. At low exercise intensity, carbohydrate utilization accounts for a large proportion of total ATP production in the first few minutes of exercise (Richards et al., 2002b), but this appears to be a transient state. As exercise duration increases, PDH activity eventually returns to control values, indicating a shift towards predominantly lipid-based ATP production (Richards et al., 2002b). Furthermore, while glucose flux appears to be stimulated by high intensity exercise in rainbow trout (Choi and Weber, 2016), endurance exercise causes a reduction of hepatic glucose production (-33%; Shanghavi and Weber, 1999); a very different response from mammals (Coggan et al., 1995; Weber et al., 1996a). This, along with the finding of Richards et al. (2002b), suggests that circulating carbohydrates may only play a minor role in fueling ATP production during sustained aerobic exercise. Because proteins also likely only contribute in small portion as an oxidative fuel during exercise (i.e. <20%; Lauff and Wood, 1996; Alsop and Wood, 1997; Kieffer et al., 1998; Richards et al., 2002b), the importance of lipids for energy metabolism in swimming fish cannot be overstated.

Fish rely on lipids as a major fuel for endurance exercise (Lauff and Wood, 1996; Hendry and Berg, 1999; Richards et al., 2002b; Magnoni et al., 2006; Magnoni and Weber, 2007). Measurements of RQ in swimming trout have shown that lipids are the major fuel for aerobic exercise up to 80% of critical swimming speed (U_{crit}; Lauff and Wood, 1996). This is supported by the observations of Richards et al. (2002b), which show that β-oxidation is stimulated during sustained aerobic exercise below 90% U_{crit}, as demonstrated by increases in the CoA/CoA-SH ratio, acetyl-carnitine and long-chain fatty acyl carnitine (LCFA) concentrations and decreases in malonyl-CoA concentration. Endurance training also causes an increase in trout red muscle

AMPK, PPARα and carnitine palmitoyltransferase-1 (CPT1) mRNA expression (Morash et al., 2014) as well as increases in hydroxyacyl CoA dehydrogenase (HOAD) activity (Farrell et al., 1991; Morash et al., 2014). All of these changes are associated with an increased capacity for lipid oxidation (Johnston and Moon, 1980a; Morash et al., 2014).

In summary, endurance exercise in fish is powered primarily by the red muscle (Johnston, 1980; Johnston and Moon, 1980a; Richards et al., 2002b) and relies on lipids as a major fuel for energy metabolism (Lauff and Wood, 1996; Richards et al., 2002b; Morash et al., 2014). Increasing exercise intensity progressively augments the role of white muscle and the reliance on carbohydrates to sustain swimming (Johnston, 1980; Richards et al., 2002a). Protein catabolism may also contribute to overall energy metabolism in fish, but it is unaffected by exercise intensity (Lauff and Wood, 1996; Alsop and Wood, 1997; Kieffer et al., 1998) and likely only becomes important when all other fuel reserves are depleted (Hendry and Berg, 1999; Weber, 2011).

Lipid mobilization in exercising salmonids

Migrating salmonids depend on lipids to fuel swimming over hundreds or even thousands of kilometers (Doucett et al., 1999; Magnoni et al., 2006) and therefore must be particularly well-adapted to store, mobilize and oxidize lipids. While mammals transport lipids from adipose tissue to muscle as free fatty acids bound to albumin (Spector, 1975; van der Vusse, 2009), circulating lipoproteins are thought to provide most of the lipids that salmonids require for ATP production during sustained swimming (Magnoni et al., 2006) and contain the most energy in plasma lipids (Magnoni et al., 2008b). In a fasted state, as most salmonids are during migration (Kadri et al., 1995; Doucett et al., 1999), the lipids contained in lipoproteins must be provided by

the animal's own lipid reserves. Salmonids store lipids in mass quantities in the form of triacylglycerol (TAG) in visceral, dorsal and abdominal adipose tissue and to a lesser extent in muscle (Kiessling et al., 1991; Kiessling et al., 2001; Kiessling et al., 2004). In order to make fatty acids available to energy metabolism, lipids reserves in adipocytes must first be mobilized through lipolysis (McClelland, 2004); the breakdown of TAG which yields free fatty acids and glycerol (Raclot and Groscolas, 1993; Frayn, 2010). Like in mammals, lipolysis of intracellular TAG in fish is accomplished by hormone sensitive lipases (HSL; van den Thillart et al., 2002; Albalat et al., 2005). However, *in vivo* regulation of lipases is not as well understood in fish and may drastically differ from mammals (van den Thillart et al., 2002; Magnoni et al., 2008a). Furthermore, the effects of exercise on lipid mobilization in fish are only poorly understood.

In vivo lipolysis in exercising fish has only been studied once before. Bernard et al. (1999) measured the rates of appearance of glycerol (R_a glycerol) and of fatty acids (R_a Palmitate and R_a NEFA) in the blood of rainbow trout during endurance exercise for 1 hour at 1.5 or 4 days at 1.0 body lengths per second (BL s^{-1}). In both treatments, they found that baseline R_a glycerol, R_a Palmitate and R_a NEFA (calculated based on R_a Palmitate) were unaffected by endurance exercise. From these findings, they concluded that contrary to mammals that are known to stimulate lipolysis during exercise (Issekutz et al., 1975; Wolfe et al., 1990; Weber et al., 1993; Klein et al., 1996), rainbow trout maintain baseline lipolytic rate in constant excess of the requirements for energy metabolism; a strategy they proposed could allow ectotherms to rapidly reorganize membrane phospholipids as a component of the homeoviscous response.

Research objectives

The existing literature on lipid mobilization in exercising salmonids fails to address several important questions about fuel metabolism in these animals. In mammals, exercise intensity is known to have a profound effect on the rate at which lipids are mobilized (Issekutz et al., 1975; Wolfe et al., 1990; Weber et al., 1993; Klein et al., 1996). It remains unclear whether or not this applies to salmonids because information on *in vivo* lipolytic rate in exercising trout is limited to relatively low exercise intensities (see Bernard et al., 1999). It is also well known that different species of salmonids express vastly different life history traits (Klemetsen et al., 2003; Quinn and Myers, 2004; Spares et al., 2015). Therefore, it would be reasonable to assume that differences in life history may elicit equally diverse metabolic phenotypes. Yet, our current knowledge of lipid mobilization in exercising salmonids is limited to a single species.

The overarching goal of my research has been to address this paucity of information on *in vivo* lipid fluxes in exercising salmonids. The first objective of my work was to expand upon the foundation set by Bernard et al. (1999) and provide insight on the modulation of lipolytic rate during graded exercise from rest to U_{crit} in rainbow trout to better understand the effects of exercise intensity on lipid mobilization. As a second objective, I sought to study fatty acid mobilization during exercise in a species known to undergo long migrations in nature; the Atlantic salmon (*Salmo salar*). To date, the vast majority of studies on energy metabolism in salmonids have used rainbow trout as the ubiquitous model for salmonids. However, it could be argued that domesticated trout are far less impressive athletes than their anadromous, migratory form (commonly referred to as steelhead trout) and other salmonids (McCormick et al., 1998; Quinn and Myers, 2004; Magnoni et al., 2006; Hayes and Kocik, 2014; Spares et al., 2015). In this regard, studying energy metabolism in Atlantic salmon (even aquaculture-reared) to make

specific comparisons between trout and salmon will help to deepen our understanding of the physiology of true long-distance migrant fish.

CHAPTER 2

LEAN, MEAN, LIPOLYTIC MACHINES:

LIPID MOBILIZATION IN RAINBOW TROUT DURING GRADED SWIMMING

Based on a manuscript by the same title

Written by

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Introduction

Fish store more than 85% of their energy reserves as triacylglycerol (TAG), mainly because lipids pack more joules per gram than any other fuel (Weber, 2011). They make predominant use of these lipids to support swimming (Richards et al., 2002b; Magnoni et al., 2006), particularly when it is sustained during long migrations (Mommsen et al., 1980; Magnoni et al., 2006). Stored TAG are made available to energy metabolism through lipolysis: an essential process regulating the strategic release of the constituent fatty acids that fuel prolonged work (Tocher, 2003; Frayn, 2010). *In vivo* lipolysis is measured by continuous tracer infusion as the rate of appearance of glycerol in the circulation (R_a glycerol; Wolfe and Chinkes, 2005). This approach is routinely used in human medicine (Kim et al., 2015) and it has been adapted for fish (Haman and Weber, 1996; Bernard et al., 1999).

Only two studies investigating *in vivo* lipolysis in rainbow trout are presently available (Bernard et al., 1999; Magnoni et al., 2008a). They reveal that circulating norepinephrine inhibits the lipolytic rate of trout instead of activating it as in mammals, whereas epinephrine has a stimulating effect in both groups of animals. Fish may therefore rely entirely on the counterplay between the two catecholamines to modulate lipolysis (Montpetit and Perry, 1998; Magnoni et al., 2008a). Unfortunately, lipid mobilization of exercising trout has only been measured during sustained, low-intensity swimming. Steady exercise at ~50% of critical swimming speed (U_{crit}) has no stimulating effect on baseline R_a glycerol, even when it is sustained for 4 days (Bernard et al., 1999). This observation is very surprising because mammals performing equivalent exercise show a 3 to 5-fold increase in lipolysis (Issekutz et al., 1967; Wolfe et al., 1990; Klein et al., 1996). They are also known to reach a maximal lipolytic rate at intermediate work intensities, before showing a marked reduction when exercise becomes more strenuous (Romijn et al.,

1993b). This lipolytic peak occurs when the rates of mammalian fatty acid oxidation are the highest (Brooks, 1998). For fish, no information is available on the relationship between exercise intensity and the rates of lipolysis or lipid oxidation. It is unknown whether intense exercise causes the inhibition of R_a glycerol in fish as it classically occurs in mammals. Therefore, the goals of this study were to quantify the lipolytic rate of rainbow trout during graded swimming from rest to U_{crit}, and to determine whether fish and mammals modulate lipolysis similarly to support exercise of different intensities. We hypothesized that rainbow trout would stimulate lipolysis to a peak with increasing work intensity, but subsequently lower its rate when reaching U_{crit}, as ATP production from carbohydrates becomes dominant (Richards et al., 2002b).

Methods

Animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) with a Fulton's condition factor (K) of 1.14±0.02 (N=16) were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada; see Table 2.1 for physical characteristics). The fish were held in a 1200 L flow-through tank in dechlorinated Ottawa tap water maintained at 13°C, on a 12 h: 12 h light:dark photoperiod, and were fed 5 days a week with Profishent floating fish pellets (Martin Mills, Elmira, ON, Canada). They were acclimated to these conditions for a minimum of 2 weeks before experiments. The animals were randomly divided into a control group kept at rest and an exercise group that performed graded swimming. All procedures were approved by the Animal Care Committee of the University of Ottawa in accordance with the guidelines established by the Canadian Council on Animal Care.

Catheterization

Fish were anesthetized with ethyl 3-aminobenzoate methanesulfonate (MS-222; 60 mg l^{-1} buffered with sodium bicarbonate, 0.2 g l^{-1}) and doubly cannulated in the dorsal aorta with BTPE-50 catheters (Instech Laboratories, Plymouth Meeting, PA, USA) as previously described (Haman and Weber, 1996). During surgery, the catheters were kept patent by flushing with Cortland saline containing 50 U ml^{-1} heparin (Sigma-Aldrich, St Louis, MO, USA). Lower heparin levels of 25 U ml^{-1} were used during metabolic measurements to avoid stimulating lipolysis. Fish were left to recover overnight while resting at the bottom of the swim-tunnel respirometer chamber at a water velocity of 0.5 body lengths per second (BL s^{-1}): a weak current that does not require swimming (Choi and Weber, 2015). Plastic beads attached to the ends of

the catheter extensions avoided the need to tie down the catheters during recovery, reducing tension on the lines while still preventing these from being pulled through the port in the lid of the swim tunnel. This eliminated having to retrieve the catheters from within the swim tunnel chamber at the start of the experiments, thereby limiting handling stress.

Respirometry

All the experiments (resting controls and exercise) were performed in a 90 L swim tunnel respirometer (Loligo Systems, Tjele, Denmark) supplied with the same quality water as the holding tank and kept at 13°C. Previous experience has shown that control (resting) measurements are more reliable in a swim tunnel because a slight current that does not require swimming (0.5 BL/s) reduces stress in trout. Metabolic rate ($\dot{M}O_2$) was measured by intermittent flow respirometry using galvanic oxygen probes connected to a DAQ-PAC-G1 instrument controlled with AutoResp software (version 2; Loligo Systems). The probes were calibrated before each experiment using air-saturated water (20.9% O_2).

Continuous tracer infusion

The catheters were made accessible above the swim tunnel lid by channeling them through a water-tight port. The rate of appearance of glycerol (R_a glycerol) was quantified by continuous infusion of 2-[^{3}H] glycerol (American Radiolabelled Chemicals, St. Louis, MO, USA; 925 GBq mmol^{-1} (see Wolfe and Chinkes, 2005), using procedures validated for measuring the lipolytic rate of rainbow trout *in vivo* (Bernard et al., 1999; Magnoni et al., 2008a). Infusates were freshly prepared immediately before each experiment by drying an aliquot of the solution obtained from the supplier under N_2 and resuspending in Cortland saline. Glycerol kinetics were

measured by administering the tracer through the infusion catheter using a calibrated syringe pump (Harvard Apparatus, South Natick, MA, USA)(Haman and Weber, 1996). Exact infusion rate (~1 ml/h) was determined individually for each fish to adjust for differences in body mass. Infusion rates averaged 10,707 ± 485 Bq kg^{-1} min^{-1} (N=16) and allowed to reach isotopic steady state in less than 45 min (Magnoni et al., 2008a). These trace amounts only accounted for <0.001% of endogenous glycerol production. Graded swimming experiments consisted of step-wise critical swimming speed (U_{crit}) tests as detailed previously (Teulier et al., 2013). Fish from the exercise group were kept at a resting water velocity (0.5 BL s^{-1}) during the first hour of tracer infusion before starting to swim. Exercise was initiated at 0.8 BL s^{-1} and intensified by 0.2 BL s^{-1} every 20 min in a step-wise manner until exhaustion. The point of exhaustion was determined when the fish could no longer sustain swimming, and was unable to remove itself from the rear grate of the swim-tunnel. Metabolic rate and glycerol kinetics of the control group were monitored at rest for the same total duration as for the exercise group, but at a constant, low water velocity of 0.5 BL s^{-1} that does not elicit swimming.

Blood sampling and analyses

Blood samples (0.1ml each) were drawn 50, 55 and 60 min after starting the tracer infusion (to determine baseline glycerol kinetics) and every 20 min thereafter (prior to each increase in swimming speed) until the end of each experiment. The amount of blood sampled from individual fish accounted for <10% of total blood volume. Each blood sample was immediately deproteinized in 0.2 ml perchloric acid (6% wt/wt) and centrifuged (5 min; 13,000 g). Supernatants were then kept at -20°C until analyses. Each sample was divided into 3 aliquots to measure lactate concentration, glycerol concentration and glycerol activity. Glycerol and

lactate concentrations were measured by spectrophotometry as previously described (Weber et al., 1993; Teulier et al., 2013). Glycerol activity was quantified by scintillation counting (Perkin-Elmer TriCarb 2910TR, Perkin-Elmer, Inc., Waltham, MA, USA). Infusion of 2-[^{3}H]glycerol only leads to significant radioactivity in circulating H_2O, glucose and glycerol (Bernard et al., 1999). Glucose activity was eliminated by incubating the samples with ATP and hexokinase to phosphorylate free glucose that was trapped by ion-exchange column chromatography (see Appendix 1). The samples were then dried under N_2 to eliminate tritiated H_2O and resuspended in dH_2O, leaving [2-^{3}H]glycerol as the only labeled compound to quantify by scintillation counting. Preliminary measurements with known amounts of labeled glycerol showed that 10.6% was lost during the ion-exchange separation by column chromatography and all glycerol activity values were corrected accordingly.

Calculations and statistics

The steady-state equation of Steele (Steele, 1959; see Appendix 2) was used to calculate lipolytic rate (R_a glycerol) because specific activity varied little over time and the high turnover rate relative to pool size allows for rapid equilibration of the tracer within the glycerol pool. Under these conditions, the steady-state equation provides the most accurate estimates of glycerol flux (Beylot et al., 1987; Wolfe et al., 1990; Magnoni et al., 2008a). Two-tailed t-tests were used to compare mean values for $\dot{M}O_2$ and glycerol metabolism between control and exercise groups (Table 2.2). The effects of time (resting group) or swimming speed (exercise group) on $\dot{M}O_2$, cost of transport, metabolite concentrations, and R_a glycerol were assessed by one-way analyses of variance with repeated measures (RM ANOVA) followed by the Holm-Sidak post-hoc test to determine which values were significantly different from baseline. When

the assumptions of normality or equality of variances were not met, the data were normalized by transformation (log_{10}, square, or square root) before parametric analysis. When transformation was unsuccessful, Friedman's nonparametric RM-ANOVA on ranks was used. All values presented are means ± s.e.m, and a level of significance of $P<0.05$ was used in all tests.

Table 2.1: Mean physical characteristics and hematocrit of adult rainbow trout. Values are means ± s.e.m.

	Control group	Exercise group	All fish
Sample size (N)	8	8	16
Body mass (g)	343 ± 32	391 ± 22	367 ± 20
Length (cm)	31.3 ± 1.0	32.0 ± 0.5	31.7 ± 0.5
Hematocrit (%)	16.1 ± 2.0	18.1 ± 1.5	17.1 ± 1.2

Results

Metabolic rate, blood lactate concentration and cost of transport

Metabolic rate ($\dot{M}O_2$) and blood lactate concentration of rainbow trout during graded swimming and during control, resting experiments are presented in Fig. 2.1. The first $\dot{M}O_2$ value indicated for the exercise treatment (at a "non-swimming" water velocity of 0.5 BL s^{-1}) is the resting metabolic rate for this group of fish. Graded exercise caused a large increase in $\dot{M}O_2$ from a baseline value of 57.5 ± 2.4 μmol O_2 kg^{-1} min^{-1} to a maximum of 153.5 ± 16.5 μmol O_2 kg^{-1} min^{-1} at 3.0 BL s^{-1} (P<0.05, top panel Fig. 2.1).The $\dot{M}O_2$ of resting controls remained stable at 49.4 ± 0.4 μmol O_2 kg^{-1} min^{-1} throughout the experiment (P>0.05; bottom panel Fig. 2.1). Blood lactate concentration remained unchanged for both groups (P>0.05; Fig. 1) and averaged 1.8 ± 0.2 μmol ml^{-1} in the control fish and 1.7 ± 0.1 μmol ml^{-1} in the exercising fish. Total oxygen cost of transport (COT; see Appendix 2) was calculated from $\dot{M}O_2$ as a function of swimming speed from 0.8 to 3.2 BL s^{-1} (Fig. 2.2). A maximum COT of 5.9 ± 0.4 μmol O_2 kg^{-1} m^{-1} was observed at the lowest swimming speed. Increasing exercise intensity then caused a progressive decrease in COT to reach a minimal value of 2.3 ± 0.2 μmol O_2 kg^{-1} m^{-1} at 2.2 BL s^{-1} (P<0.001). No further decline in COT was elicited as swimming speed was increased from 2.2 to 3.2 BL s^{-1}.

Table 2.2: Resting metabolic rate (MO_2) and parameters of glycerol metabolism in the control and exercise groups. Values are means ± s.e.m. (N=8). * P<0.05.

	Control group	Exercise group
Resting MO_2 (µmol O_2 $kg^{-1}min^{-1}$)	49.4 ± 0.4	57.5 ± 2.4*
Resting [Glycerol] (µmol ml^{-1})	0.18 ± 0.01	0.13 ± 0.01
Resting R_a Glycerol (µmol $kg^{-1}min^{-1}$)	1.67 ± 0.18	1.49 ± 0.37

Fig. 2.1. Metabolic rate and blood lactate concentration of swimming trout (top panel) and resting controls (bottom panel). Values are means ± s.e.m. (N for individual means of swimming fish are given on the graph; N=8 for controls). Time scale of resting controls (bottom panel) also applies to swimming fish (top panel). * indicates significant differences from baseline values (0.5 BL s^{-1} or t = 0; $P<0.05$).

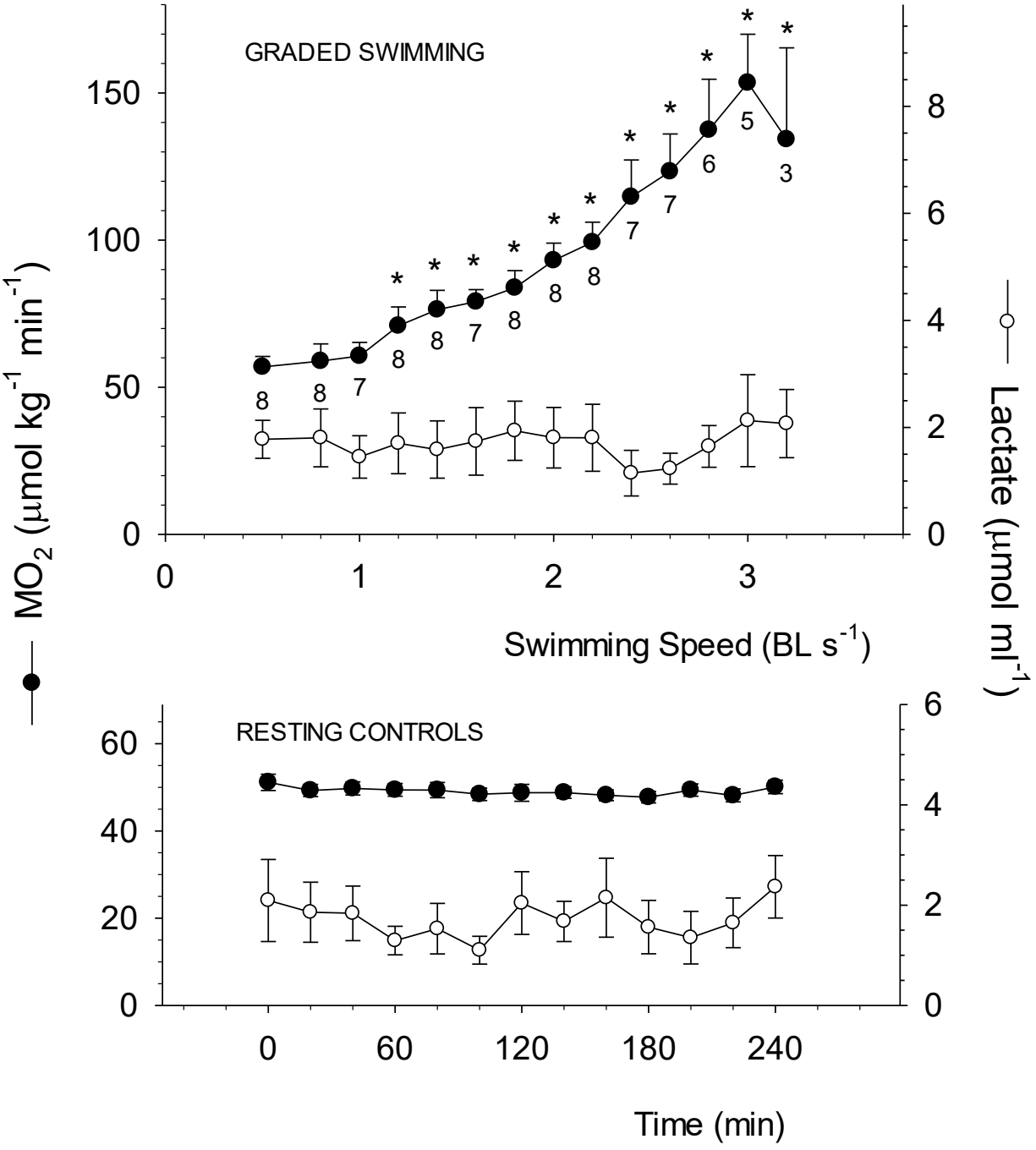
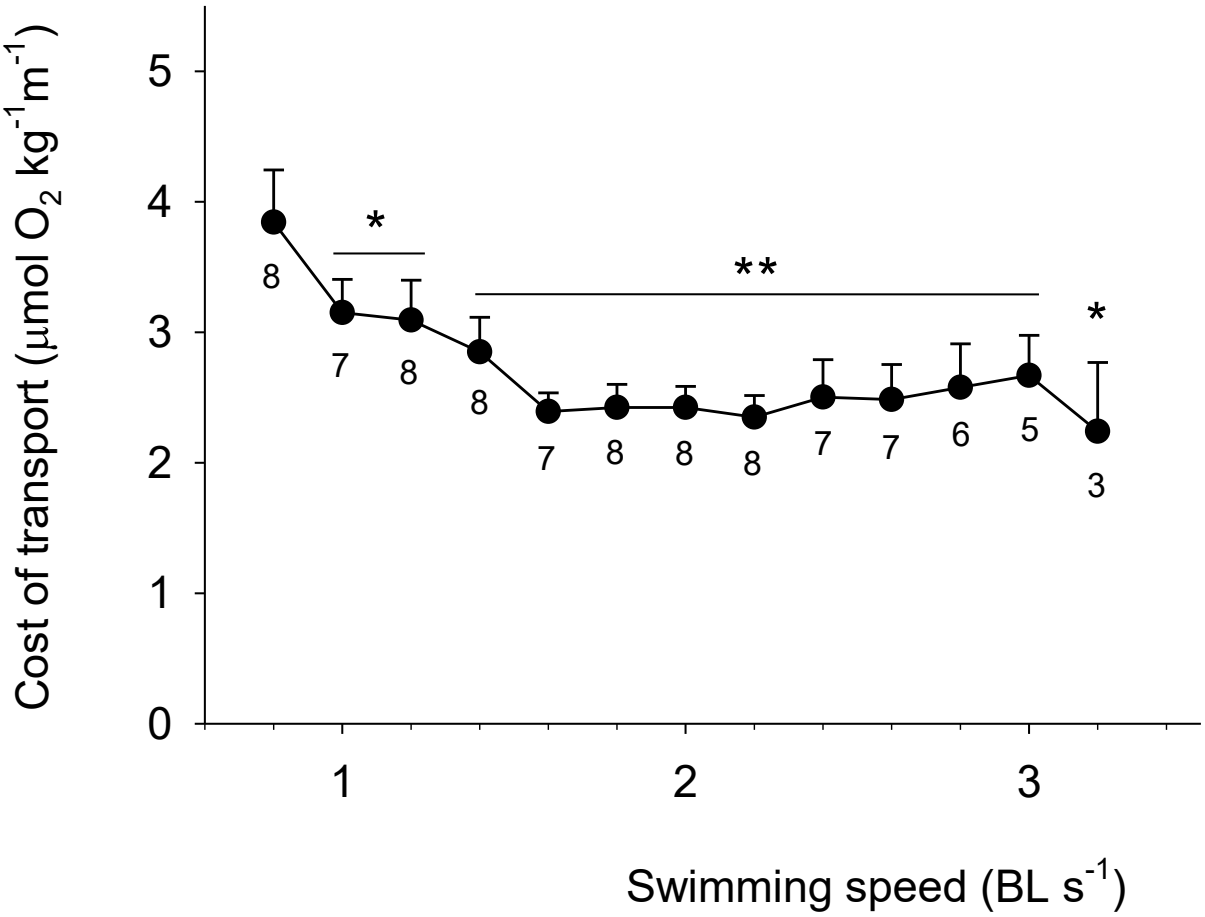

GRADED SWIMMING
RESTING CONTROLS
MO_2 (μmol kg^{-1} min^{-1})
Lactate (μmol ml^{-1})
Swimming Speed (BL s^{-1})
Time (min)
0
50
100
150
2
4
6
8
1
3
20
40
60
60
120
180
240
*
8
7
6
5
3

Fig. 2.2. Total cost of transport during graded exercise from the lowest swimming speed to U_{crit}. Values are means ± s.e.m. with sample size N. Significant differences from cost of transport at the lowest speed of 0.8 BL s^{-1} are indicated as * (P<0.05) or ** (P<0.001).


Cost of transport (μmol O2 kg-1 m-1)
0
1
2
3
4
5
1
2
3
Swimming speed (BL s-1)
*
**
*
8
7
8
8
7
8
8
8
7
7
6
5
3

Glycerol metabolism

The rate of appearance of glycerol in the circulation (R_a glycerol) and plasma glycerol concentration were quantified in controls (Fig. 2.3) and swimming fish (Fig. 2.4). In control animals, R_a glycerol averaged 1.67 ± 0.18 μmol kg^{-1} min^{-1} and glycerol concentration 0.18 ± 0.01 μmol ml^{-1} throughout the 4 h of resting measurements. Only minor differences from the initial values of 2.41 ± 0.42 μmol kg^{-1} min^{-1} (R_a glycerol) and 0.17 ± 0.02 μmol ml^{-1} (glycerol concentration) were observed over time (Fig. 2.3). The R_a glycerol of controls was lower at 160 and 220 min ($P<0.05$), but not different from initial fluxes at all other times ($P>0.05$). Glycerol concentration remained steady at all times ($P>0.05$), except at 120 min when it increased to 0.37 ± 0.05 μmol ml^{-1} from baseline ($P<0.05$). In exercising fish (Fig. 2.4), R_a glycerol averaged 1.24 ± 0.10 μmol kg^{-1} min^{-1} and glycerol concentration 0.13 ± 0.01 μmol ml^{-1} throughout the experiments. Graded exercise had no effect on R_a glycerol at any swimming speed up to U_{crit} ($P>0.05$). Glycerol concentration was slightly increased with exercise intensity ($P<0.05$ for effect of speed in overall ANOVA), but the post-hoc test was unable to identify which mean was significantly different from the resting value. Mean R_a glycerol and mean plasma glycerol concentration throughout the experiments were not different between the controls and the exercise group ($P>0.05$; Table 2.2).

Fig. 2.3. Glycerol metabolism of resting (control) trout. Rate of appearance of glycerol or lipolytic rate (R_a glycerol; top panel) and blood glycerol concentration (bottom panel). R_a glycerol was measured by continuous infusion of 2-[^{3}H] glycerol. Values are means ± s.e.m. (*N*=8). * indicates significant differences from baseline (t=0; $P<0.05$).

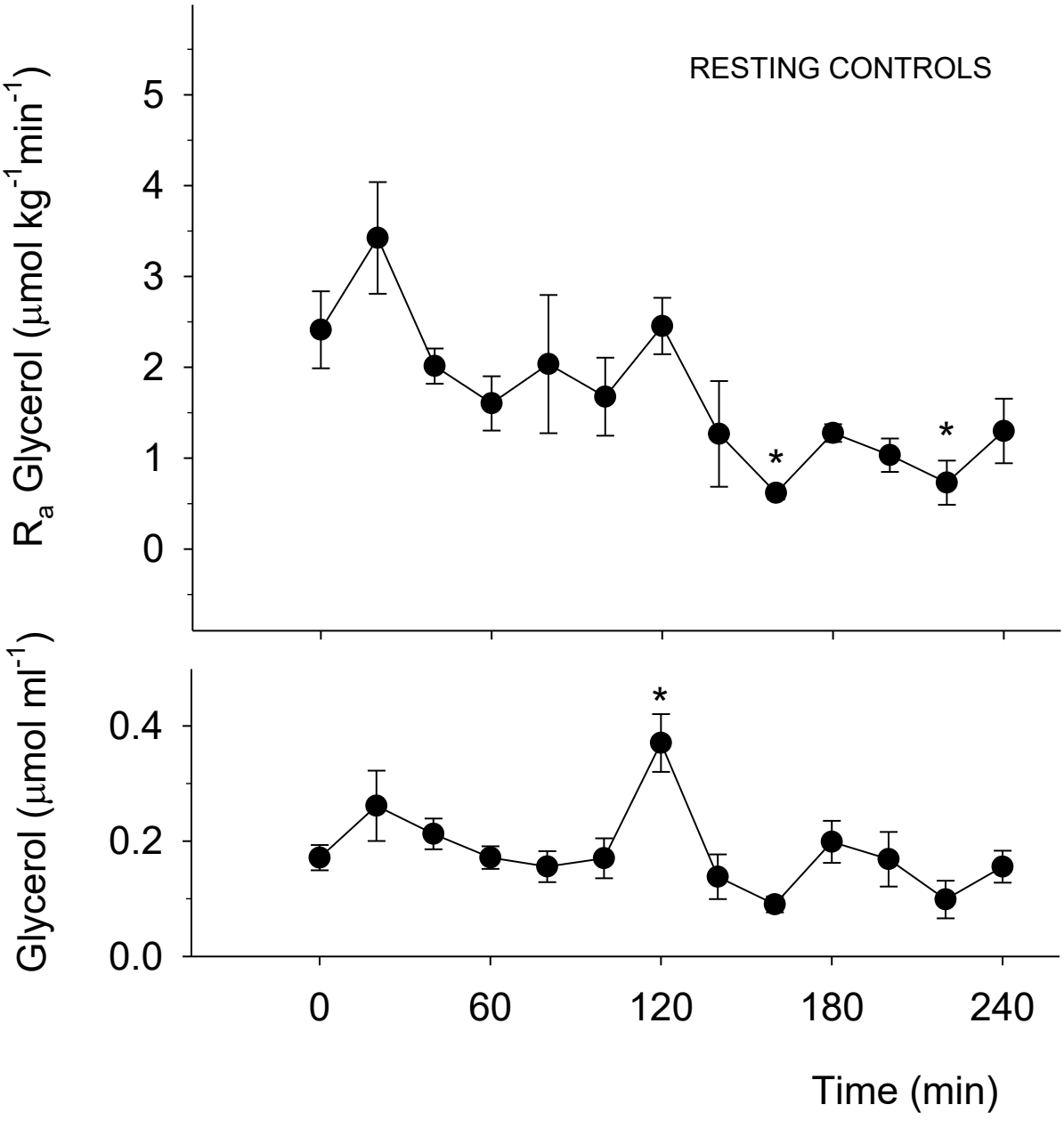
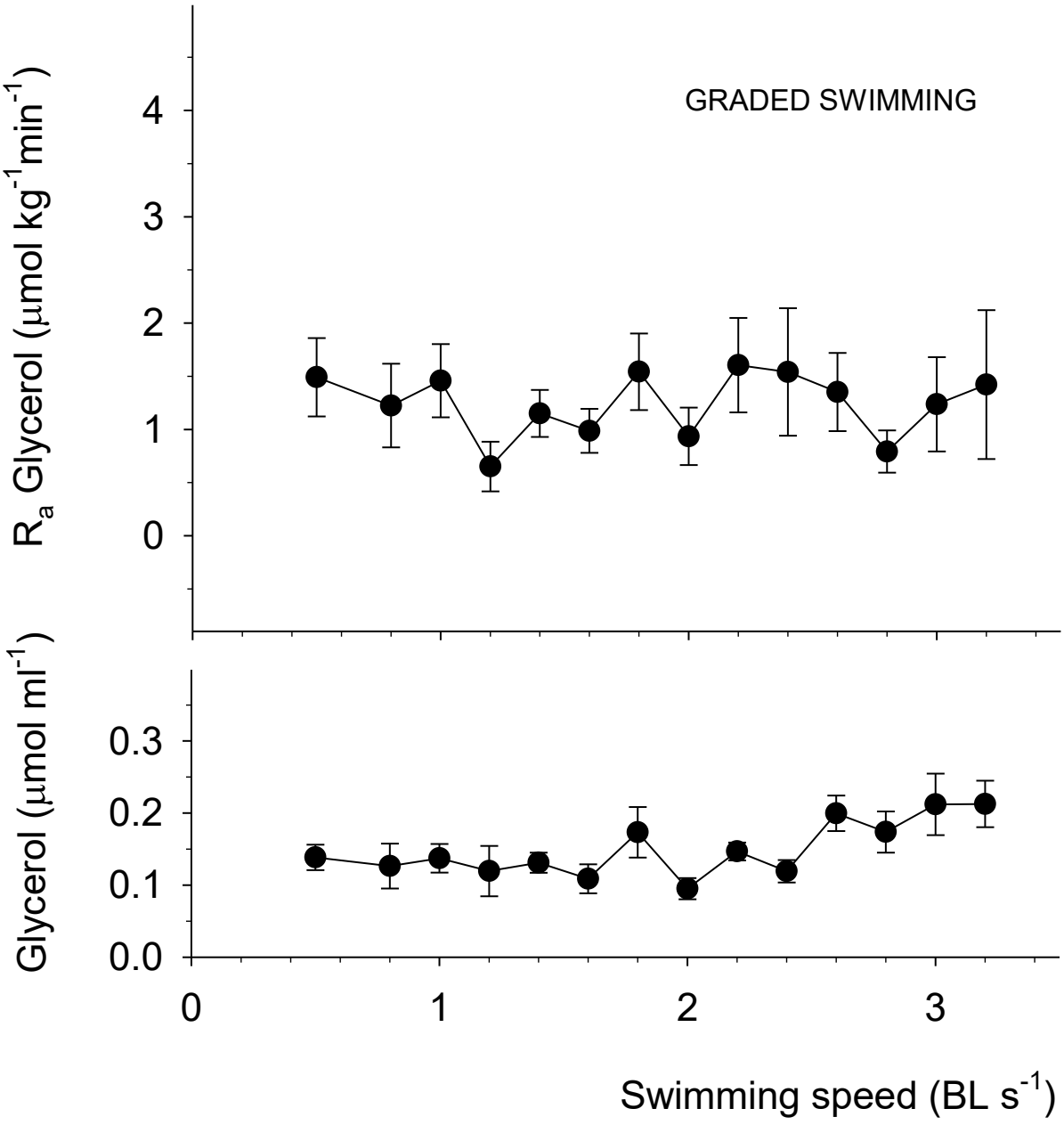

RESTING CONTROLS
R_a Glycerol (µmol kg^{-1}min^{-1})
5
4
3
2
1
0
*
*
Glycerol (µmol ml^{-1})
0.4
0.2
0.0
*
0
60
120
180
240
Time (min)

Fig. 2.4. Glycerol metabolism of trout during graded exercise up to critical swimming speed (U_{crit}). Rate of appearance of glycerol or lipolytic rate (R_a glycerol; top panel) and blood glycerol concentration (bottom panel). R_a glycerol was measured by continuous infusion of 2-[^{3}H] glycerol. Values are means ± s.e.m. (individual sample sizes N are the same as in top panel of Fig. 2.1). No significant effect of exercise was detected ($P>0.05$).

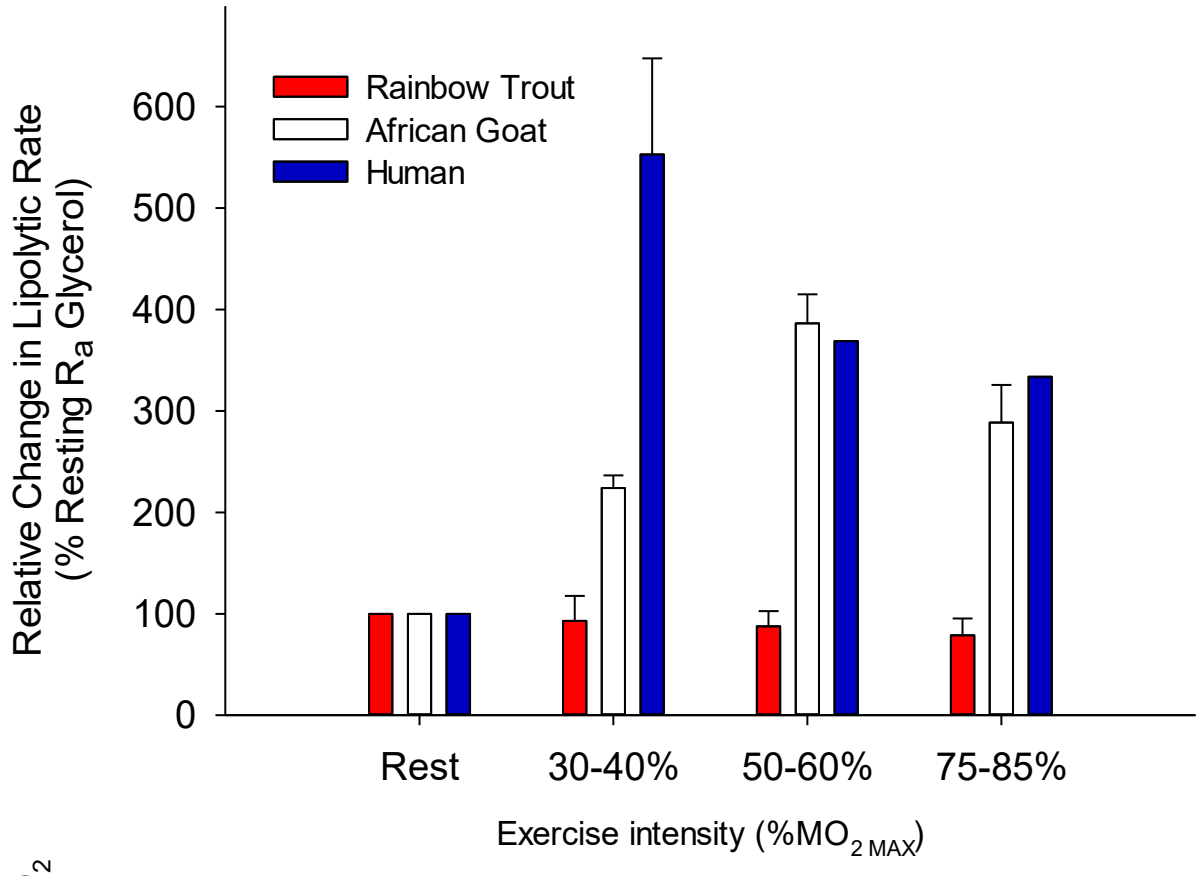

GRADED SWIMMING
R_a Glycerol (µmol kg^{-1}min^{-1})
Glycerol (µmol ml^{-1})
Swimming speed (BL s^{-1})
0
1
2
3
4
0.0
0.1
0.2
0.3

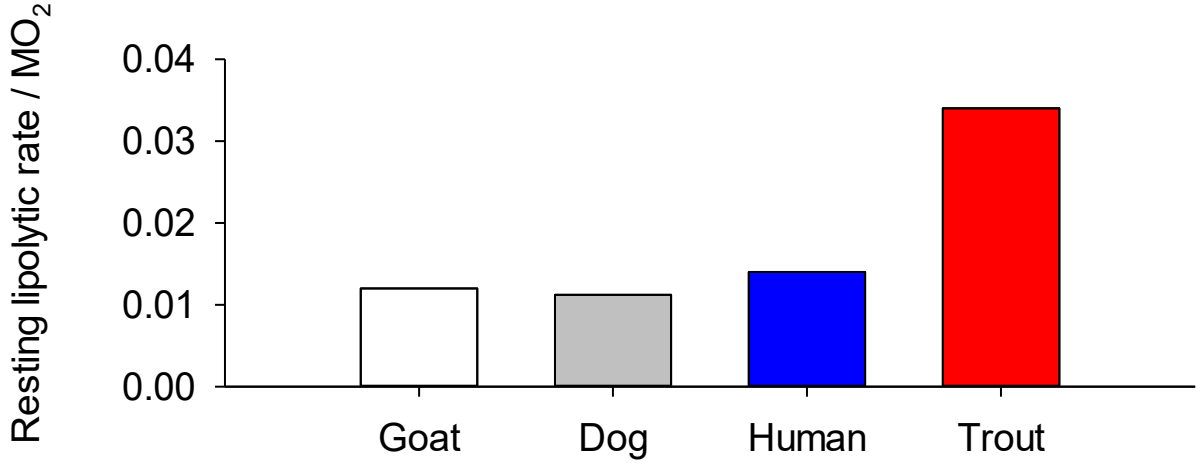
Comparison of lipolytic response in trout and mammals

Fundamental differences in the regulation of R_a glycerol between trout and mammals are illustrated in Fig. 2.5. Graded exercise causes a 3 to 5-fold increase in the lipolytic rate of goats and humans, but has no stimulating effect in rainbow trout (top panel Fig. 2.5). In mammals, maximal rates of lipolysis are reached at intermediate exercise intensities of 30-60% $\dot{M}O_{2\,MAX}$. Because the metabolic fuel demands of fish and mammals greatly differ (ectotherm *vs* endotherm, and effects of body mass), baseline lipolytic rates were standardized as resting R_a glycerol / resting $\dot{M}O_2$ to provide energetically fair comparison (bottom panel Fig. 2.5). Standardized baseline lipolytic rate of rainbow trout is 2.4 to 3 times higher than for mammals.

Fig. 2.5. Top panel: Mammal-trout comparison of relative changes in lipolytic rate as a function of exercise intensity expressed as a percentage of resting R_a glycerol. Values are presented for rainbow trout (this study), African goat (calculated from Weber et al., 1993), and humans (calculated from Wolfe et al., 1990; Klein et al., 1996; Coggan et al., 2000). Mammals show a 3- to 5-fold increase in lipolytic rate during exercise, whereas trout maintain a steady, baseline rate at all exercise intensities. Values are means (± s.e.m. when it could be calculated from the source papers).

Bottom panel: Comparison of standardized baseline lipolytic rate [calculated as resting R_a glycerol / resting metabolic rate] between mammals and trout. Values were calculated for goat (Weber et al., 1993), dog (resting metabolic rate: Issekutz et al., 1967; R_a glycerol: Issekutz et al., 1975), human (Wolfe et al., 1990) and trout (this study). Trout maintain a much higher baseline lipolytic rate relative to their metabolic rate than mammals. This may explain why trout do not stimulate R_a glycerol during exercise, whereas mammals do.

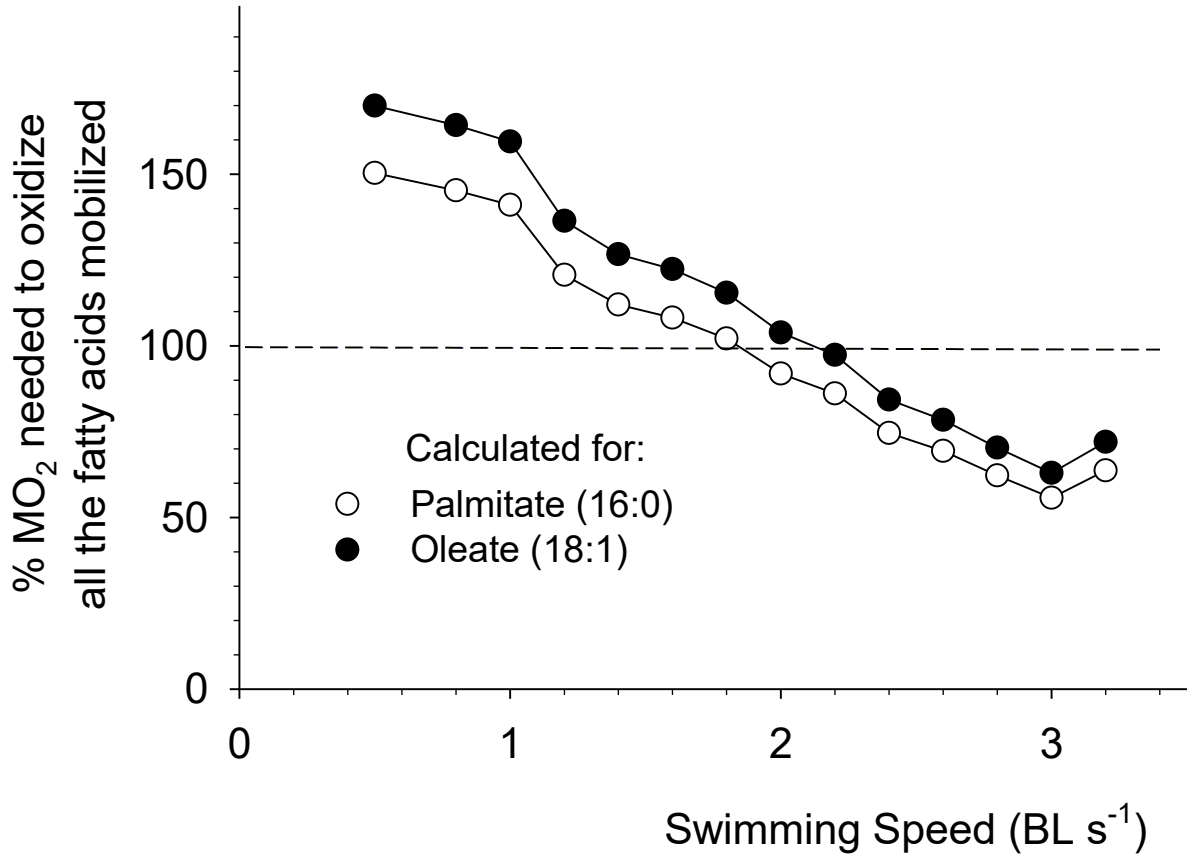

Rainbow Trout
African Goat
Human
Relative Change in Lipolytic Rate
(% Resting R_a Glycerol)
600
500
400
300
200
100
0
Rest
30-40%
50-60%
75-85%
Exercise intensity (%$MO_{2\,MAX}$)


Resting lipolytic rate / MO_2
0.04
0.03
0.02
0.01
0.00
Goat
Dog
Human
Trout

Contribution of trout lipolysis to total oxidative fuel needs during exercise

In Fig. 2.6, the contribution of fatty acids to energy metabolism was calculated as a function of exercise intensity by assuming that all the fatty acids released by lipolysis were oxidized (= 3 times mean R_a glycerol during exercise = 3.72 μmol fatty acid kg^{-1} min^{-1}). The percentage of $\dot{M}O_2$ accounted for by fatty acid oxidation was then computed for the 2 most abundant fatty acids available in trout plasma: palmitate (16:0) and oleate (18:1)(Bernard et al., 1999). These calculations show that lipolytic rate supplies enough fatty acids to support total metabolic rate up to 2 BL s^{-1} (values >100% $\dot{M}O_2$), and 50% $\dot{M}O_2$ at U_{crit} (Fig. 2.6).

Fig. 2.6. Capacity of trout lipolysis to fuel oxidative metabolism with fatty acid fuel as a function of exercise intensity. Values are expressed as percentage of the fish's metabolic rate accounted for by the oxidation of all the fatty acids made available by lipolysis (=3 times mean R_a glycerol of swimming fish or 3.72 μmol of fatty acids kg^{-1} min^{-1}). These values were calculated as if all the fatty acids oxidized were either palmitate (16:0) or oleate (18:1): the 2 most abundant fatty acids in trout lipid reserves. Lipolysis can provide all the energy necessary to support total metabolic rate up to 2 BL s^{-1}, and more than 50% of the energy needed at critical swimming speed (U_{crit}).


% MO_2 needed to oxidize all the fatty acids mobilized
150
100
50
0
0
1
2
3
Swimming Speed (BL s^{-1})
Calculated for:
Palmitate (16:0)
Oleate (18:1)

Discussion

This study shows that rainbow trout do not modulate *in vivo* lipolysis to cope with exercise of any intensity. Baseline lipolytic rate is steadily maintained throughout graded swimming from rest to U_{crit}. When standardized to metabolic rate, the resting lipolytic rate of trout is extremely high compared to mammals, but it is neither stimulated by submaximal exercise, nor inhibited by intense work. Baseline trout lipolysis releases enough fatty acids from lipid stores to power swimming up to 2 BL/s, and it can provide over 50% of the oxidative fuel required at U_{crit}. Therefore, these athletic fish show a completely different strategy for lipid mobilization from mammals that boost lipolytic rate several fold above baseline at intermediate exercise intensities.

Trout lipolysis is not affected by exercise

Submaximal and intense exercise have no impact on the intrinsically high lipolytic rate of rainbow trout. Throughout graded swimming, the R_a glycerol of exercising fish averages 1.24 μmol kg^{-1} min^{-1} and remains unchanged from baseline values (Fig. 2.4) of the same individuals or from separate control fish kept in the resting state for the same duration (Fig. 2.3 and Table 2.2). These results highlight an essential metabolic difference in the way fish and mammals regulate fuel selection. During the transition from rest to moderate exercise, dogs (Issekutz et al., 1975), goats (Weber et al., 1993) and humans (Wolfe et al., 1990; Klein et al., 1996; Coggan et al., 2000) stimulate lipolytic rate by 3 to 5-fold to provide more fatty acids to working muscles as their principal oxidative fuel (top Fig. 2.5). When exercise becomes more strenuous, ATP production starts to rely more on carbohydrates (Roberts et al., 1996; Brooks, 1998) and lipolysis

becomes inhibited (Romijn et al., 1993b). This pattern is not observed in fish because they keep high and constant lipolytic rates at all times (Fig. 2.4 and 2.5).

High sustained lipolytic rate in trout

The ratio of R_a glycerol to MO_2 calculated for resting conditions is 2.4 to 3 times higher in trout than in all mammals measured to date, from sedentary goats to highly aerobic dogs (bottom panel Fig. 2.5). Such proportionately high rates of resting lipolysis are actually sufficient to cover the energy needs of trout exercise up to 2 BL/s, thereby meeting all oxidative fuel requirements for routine swimming (Fig. 2.6). Baseline trout lipolysis can even provide over 50% of the energy required at U_{crit}, suggesting that no stimulation of R_a glycerol is ever necessary in exercising salmonids. By contrast, mammals may be forced to stimulate R_a glycerol by several fold to support exercise because of their comparatively low resting lipolytic rate (standardized to resting MO_2; see bottom panel Fig. 2.5). Multiple papers now show that rainbow trout sustain much higher fluxes of glycerol (this study; Bernard et al., 1999; Magnoni et al., 2008a), fatty acids (Bernard et al., 1999; Weber et al., 2003), and triacylglycerol (Magnoni et al., 2008b) than strictly necessary to fuel energy metabolism (Weber et al., 2016). Together, these results demonstrate that a large fraction of the released fatty acids are therefore reesterified to prevent exceeding the solubilizing capacity of albumin-like proteins. This is particularly true at rest, but also while swimming up to 2 BL/s (top panel Fig. 2.6) because oxidizing 100% of the fatty acids made available by lipolysis would require much higher metabolic rates than actually measured here *in vivo* (Fig. 2.1 and Table 2.2).

Potential role of sustained reesterification

Simultaneous lipolysis and reesterification form the TAG/fatty acid cycle, a substrate cycle that consumes ATP (Reidy and Weber, 2002). It is unclear why resting fish would need to keep this cycle active, but they are unlikely to spend energy on such a process if it has no physiological purpose. One intriguing hypothesis for the "excess lipolysis" observed in fish is that it continually supplies fatty acids of various chain lengths and levels of unsaturation that could be conveniently used to remodel membrane phospholipids. Ectothermy is a ubiquitous fish trait that must be associated with adequate acclimation mechanisms to cope with fluctuations in body temperature (Hulbert and Else, 2000; Anttila et al., 2015). Anadromy is also characteristic of salmonids, but it is differentially expressed across species (Quinn and Myers, 2004; Spares et al., 2015). The changes in salinity happening with saltwater to freshwater transitions during migration create a serious osmotic stress (McCormick et al., 1998) that can trigger the remodelling of membrane lipids in fish (Gonzalez et al., in preparation). Under changing thermal and osmotic conditions, the ability to stabilize membrane fluidity and the activity of membrane-bound proteins is crucial for fish survival (Williams and Hazel, 1995; Guderley et al., 1997; Gonzalez et al., 2013). Rainbow trout may therefore maintain high rates of lipolysis to ensure rapid and continuous restructuring of membrane phospholipids (Bernard et al., 1999; Magnoni et al., 2008b). Conveniently, such a strategy has the added advantage that no upregulation of lipolysis is necessary to support swimming.

Opposing effects of epinephrine and norepinephrine may explain steady lipolysis

In mammals, epinephrine and norepinephrine stimulate lipolysis (Romijn et al., 1993b) and the circulating levels of both hormones increase during exercise (Pedersen and Hoffman-

Goetz, 2000). Catecholamines were not measured here due to limitations in the total volume of blood that could be drawn. However, previous studies have characterized the effects of swimming on fish catecholamines. In trout, blood epinephrine and norepinephrine levels fall below resting values at low to moderate exercise intensities (Butler et al., 1986; Shanghavi and Weber, 1999), and both show a sharp increase during and after more strenuous swimming (Butler et al., 1986; Milligan, 1996). Because trout lipolysis is stimulated by epinephrine (Fabbri et al., 1998; Shanghavi and Weber, 1999; Magnoni et al., 2008a), but reduced by norepinephrine (Magnoni et al., 2008a) via inhibitory β-adrenoceptors in adipocytes (van den Thillart et al., 2002; Vianen et al., 2002), the coordinated changes in these hormone levels during swimming may leave trout lipolytic rate independent of exercise at any intensity. Constant lipolysis may be maintained *in vivo* via differential activation of adrenoceptor subtypes (van den Thillart et al., 2002) and tissue specific differences in receptor expression (Fabbri et al., 1998).

Critical swimming speed (U_{crit}) and cost of transport (COT)

Exercising rainbow trout reached a U_{crit} of 3.4 ± 0.1 BL s^{-1} (N=8). It could be argued that their true U_{crit} may be higher because no significant increase in blood lactate concentration was observed at the highest swimming speeds (top panel Fig. 2.1). However, it is unlikely that U_{crit} was underestimated here because lower values were measured in previous studies on doubly cannulated trout of the same size, tested with the same swimming protocol, and when circulating lactate concentrations were increased: 2.9 ± 0.2 BL s^{-1} (N=7)(Omlin et al., 2014), 2.8 ± 0.1 BL s^{-1} (N=7)(Teulier et al., 2013) and 2.3 ± 0.1 BL s^{-1} (N=10)(Choi and Weber, 2016).

The cost of transport throughout graded swimming in this study reflected trends described previously in doubly-cannulated trout (Teulier et al., 2013; Choi and Weber, 2016),

whereby the total cost of transport decreases from the highest values observed at low speeds, with a minimal cost of transport occurring at intermediate exercise intensities. The lowest costs of transport reported here (1.6 – 2.2 BL s^{-1}, Fig. 2.2) also coincide with optimal swimming speeds (U_{opt}) of between 1 – 2 BL s^{-1} previously reported in trout and other salmonids (Brett, 1964; Webb, 1971a, b; Tudorache et al., 2011). In brook charr (*Salvelinus fontinalis* Mitchill, 1874) at least, preferred swimming speeds have been shown to strongly correlate with U_{opt} (Tudorache et al., 2011). This likely explains why migration tends to occur at these swimming speeds; swimming more slowly incurs a higher total cost of transport with longer migration times and higher speeds come with the added detriment of exhausting fuel reserves more rapidly. This is a particularly important consideration for species that do not eat during migration (Kadri et al., 1995; McCormick et al., 1998; Quinn and Myers, 2004; Spares et al., 2015).

Conclusions

Results show that unlike mammals, rainbow trout maintain their resting lipolytic rate irrespective of exercise intensity. Baseline lipolytic rate in trout is high and in constant excess of what is necessary to accommodate the demand of energy metabolism for fatty acids during swimming. This eliminates the need to stimulate lipolysis to fuel exercise. The counteracting effects of epinephrine and norepinephrine could be essential to maintain steady lipolysis at all times. Finally, high rates of TAG:FA cycling consume energy and are unlikely to be sustained without a specific physiological purpose. These high rates of TAG:FA cycling may be an evolutionary strategy of migratory anadromous ectotherms allowing for rapid homeoviscous adjustment of membranes to cope with thermal and osmotic stress in variable environments.

CHAPTER 3

FUELING AN UPSTREAM MARATHON:

PALMITATE KINETICS IN ATLANTIC SALMON DURING PROLONGED ENDURANCE SWIMMING

Introduction

Long-distance migration is a spectacular feat of endurance only performed by the greatest animal athletes. To meet the energetic requirements of migration, these animals depend on lipids as their primary source of fuel because they pack more joules per gram than any other fuel (Weber, 2011). Migrating salmonids are especially reliant on lipids as a major source of energy (Lauff and Wood, 1996; van den Thillart et al., 2002; Magnoni et al., 2006), because for many species, the onset of migration is accompanied by a strong reduction or even cessation of feeding (Jonsson et al., 1991; Kadri et al., 1995; Doucett et al., 1999; Klemetsen et al., 2003; Spares et al., 2015). Therefore, these fish must be well-adapted to store, mobilize and oxidize lipids efficiently to ensure successful migration. Like mammals, salmonids store lipids in adipose tissue as esterified fatty acids in triacylglycerol (TAG). From here, lipids are mobilized through lipolysis: the breakdown of TAG into its constituent fatty acids and glycerol (Frayn, 2010). Following lipolysis, fatty acids may be exported into the circulation or reesterified back into TAG *in situ* (Wolfe et al., 1990; Weber et al., 1993). During endurance exercise, mammals stimulate lipolysis (Romijn et al., 1993b; Weber et al., 1993; Klein et al., 1994; Klein et al., 1996; Jeukendrup, 2003) and decrease reesterification (Newsholme and Crabtree, 1976; Wolfe et al., 1990; Campbell et al., 1992; Jeukendrup, 2003) to increase the amount of fatty acids made available to energy metabolism. This results in an elevated rate of fatty acid release to the circulation (Romijn et al., 1993b; Romijn et al., 1993a), which can be measured *in vivo* by continuous tracer infusion as the rate of appearance of non-esterified fatty acids (R_a NEFA; Bernard et al., 1999; Wolfe and Chinkes, 2005).

To date, only one study has measured *in vivo* fatty acid kinetics during exercise in salmonids. Bernard et al. (1999) showed that lipolytic rate (R_a glycerol) and the rate of fatty acid mobilization (R_a NEFA) of rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) are unaffected by endurance swimming, even when it is sustained for 4 days. However, the issue of whether this is a common trait of all salmonids remains unresolved. A study by Anttila et al. (2010) revealed that exercise causes a significant decrease in plasma fatty acid concentration in sea trout (anadromous brown trout; *Salmo trutta* Linnaeus 1758). Though fatty acid kinetics were not measured and therefore inferring changes in flux is problematic (Haman et al., 1997), this conflicts with the findings of Bernard et al. (1999) in swimming rainbow trout because a decrease in plasma fatty acids is unlikely to be associated with an increase in their oxidation (increased R_d NEFA), an inhibition of lipolysis (reduced R_a glycerol) or an increase in fatty acid reesterification (increased TAG:FA cycling thereby reducing R_a NEFA; Weber and Reidy, 2012). This suggests that lipid metabolism may be differentially regulated across species or in response to differences in diet and training (Anttila et al., 2010). Moreover, the swimming speed at which prolonged swimming was performed when NEFA fluxes were measured in trout (1.0 BL s^{-1}; Bernard et al., 1999) is not appropriate in the context of salmonid migration because: a) it is at the low end of the range of optimal swimming speeds previously reported in other salmonids (Brett, 1964; Webb, 1971a, b; Hinch and Rand, 2000; MacNutt et al., 2006; Tudorache et al., 2008; Tudorache et al., 2011), b) it incurs a relatively high cost of transport (Teulier et al., 2013; Choi and Weber, 2016; chapter 2 of this thesis), and c) fatty acids remain the major fuel for oxidative energy metabolism at higher exercise intensities in trout (Lauff and Wood, 1996; Richards et al., 2002b). Therefore, the possible stimulation of lipolysis or reduction of reesterification to make more fatty acids available during migration has not been assessed.

The goals of this study were to investigate the effects of endurance exercise on fish lipid mobilization by measuring fatty acid kinetics during prolonged swimming, but in a salmonid known to undergo long migrations in nature: the Atlantic salmon (*Salmo salar*). Because fatty acids are the major fuel for prolonged endurance exercise, I hypothesized that fatty acid mobilization increases during exercise to make more of this fuel available to energy metabolism. Furthermore, to characterize how fatty acid availability might limit the migration capacity of salmon, metabolic rate and cost of transport were also assessed, and total lipid reserves were measured in a separate group of fish. Overall, the aim was to combine these measurements to calculate to what extent released fatty acids are oxidized and reesterified, and to estimate maximal migration distance and duration.

Methods

Animals

Juvenile (~10g) Atlantic salmon (*Salmo salar* Linnaeus 1758) were obtained from the USDA National Cold Water Marine Aquaculture Center (Orono, ME, USA). The fish were held in a 1200 liter flow-through tank in dechlorinated Ottawa tap water maintained at 13°C, and were exposed to a 12 h: 12 h light:dark photoperiod. They were acclimated to these conditions and grew to experimental size from December 2015 through February 2017 (see Tables 3.1-3.3 for physical characteristics) when they were generously donated to our laboratory by Dr. Steve Perry (University of Ottawa). Throughout growth and until three weeks prior to experimentation, the diet consisted of feeding to satiation 5 days a week with Skretting Nutra RC pellets for salmonids (Skretting, St. Andrews, N.B., Canada). The diet was then changed to Profishent floating fish pellets (Martin Mills, Elmira, ON, Canada), also fed to satiation 5 days a week. Fish were fasted for a minimum of 24 hours prior to swimming and were not fed during experiments to minimize the influence of feeding and digestion on the results. Fish were randomly divided into groups for critical swimming speed tests (N=8, Table 3.2), whole body lipid determination (N=8, Table 3.3) and measurements of fatty acid kinetics during prolonged swimming (N=4, Table 3.1). At the end of critical swimming speed tests, fish were euthanized and plasma was harvested as donor plasma for the preparation of infusates for fatty acid kinetics (see *Continuous tracer infusions* below). All procedures were approved by the Animal Care Committee of the University of Ottawa in accordance with the guidelines established by the Canadian Council on Animal Care.

Catheterization

Fish used for measuring fatty acid kinetics (N=4, Table 3.1) were anesthetized with benzocaine (0.05g l^{-1} in the same quality water as the holding tank, aerated with pure O_2) and doubly cannulated with BTPE-50 catheters (Instech Laboratories, Plymouth Meeting, PA, USA) as previously (Haman and Weber, 1996). During surgery, the catheters were kept patent by flushing with Cortland saline containing 50 U ml^{-1} heparin (Sigma-Aldrich, St Louis, MO, USA). Lower heparin levels of 25 U ml^{-1} were used during experiments to avoid stimulating lipolysis. Fish were left to recover overnight while resting at the bottom of the swim-tunnel respirometer chamber at a water velocity of 0.5 body lengths per second (BL s^{-1}): a weak current that does not elicit swimming (Choi and Weber, 2015). Plastic beads attached to the ends of the catheter extensions eliminated the need to tie down the catheters during recovery, reducing tension on the lines while also preventing these from being pulled through the port in the lid of the swim tunnel. This avoided having to retrieve the catheters from within the swim tunnel chamber at the start of the experiments, thereby limiting handling stress.

Table 3.1: Mean physical characteristics, resting metabolic rate and hematocrit of Atlantic salmon used in fatty acid kinetics experiments (N=4). Values are means ± s.e.m.

Weight (g)	495.0 ± 11.6
Length (cm)	37.8 ± 0.5
Fulton's Condition Factor (K)	0.92 ± 0.03
Resting MO_2 ($\mu mol\ O_2\ kg^{-1}min^{-1}$)	47.7 ± 1.6
Hematocrit (%)	20.4 ± 2.8

Respirometry

All experiments were performed in a 90 L swim tunnel respirometer (Loligo Systems, Tjele, Denmark) supplied with the same quality water as the holding tank and kept at 13°C. Metabolic rate ($\dot{M}O_2$) was measured by intermittent flow respirometry using galvanic oxygen probes connected to a DAQ-PAC-G1 instrument controlled with AutoResp software (version 2; Loligo Systems). The probes were calibrated before each experiment using air-saturated water (20.9% O_2)

Critical swimming speed

Graded swimming experiments were performed on non-cannulated fish (N=8, Table 3.2) to determine critical swimming speed (U_{crit}) and evaluate the total cost of transport (COT; see Appendix 2) at different swimming speeds. This also served to determine the ideal speed at which to perform the prolonged swimming experiments. These tests were carried out as previously described (Teulier et al., 2013; chapter 2 of this thesis). Swimming was initiated at 0.8 BL s^{-1} and water velocity was increased by 0.2 BL s^{-1} every 20 min in a step-wise manner until exhaustion. Fish were deemed exhausted when they could no longer sustain swimming and were unable to remove themselves from the rear grate of the swim-tunnel. At the highest water velocities nearing U_{crit}, the oxygen probes could not accurately measure metabolic rate. This may be due to increased turbulence at high speeds or water velocity causing pressure in excess of the capacity for the water tight port used to feed catheters through the lid of the swim tunnel (or possibly the housings for the probes) to prevent an influx of oxygenated water. It was determined that $\dot{M}O_2$ measurements were only reliable up to ~4.2 BL s^{-1}, even though U_{crit} exceeded these speeds in some individuals (Table 3.2 and Fig. 1).

Table 3.2: Mean physical characteristics, resting metabolic rate and U_{crit} of non-cannulated adult Atlantic salmon (N=8). Values are means ± s.e.m.

Weight (g)	440.3 ± 49.5
Length (cm)	35.5 ± 0.9
Fulton's Condition Factor (K)	0.95 ± 0.04
Resting MO_2 (μmol O_2 $kg^{-1}min^{-1}$)	45.7 ± 6.3
U_{crit} (BL s^{-1})	4.69 ± 0.27

Continuous tracer infusion

Labelled palmitate was infused as previously described (Haman and Weber, 1996; Bernard et al., 1999). The infusate was prepared on the first day of experiments and kept refrigerated, with aliquots taken daily for each infusion. 1-[^{14}C]palmitate (Perkin Elmer, Boston, MA, USA; 2.076 GBq mmol^{-1}) was supplied in ethanol, dried under nitrogen and resuspended in 400µl of plasma obtained from donor individuals from the same batch of fish, and vortexed before dissolving in Cortland saline. Donor plasma was used as a source of albumin-like proteins to solubilize the labelled palmitate as previously (Weber et al., 2003). One hour prior to swimming, 0.1 ml of blood were drawn to determine hematocrit (Table 3.1) and infusion of the isotope was started using a calibrated syringe pump (Harvard Apparatus, South Natick, MA, USA). Exact infusion rates (~1 ml/h) were determined for each fish to account for differences in body mass and averaged 256.2 ± 107.7 Bq kg^{-1} min^{-1} (N=4). This infusion rate corresponds to approximately 0.01% of endogenous R_a palmitate.

Prolonged swimming and blood sampling

After one hour of tracer infusion at rest, 0.2ml of blood were drawn to determine baseline fatty acid kinetics (t=0, Fig. 3.5 and 3.6). Swimming was then initiated at 1.8 BL s^{-1} (~40% U_{crit}, see Table 3.2) and maintained until the end of the experiment, with 0.2 ml of blood being drawn after 1, 2 and 3 hours of infusion and swimming and then again after 24, 48 and 72 hours of swimming; each of these following additional 1 hour infusions. Immediately after being drawn, the blood was centrifuged and plasma was separated and stored at -20°C until further analysis.

Fatty acid analysis

Lipids were extracted by mixing 100 µl of plasma in 5 ml of chloroform:methanol (Folch 2:1), centrifuging and filtering the supernatant into 3.75 ml of 0.25% v/v KCl and repeating the extraction of the pellet in an additional 5 ml of chloroform:methanol as previously (Bernard et al., 1999). The resulting mixture was then centrifuged to separate the aqueous and organic phases. The aqueous phase was discarded and the organic phase was collected and dried using a rotating evaporator (Büchi RE 121 Rotavapor). Lipids were then resuspended in chloroform and neutral lipids (NL), non-esterified fatty acids (NEFAs) and phospholipids (PL) were separated by filtration through Supelclean solid-phase extraction tubes (100mg LC-NH2, Sigma, St Louis, MO, USA) as previously (Bernard et al., 1999; Gonzalez et al., 2015). The relative abundance and concentrations of the different fatty acids present in the NEFA fraction were quantified by gas chromatography (6890N gas chromatograph, Agilent Technologies, Mississauga, ON, Canada), using margaric acid (17:0; 30mg/100ml; Sigma, St Louis, MO, USA) as an internal standard. Blank runs (using only the extraction solvents) were also performed and consistently revealed contamination by 16:0 and 18:0 from an unknown source. To correct for this contamination, blanks were included with every analysis and all plasma results were corrected accordingly. Preliminary measurements also showed that dilution through the extraction process and to a lesser degree, retention of fatty acids by components used during the lipid extraction, caused the already low activity in samples to fall to near background levels, making it difficult to accurately quantify palmitate specific activity from the resulting NEFA fraction. Therefore, 50µl of the remaining plasma were used for scintillation counting to measure palmitate activity because no ^{14}C is incorporated into fatty acids other than palmitate (Bernard et al., 1999).

Whole body lipid reserves

Total body lipids were quantified using Smedes' method for lipid determination (Smedes, 1999). Whole salmon (N=8, Table 3.3) were cut and ground using a Cabela's professional electric meat grinder (Cabela's Inc., Sidney, NE, USA). All components were washed with ethanol (95% v/v) and dried prior to use. Ground biota was mixed and processed several times, gradually reducing cutting plate diameter to maximize homogeneity. Lipid determination was subsequently performed using 500 mg of wet biota from each fish following Smedes' method (Smedes, 1999). Samples were homogenized in 14.5 ml 8:10:11 2- propanol: cyclohexane: distilled water using a Polytron homogenizer (model PT10-35, KINEMATICA AG, Lucerne, Switzerland) and centrifuged. The supernatant was collected and transferred to a pre-weighed metal weighing dish. The remaining mixture was further homogenized in an additional 9 ml 8:10 v/v 2-propanol:cyclohexane, centrifuged and re-extracted. Samples were covered and left to dry overnight and the weight of the dried sample was recorded to determine the mass of lipids in the sample. Total lipids (%) were then calculated by comparing the mass of lipids in the sample to the original mass of biota used.

Table 3.3: Mean physical characteristics and body lipid composition of adult Atlantic salmon used in whole body lipid determination (N=8). Values are means ± s.e.m.

Weight (g)	375.6 ± 20.1
Length (cm)	34.8 ± 0.6
Fulton's Condition Factor (K)	0.89 ± 0.01
Total Lipids (%)	5.38 ± 0.61

Calculations and statistics

R_a palmitate was calculated using the steady-state equation of Steele (Steele, 1959; see Appendix 2). R_a NEFA could then be determined by dividing R_a palmitate by the fractional contribution of palmitate to total NEFAs (see Table 3.4). Statistical differences were assessed by one-way analysis of variance (ANOVA) followed by the Holm-Sidak post-hoc test to determine which means were significantly different from each other. When the assumptions of normality or equal variance were not met and transformation failed to normalize the data, Friedman's nonparametric analysis of variance on ranks was performed. When results were percentages, arcsine transformation of the data was performed prior to statistical analysis. All values presented are means ± s.e.m., and a level of significance of $P<0.05$ was used in all tests.

Results

Metabolic rate, U_{crit} and cost of transport

Metabolic rate ($\dot{M}O_2$) and total oxygen cost of transport during graded swimming and prolonged swimming are presented in Fig. 3.1, 3.2 and 3.3, respectively. In the graded swimming experiments, speed had a significant effect on $\dot{M}O_2$ ($P<0.001$) and caused a progressive increase from a baseline value of 45.7 ± 6.3 μmol O_2 $kg^{-1}min^{-1}$ (at a "non-swimming" water velocity of 0.5 BL s^{-1}) to a maximum recorded $\dot{M}O_2$ of 160.5 ± 23.3 μmol O_2 $kg^{-1}min^{-1}$ at 3.8 BL s^{-1} ($P<0.05$, top panel Fig. 3.1; a higher value of 184.6 μmol O_2 $kg^{-1}min^{-1}$ occurs at 4.2 BL s^{-1}, though this is the $\dot{M}O_2$ of a single fish). The fish most likely reach higher $\dot{M}O_2$ than reported here, but this could not be measured here due to technical limitations (see *Critical swimming speed* in Methods). The critical swimming speed (U_{crit}) of these fish was also assessed and it averaged 4.69 ± 0.27 BL s^{-1} (Table 3.2). The $\dot{M}O_2$ of salmon during sustained swimming at 1.8 BL s^{-1} (Fig. 3.2) was also significantly higher than the baseline value of 47.7 ± 1.6 μmol O_2 $kg^{-1}min^{-1}$ in this group, averaging 100.5 ± 3.8 μmol O_2 $kg^{-1}min^{-1}$ ($P<0.05$). This $\dot{M}O_2$ remained unchanged throughout swimming for 72 hours ($P>0.05$). During graded exercise, the total oxygen cost of transport (Fig. 3.1, bottom panel) remained constant, although a non-significant trend towards a decrease from the highest mean value of 3.59 ± 0.69 μmol O_2 $kg^{-1}m^{-1}$ (at 0.8 BL s^{-1}) was observed ($P=0.174$). During sustained swimming however, COT (Fig. 3.3) was found to be significantly lower than baseline values (at 0.5BL s^{-1}; $P<0.05$) of 4.27 ± 0.35 and remained relatively constant throughout swimming ($P>0.05$), averaging 2.46 ±0.08 μmol O_2 $kg^{-1}m^{-1}$. In the prolonged swimming experiments, all values at 72 hours represent a sample size of only 3 fish because one fish tangled its catheters and died overnight between the 48 and 72 hour time points.

Fig. 3.1. Metabolic rate (top panel) and total cost of transport (bottom panel) of non-cannulated Atlantic salmon during graded exercise from rest to ~90% U_{crit}. Values are means ± s.e.m. (N for each mean are given on the graph). * indicates significant difference from baseline values (0.5 BL s^{-1} for metabolic rate, 0.8 BL s^{-1} for cost of transport; $P<0.05$).

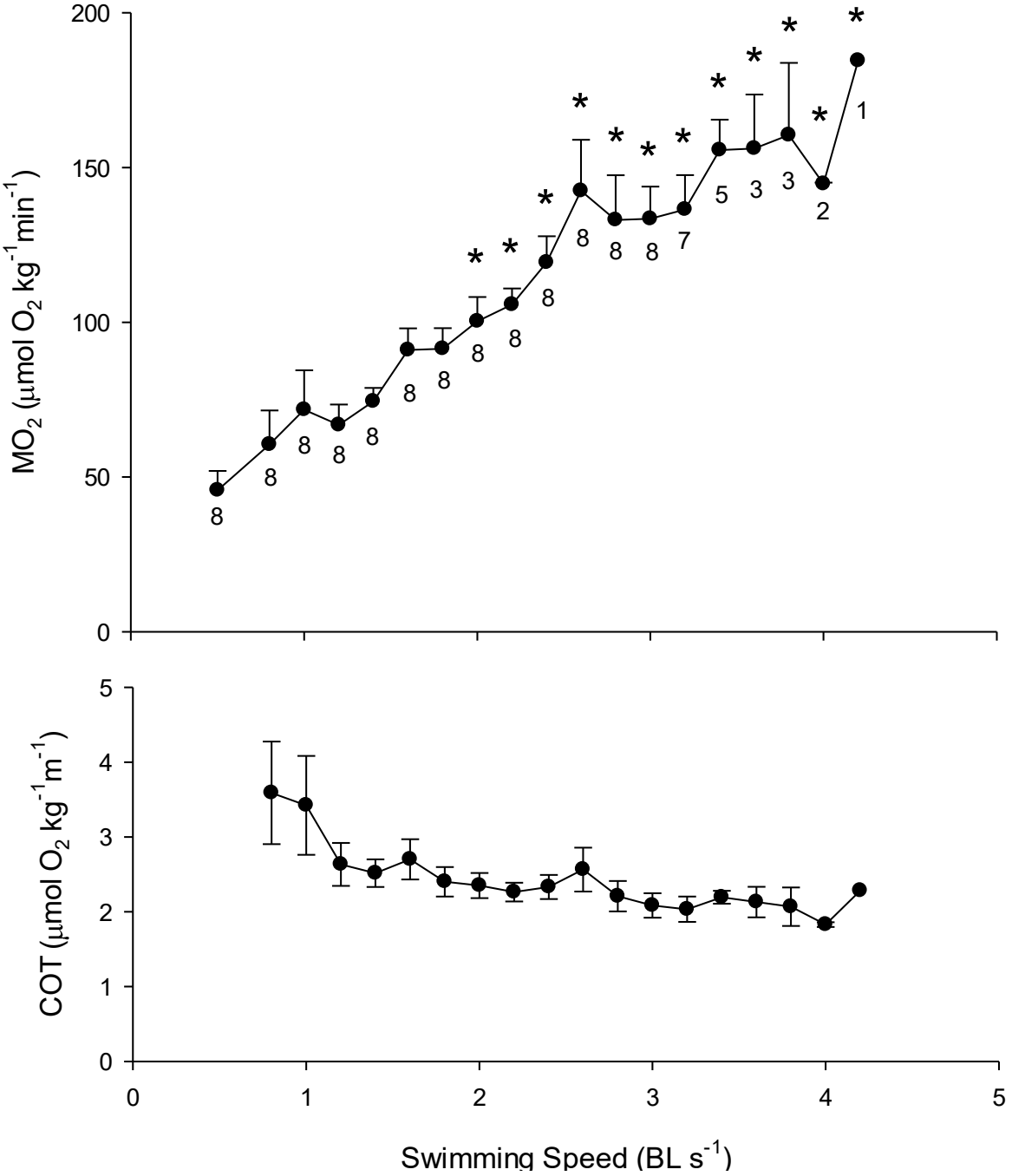

200
150
100
50
0
MO_2 (µmol O_2 $kg^{-1}min^{-1}$)
8
8
8
8
8
8
8
8
8
8
8
8
8
7
5
3
3
2
1
*
5
4
3
2
1
0
COT (µmol O_2 $kg^{-1}m^{-1}$)
0
1
2
3
4
5
Swimming Speed (BL s^{-1})

Fig. 3.2. Metabolic rate of cannulated Atlantic salmon at rest (t=0) and during sustained swimming at 1.8 BL s^{-1} (~40% U_{crit}). Values are presented as means ± s.e.m. of the metabolic rate during periods of infusion and blood sampling (*N*=4 except at 72 hours where *N*=3; top panel) and as individual traces for each fish with metabolic rate measurements taken every 20 minutes throughout swimming (bottom panel). * indicates significant differences from baseline values (0.5 BL s^{-1} or t = 0; $P<0.05$).

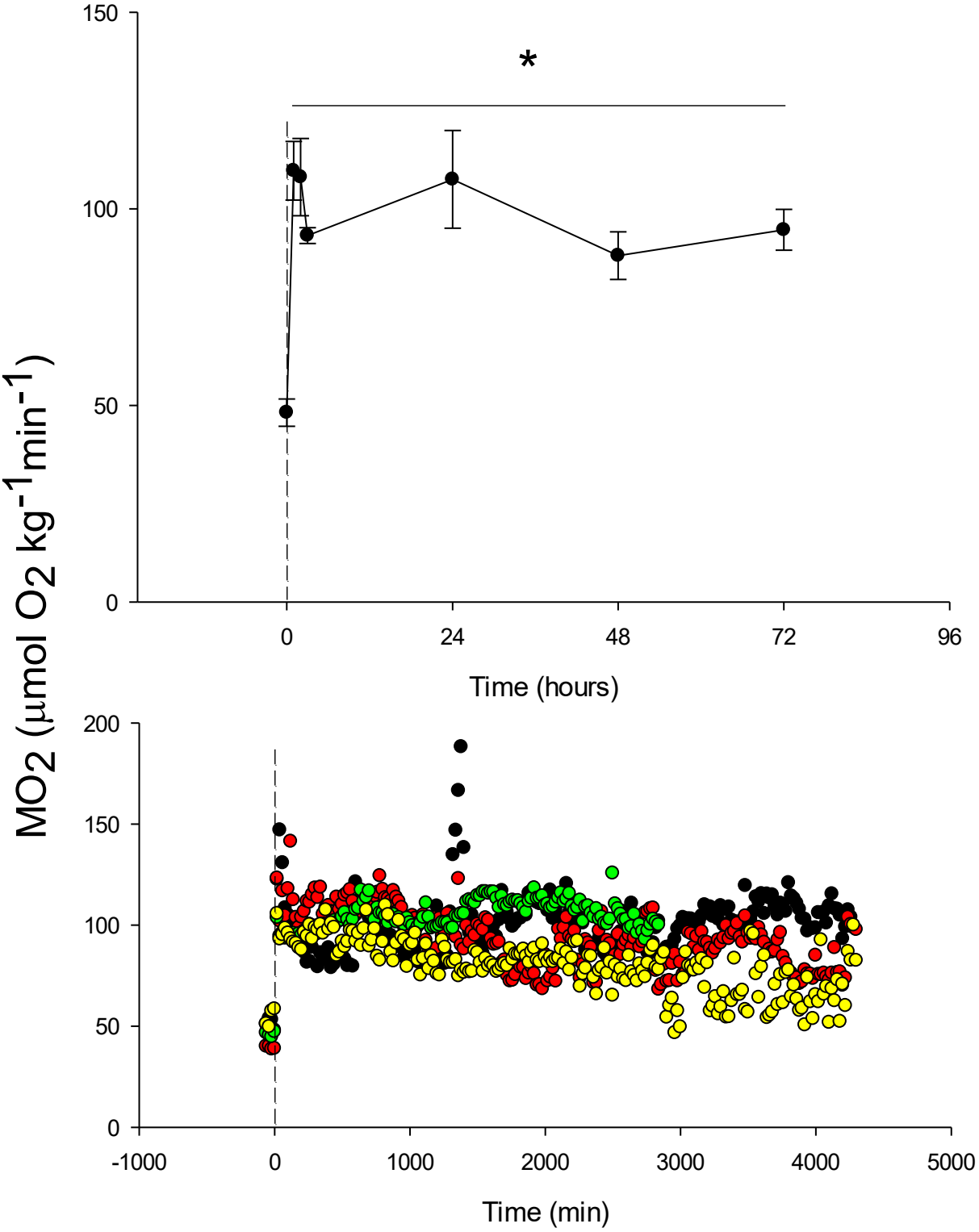
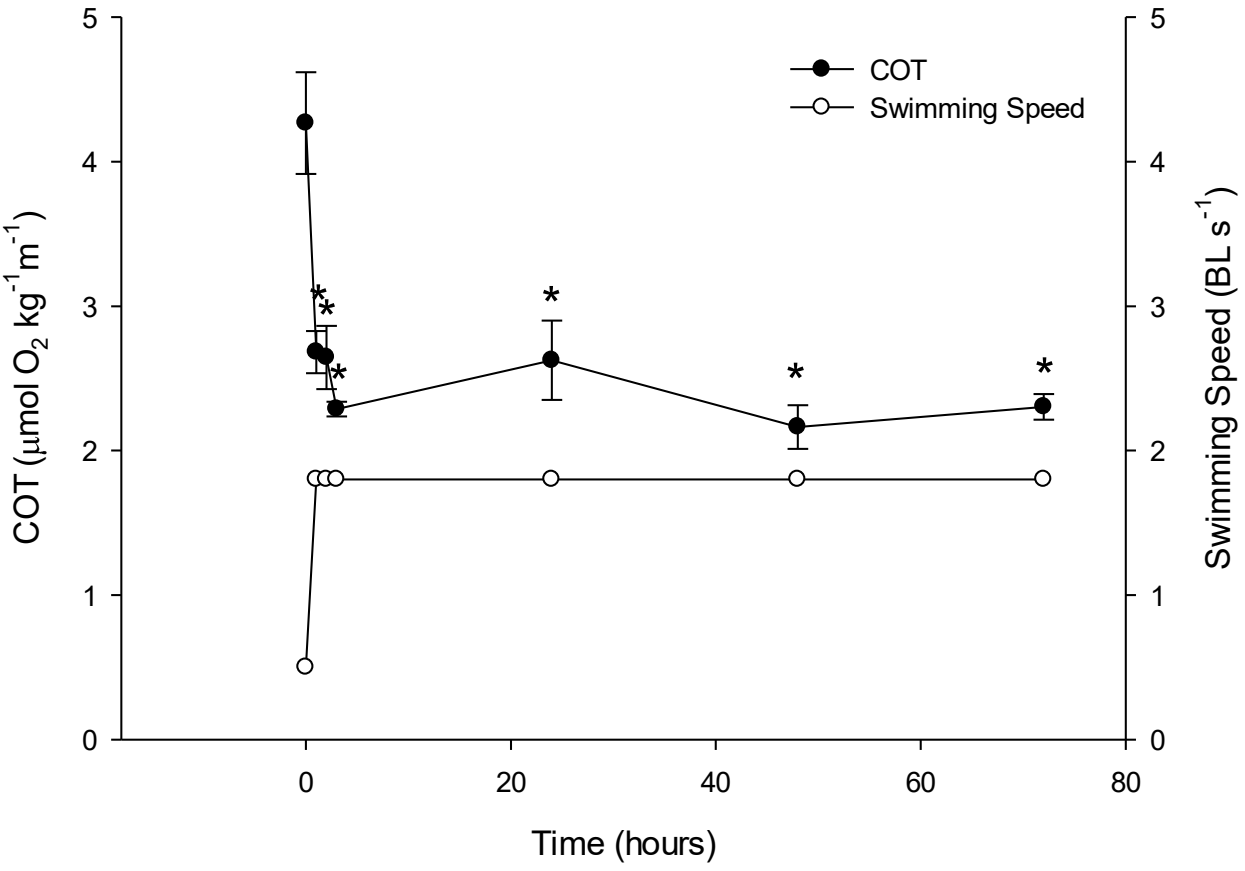

150
100
50
0
*
0
24
48
72
96
Time (hours)
MO2 (µmol O2 kg-1min-1)
200
150
100
50
0
-1000
0
1000
2000
3000
4000
5000
Time (min)

Fig. 3.3. Total cost of transport of cannulated salmon at resting water velocities (0.5 BL s^{-1}, t = 0) and during sustained swimming at 1.8 BL s^{-1} (~40% U_{crit}). Values are means ± s.e.m. (*N*=4 except at 72 hours where *N*=3). * indicates significant difference from baseline (0.5 BL s^{-1} or t = 0; $P<0.05$).


COT
Swimming Speed
COT (µmol O2 kg-1m-1)
Swimming Speed (BL s-1)
Time (hours)
0
1
2
3
4
5
0
20
40
60
80
*

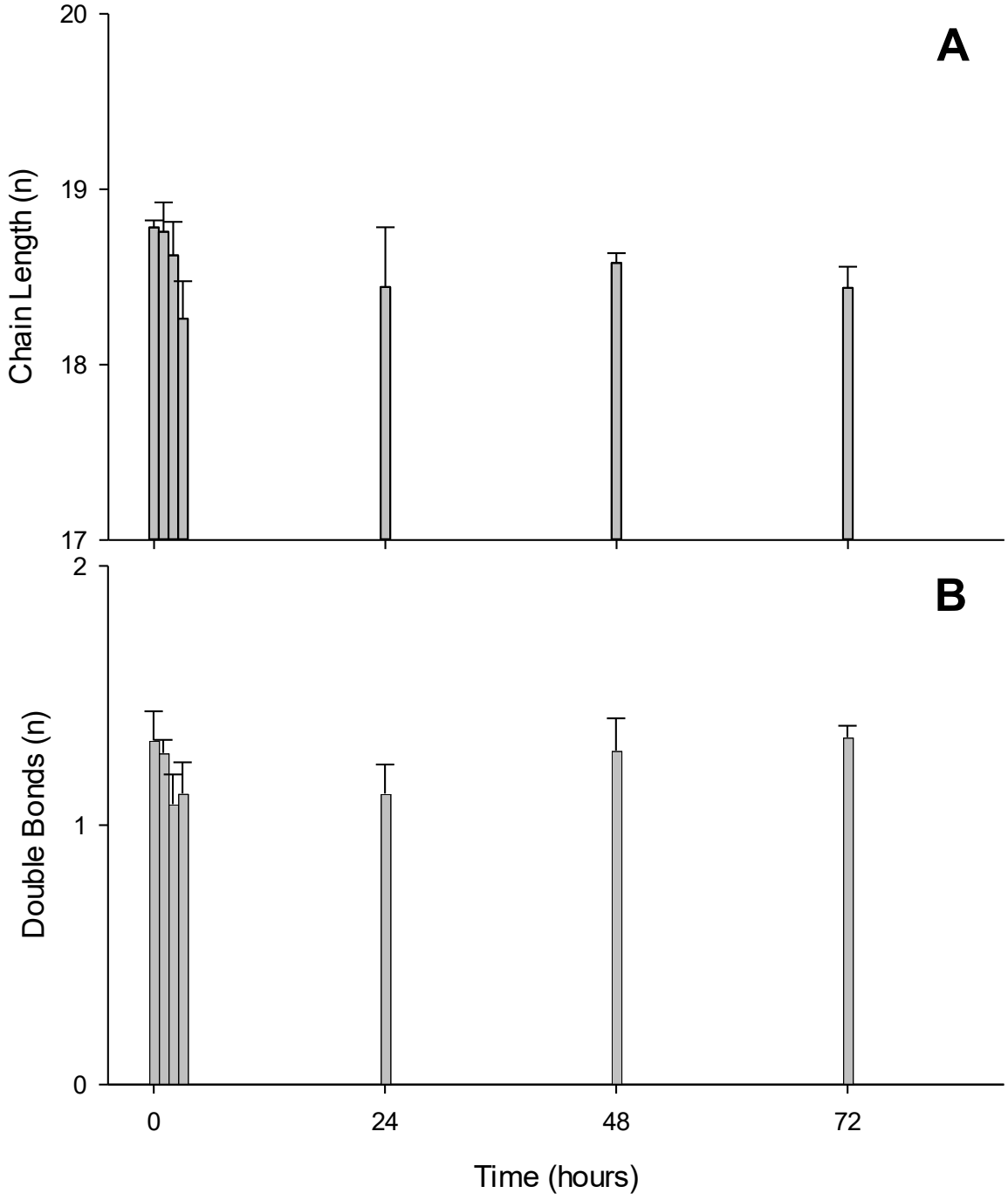
Whole body lipids and plasma NEFA composition

Total lipids were quantified from whole body homogenates and the results are presented in Table 3.3. Lipids represent 5.38 ± 0.61% of total body mass, or an average of 20.31 ± 2.53g of total lipids. Plasma NEFA composition was also assessed in cannulated fish and is presented in Table 3.4. Oleate (18:1 (n-9)) is by far the most abundant fatty acid, representing 36.11 ± 2.37% of total NEFAs, followed by 18:2 (lineoleate), 18:3 (linolenate) and 16:0 (palmitate); each representing approximately 10% of total NEFAs in plasma. At no point during swimming did plasma NEFA composition differ significantly from baseline values (at rest, t=0; $P>0.05$). Average chain length (18.6 ± 0.1) and number of double bonds (1.21 ± 0.04) of plasma NEFA were also unaffected by time or swimming (Fig. 3.4; $P>0.05$).

Table 3.4: Fatty acid composition as a percentage of total non-esterified fatty acids (NEFA) in Atlantic salmon plasma before and during sustained swimming at 1.8 BL s^{-1} (40% U_{crit}). Values presented are means ± s.e.m. (N=4).

		Swimming period (hours)						
NEFA	Rest	1	2	3	24	48	72	Combined data
16:0	8.14±3.81	5.75±1.44	7.04±0.66	16.08±7.22	7.58±4.38	10.37±3.29	8.40±2.79	9.11±1.65
16:1	3.75±1.34	4.76±1.03	5.06±1.90	3.84±0.94	8.04±4.92	5.22±1.28	3.87±0.38	4.94±0.86
18:0	2.87±0.79	7.92	7.71±2.51	11.69±0.05	5.10±1.24	7.45±1.20		6.77±0.97
18:1(n-9)	24.99±3.11	33.62±5.62	37.22±4.98	35.68±6.04	41.58±6.91	33.02±5.09	46.48±4.37	36.11±2.37
18:1(n-7)		3.17						3.17
18:2	14.51±1.14	13.33±2.50	9.12±2.52	12.07±2.96	11.04±2.81	12.81±2.92	11.12±4.00	11.94±1.11
18:3	11.95±3.52	11.09±0.79	8.98±0.93	5.68±2.78	12.36±0.40	9.21±0.42	10.61	10.14±0.99
20:0	5.27±1.06	6.97±1.68	6.38±0.51	3.56	5.56±1.73	6.73±0.45	3..34	5.78±0.59
20:1	11.97±2.93	8.29±1.32	8.82±0.54	9.03±2.75	5.88±1.09	8.85±2.10	11.04±3.99	8.94±0.89
20:2	2.60±0.18	3.80±1.08	3.32±0.14	3.54±1.22	3.31±0.53	3.78±1.20	5.08±1.77	3.63±0.42
20:3		4.27	2.78	4.25	1.91			3.30±0.50
20:5	1.34	2.65		1.49±0.28	0.59	2.06±0.14	2.42±0.37	1.84±0.23
22:0	7.97±3.33	7.42±3.35	15.96±4.04	3.14	9.22±1.56	9.78±4.13	5.11	8.97±1.53
22:1	7.15±1.16	7.24±1.34	7.54±0.18	8.45±2.90	7.87±1.74	5.25±1.38	4.42±1.11	6.98±0.78
22:6	3.44±0.01	2.34±0.21	2.41±0.17	2.80±0.35	1.54±0.29	4.80±2.27	3.09±0.76	2.95±0.48

Fig. 3.4. Average chain length (A) and number of double bonds (B) of fatty acids in the blood of Atlantic salmon at rest (t=0) and during sustained swimming. Values are means ± s.e.m. (N=4 except at 72 hours where N=3). * indicates significant differences from baseline (t=0; P<0.05).


A
B
Chain Length (n)
Double Bonds (n)
20
19
18
17
2
1
0
0
24
48
72
Time (hours)

Fatty acid kinetics

The rate of appearance of palmitate in the circulation (R_a palmitate) and plasma palmitate concentration were quantified at rest (t=0) and during sustained swimming at 1.8 BL s^{-1} (Fig. 3.5). Time had a significant effect on R_a palmitate ($P<0.05$), with the most dramatic difference observed between resting values of 0.75 ± 0.12 μmol $kg^{-1}min^{-1}$ and the minimum value of 0.12 ± 0.03 μmol $kg^{-1}min^{-1}$ occurring during swimming at t=24 h ($P<0.05$). R_a palmitate was not different from resting values at any other time point, though a non-significant decrease from baseline was apparent in most comparisons (e.g. $P=0.061$ at t=72 h). Furthermore, these points were not statistically different from R_a palmitate at t=24 h. Thus, the average value of 0.27 ± 0.06 μmol $kg^{-1}min^{-1}$ during swimming would correspond to a 64% decrease in R_a Palmitate from resting values. Palmitate concentration (Fig. 3.5, bottom panel) did not change from rest throughout exercise ($P>0.05$) and averaged 125.9 ± 18.0 μmol l^{-1}. R_a NEFA was calculated by dividing the fractional contribution of palmitate to total NEFAs. The relative abundance of palmitate in plasma was not affected by exercise or time ($P>0.05$) and averaged 9.11 ± 1.65% of NEFAs (Fig. 3.6, bottom panel). During swimming, R_a NEFA (Fig. 3.6, top panel) trended towards an overall decrease from resting values of 19.3 ± 7.8 μmol $kg^{-1}min^{-1}$ to an average value of 6.9 ± 2.0 μmol $kg^{-1}min^{-1}$ during swimming, but this decrease did not reach statistical significance ($P=0.100$).

Fig. 3.5. Palmitate metabolism of Atlantic salmon at rest (t=0) and during sustained swimming. Rate of appearance of palmitate (R_a palmitate; top panel) and blood palmitate concentration (bottom panel). R_a palmitate was measured by continuous infusion of 1-[^{14}C]palmitate. Values are means ± s.e.m. (*N*=4 except at 72 hours where *N*=3). * indicates significant differences from baseline (t=0; $P<0.05$).

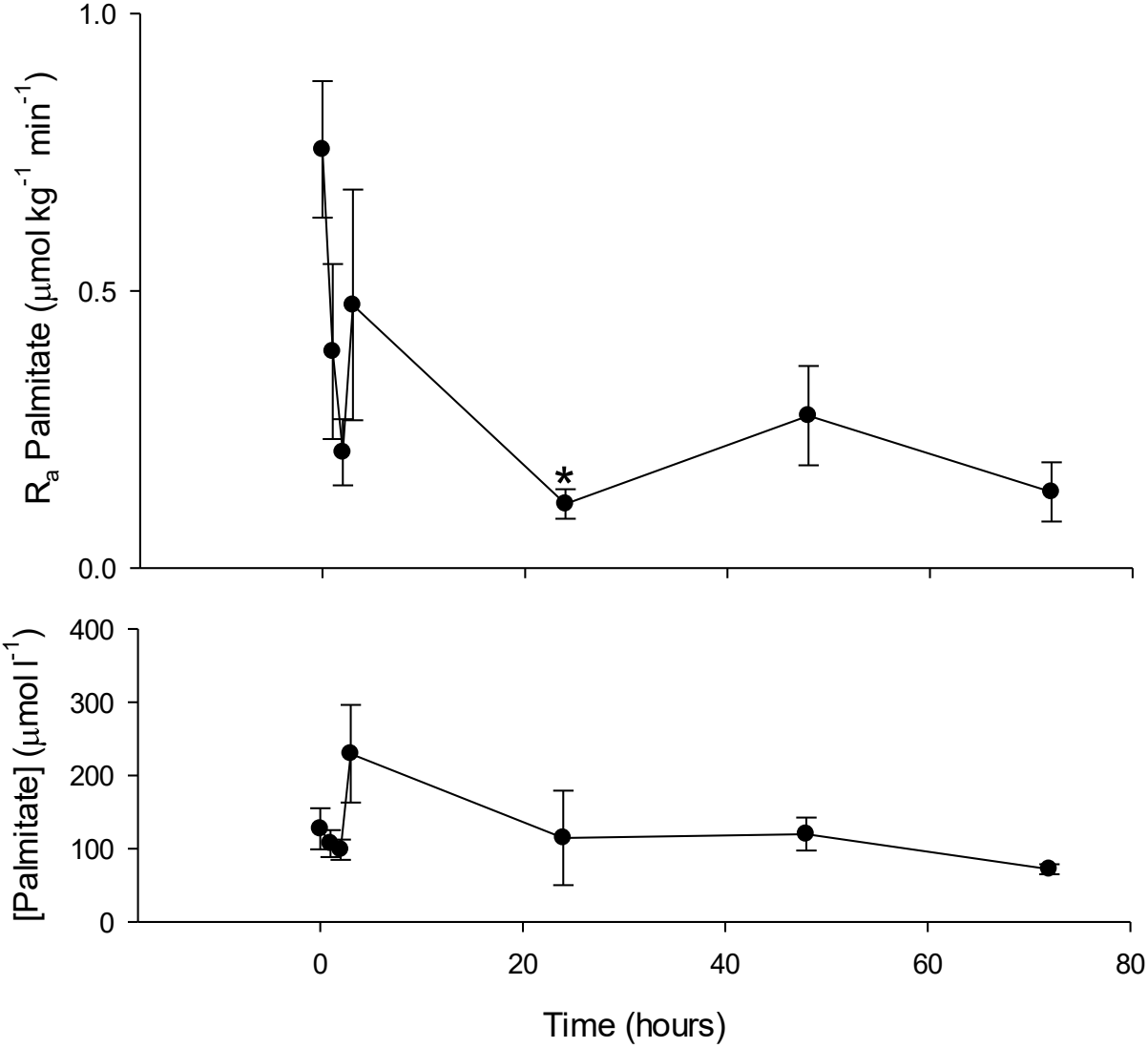
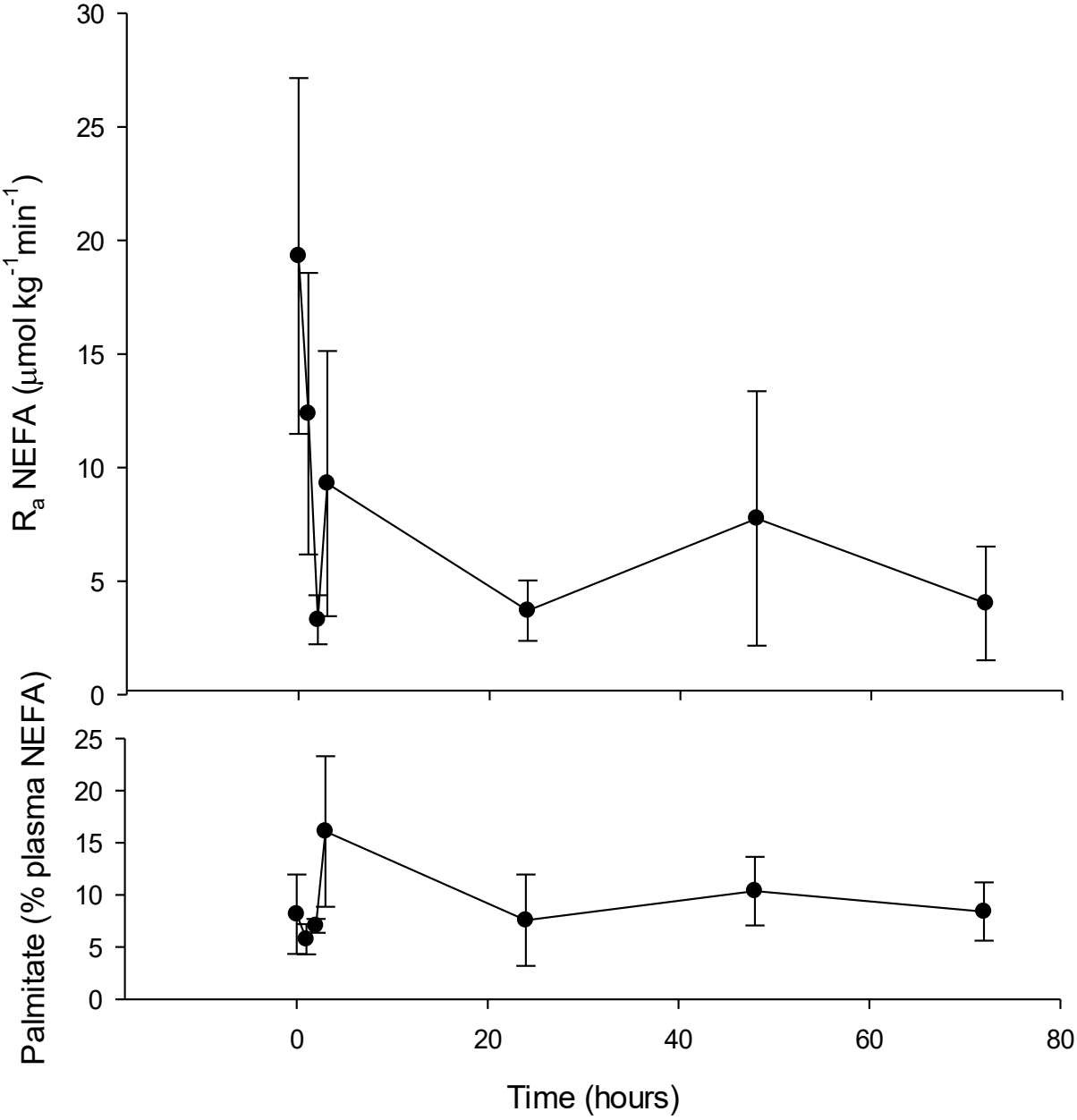

1.0
0.5
0.0
R_a Palmitate (μmol kg^{-1} min^{-1})
*
400
300
200
100
0
[Palmitate] (μmol l^{-1})
0
20
40
60
80
Time (hours)

Fig. 3.6. Rate of appearance of non-esterified fatty acids (R_a NEFA; top panel) calculated based on the fractional contribution of palmitate to total NEFA (% NEFA; bottom panel) in the blood of Atlantic salmon at rest (t=0) and during sustained swimming. Values are means ± s.e.m. (*N*=4 except at 72 hours where *N*=3). * indicates significant differences from baseline (t=0; $P<0.05$).


30
25
20
15
10
5
0
R_a NEFA (μmol $kg^{-1}min^{-1}$)
25
20
15
10
5
0
Palmitate (% plasma NEFA)
0
20
40
60
80
Time (hours)

Discussion

This study provides the first measurements of *in vivo* fatty acid kinetics in Atlantic salmon and shows that R_a palmitate is reduced during endurance exercise. 72 hours of continuous swimming at 1.8 BL s^{-1} leads to a decrease in R_a Palmitate which translates to a non-significant trend towards a decrease in R_a NEFA. A reduction in fatty acid mobilization suggests that either 1) lipolytic rate is reduced or 2) *in situ* reesterification of fatty acids is increased. This observation is different from rainbow trout which show no modulation of either lipolytic rate or fatty acid mobilization during sustained or graded exercise (Bernard et al., 1999; chapter 2 of this thesis). This is surprising because salmonids rely on lipids as their primary fuel for this type of exercise (Lauff and Wood, 1996; Richards et al., 2002b) and it is unclear what advantage would be gained by reducing the mobilization of fatty acids during prolonged swimming. However, R_a NEFA during exercise remains in excess of the requirements for energy metabolism even if ATP production relied exclusively on fatty acids. Therefore, it is possible that a partial inhibition of lipolysis may occur to reduce the energetic burden of TAG:FA cycling during prolonged exercise.

Limitations of study

Unfortunately, the results presented for fatty acid kinetics are plagued by the influence of large error bars due to low sample size (N=4). Double-cannulation surgery was initially performed using ethyl 3-aminobenzoate methanesulfonate (MS-222; 60 mg l^{-1} buffered with sodium bicarbonate, 0.2 g l^{-1}) as an anesthetic. A number of failed attempts (<40% success rate) revealed that Atlantic salmon were particularly sensitive to this drug and did not recover well

from surgery. Personal communications with members of Dr. Katie Gilmour's laboratory (University of Ottawa) suggested that Benzocaine could be used instead of MS-222 and that success rate could be further enhanced by bubbling pure O_2 into the anesthetic bath. While this increased the success rate of surgery to ~60%, actual experimental success rate remained <50% because catheters were occasionally blocked by blood clots, preventing samples from being taken. In some cases fish were also unwilling to swim, adding yet another complication towards obtaining a more adequate sample size for these experiments. As a result, reaching a larger sample size was simply beyond the reach of this thesis. Therefore, the interpretations of the results presented here should be met with caution until such a time as more experiments may be performed to increase the sample size.

Sources of lipids for energy metabolism

While mammals transport lipids to working tissues primarily as free fatty acids bound to albumin (Spector, 1975; van der Vusse, 2009), salmonids are known to rely on circulating lipoproteins as a source of lipids for energy metabolism (Magnoni et al., 2006; Magnoni and Weber, 2007) and possess exceptional capacity for hydrolyzing these lipoproteins (Magnoni et al., 2008b). Lipoprotein synthesis occurs in the liver (Sheridan, 1988, 1994; Rui, 2014) and is supplemented by lipids brought to it in chylomicrons supplied by the intestine in the post-absorptive state (Sheridan, 1988; Rui, 2014). During migration however, when salmonids reduce or even halt feeding entirely (Kadri et al., 1995; Quinn and Myers, 2004), lipoprotein synthesis does not rely on digestion, but entirely on endogenous lipid reserves. Though it has not been unequivocally demonstrated in fish, these lipids may be supplied to the liver from adipose tissue (Rui, 2014). Therefore, even if circulating lipoproteins are the primary source of lipids for

exercise, this should not influence the conclusions presented here. Whether fatty acids are supplied directly to muscle for oxidation or are first incorporated into lipoproteins in the liver, measuring R_a NEFA still assesses the rate at which lipids stored in adipose tissue are being made available to energy metabolism

Influence of the stress response

Stress hormones could not be measured here because of limitations in the amount of plasma available for analysis, but metabolic responses to stress are well documented in fish. Salmonids are sensitive to handling stress (Davis and Schreck, 1997; Gesto et al., 2015). Even very short handling stress (<10 s) can cause large increases in circulating catecholamines and cortisol (Gamperl et al., 1994) which can persist in excess of 45 minutes (VanRaaij et al., 1996; van den Thillart et al., 2002; Gesto et al., 2015). Epinephrine and norepinephrine are known to have opposing effects on lipolysis in trout (Van Raaij et al., 1995; Magnoni et al., 2008a) and different stress conditions are known to cause differentiated catecholamine release (Van Heeswijk et al., 2006). Therefore, it is important to consider the nature of the stress when interpreting its effects on catecholamines and fuel mobilization. While the utmost care was taken to try and minimize stress, at the start of these experiments, salmon may still have experienced stress caused by a number of factors including installation of the infusion pumps and taking blood to determine hematocrit. Occasionally, the catheters were also tangled and needed to be unwound before starting the infusion. These kinds of acute handling stressors can lead to a greater release of epinephrine relative to norepinephrine (van Raaij et al., 1996; Gesto et al., 2013, 2015) which may stimulate the mobilization of fatty acids (Van Raaij, 1994; van den Thillart et al., 2002; Magnoni et al., 2008a).

Plasma cortisol also increases in rainbow trout (Benfey and Biron, 2000; Gesto et al., 2013) and brook trout (*Salvelinus fontalis* Mitchill 1814; Benfey and Biron, 2000) following exposure to acute handling stress and in response to confinement and handling stress in sockeye salmon (*Oncorhynchus nerka* Walbaum 1792; Kubokawa et al., 1999). Other studies on the effects of handling stress have shown similar increases in plasma cortisol in rainbow trout and other fish following stress exposure, but the size, time and duration of the increase is variable based on the species and the nature of the stress applied (VanRaaij et al., 1996; Mommsen et al., 1999; Barton, 2002; Dindia et al., 2013; Gesto et al., 2013). In mammals, the role of cortisol in the regulation lipolysis is more complex than that of catecholamines (Bhathena, 2006) and contradiction in the literature suggests that it is only poorly understood (Peckett et al., 2011). Nonetheless, there is growing consensus that acute elevations in cortisol have a primarily lipolytic effect, whereas chronically elevated cortisol may be more lipogenic (Peckett et al., 2011; Stimson et al., 2017). There is evidence to suggest that this is also the case in fish (Sheridan, 1994; VanRaaij et al., 1996; Mommsen et al., 1999; Gesto et al., 2013).

Together, greater increases in epinephrine relative to norepinephrine and increases in circulating cortisol caused by an acute handling stress may stimulate fatty acid mobilization and be responsible for the higher R_a palmitate observed at the start of the experiment. While the influence of the stress response on the results presented here is difficult to discount, it should be noted that the salmonid stress response is typically accompanied by large increases in metabolic rate (Davis and Schreck, 1997; Sloman et al., 2000; McKenzie et al., 2012). Metabolic rate in these experiments was low prior to swimming (45.7 and 47.7 μmol O_2 $kg^{-1}min^{-1}$ in the graded exercise and prolonged swimming groups, respectively) and was consistent with resting metabolic rates previously reported in salmonids of equivalent size (Burgetz et al., 1998; Choi

and Weber, 2015; Choi and Weber, 2016; chapter 2 of this thesis). Therefore, caution should be exercised in attributing the higher R_a palmitate at the start of the experiments to stress.

Energetic requirements of swimming

Reductions in R_a palmitate and R_a NEFA can indicate either 1) an inhibition of lipolysis or 2) an increase in FA reesterification (TAG:FA cycling). Endurance exercise in salmonids is powered by the red muscle (Johnston, 1980; Johnston and Moon, 1980b; Anttila et al., 2010) and relies predominantly on lipids to fuel aerobic energy metabolism (Lauff and Wood, 1996; Richards et al., 2002b; Anttila et al., 2010). The highest rates of lipid oxidation generally occur at intermediate exercise intensities (i.e. 40-60% Ucrit; Lauff and Wood, 1996; Richards et al., 2002b) and rates of lipid uptake in red muscle remain elevated even at high exercise intensities (Anttila et al., 2010). In the prolonged swimming experiments, fish swam at 1.8 BL s^{-1}; a speed which corresponds to approximately 40% U_{crit} (Table 3.2 and Fig. 3.1). It can therefore be assumed that near-maximal rates of fatty acid oxidation occur during this exercise, meaning that a reduction of R_a NEFA due to reduced reliance on lipids as a fuel for energy metabolism is unlikely. Additionally, FA reesterification is known to increase in mammals during recovery from exercise (van Hall et al., 2002b; van Hall et al., 2002a)(presumably to replenish intracellular TAG), but exercise itself does not stimulate TAG:FA cycling (McClelland et al., 2001; van Hall et al., 2002a). While only limited information is available in fish, an increase in TAG:FA cycling is unlikely to be responsible for the observed reduction in R_a palmitate because it is an energetically costly process (Elia et al., 1987; Bernard et al., 1999; McClelland et al., 2001; Reidy and Weber, 2002) and would be counterproductive to sustaining prolonged exercise

on limited fuel reserves. A more likely scenario is a reduction of lipolysis to reduce the energetic burden of TAG:FA cycling.

If the rate of O_2 consumption for oxidation of the average fatty acid is assumed, the minimal rate of fatty acid supply to sustain energy metabolism can be calculated, assuming that energy metabolism is supported entirely by fatty acid oxidation. The average fatty acid in the plasma of these salmon is 18.6 carbons in length (Table 3.4 and Fig. 3.4) and therefore requires approximately 26 moles of O_2 per mole FA oxidized (Bernard et al., 1999). If the resting R_a NEFA (at t=0) of 19.3 μmol $kg^{-1}min^{-1}$ was maintained at all times similarly to how baseline R_a NEFA is maintained during exercise in rainbow trout (Bernard et al., 1999), oxidation of 100% of fatty acids released (assuming values for the average fatty acid) would result in a metabolic rate of ~ 500 μmol O_2 $kg^{-1}min^{-1}$. Because $\dot{M}O_2$ averages 100.5 μmol O_2 $kg^{-1}min^{-1}$ throughout swimming, only 20% of baseline R_a NEFA is therefore sufficient to meet all of the fuel requirements for aerobic ATP production. The fate of NEFA produced via lipolysis can only be subsequent oxidation or reesterification (Bernard et al., 1999; Reidy and Weber, 2002; Weber and Reidy, 2012), meaning that a minimum of 80% of NEFA released would have to be reesterified if resting (t=0) R_a NEFA was maintained during swimming. By contrast, if these same calculations are made using the average value for R_a NEFA during swimming (t >0) of 6.9 μmol $kg^{-1}min^{-1}$, 56% of this rate is sufficient to cover all the needs of energy metabolism with only 44% of fatty acids released requiring reesterification. Therefore, reducing R_a NEFA from baseline (t=0) to the rate observed during swimming (t>0) results in a decrease in the amount of fatty acids that need to be reesterified.

Energy budgets of migration and the cost of TAG:FA cycling

FA reesterification (TAG:FA cycling) is an energetically costly process (Elia et al., 1987; Bernard et al., 1999; McClelland et al., 2001; Reidy and Weber, 2002). The amount of phosphate bonds required to form 1 molecule of TAG (7-8 phosphate bonds) and the energy released by the hydrolysis of 1 mol of ATP (~75kJ) are known (Elia et al., 1987; Golding et al., 1995; Reidy and Weber, 2000), allowing to calculate the energetic cost of TAG:FA cycling. If energy metabolism relied exclusively on fatty acids and resting R_a NEFA (t=0) was maintained during swimming, 80% of fatty acids released would require reesterification into TAG. This represents an investment of approximately 3.10 J $kg^{-1}min^{-1}$, versus only 0.78 J $kg^{-1}min^{-1}$ for the reesterification of excess FAs (44%) released at the average rate observed during swimming (t>0). This implies that the reduction in R_a NEFA from baseline may lower the energetic burden of TAG:FA cycling by as much as 75% during swimming. While the absolute cost of reesterification is low and only represents a small contribution to total metabolic rate, reduced substrate cycling may be beneficial to optimize energy budgets during long migrations when fuel stores are limited and energetic input from feeding is low or non-existent.

Fueling an upstream marathon

Total lipid reserves are 5.38% of total body mass (Table 3.3) and therefore theoretical migration distances that can be covered by relying exclusively on lipids to fuel locomotion can be calculated if it is assumed that 1) lipid reserves are almost entirely TAG made up of the most abundant fatty acid found in plasma (18:1; Table 3.4 and Fig. 3.6), 2) locomotion is fueled entirely by lipids stored in adipose tissue (whether supplied to muscle directly or following incorporation into lipoproteins) and 3) lipid reserves are fully used. Total lipids reserves of 53.8g

TAG kg^{-1} body mass correspond to 0.061 moles trioleate kg^{-1} body mass (885.43 g mol^{-1}). As previously mentioned, 56% of mean R_a NEFA during swimming is sufficient to accommodate all of the energy requirements for swimming. Thus, a rate of fatty acid mobilization of 3.86 μmol kg^{-1} min^{-1} could be sustained for approximately 47 400 minutes before lipid reserves are exhausted. Swimming at 1.8 BL s^{-1}, endogenous lipids can support locomotion entirely in the average salmon (Table 3.1) in excess of 1900 km. It should be noted however that in reality, locomotion is not supported entirely by lipids and swimming would cease well before 100% of lipids are exhausted. These theoretical calculations merely serve to demonstrate that even relatively small lipid reserves (as a percent of total body mass) contain huge energetic potential and are indispensable as a fuel for long distance migration.

Conclusions

In this study, we have demonstrated that like rainbow trout, Atlantic salmon mobilize lipids in excess of the requirements for energy metabolism during exercise. However, contrary to mammals that stimulate fatty acid mobilization during prolonged endurance exercise, fatty acid mobilization appears to be reduced during sustained swimming in salmon. Furthermore, unlike trout, Atlantic salmon may partially inhibit lipolysis to reduce the energetic burden of fatty acid reesterification during swimming. This strategy could serve to optimize energy budgets during long migrations when feeding stops and locomotion relies entirely on endogenous fuels. Still, future measurements of glycerol kinetics in swimming salmon will be required to support or refute this hypothesis.

CHAPTER 4
GENERAL CONCLUSIONS

The goal of my research was to address the paucity of information on *in vivo* lipid fluxes in exercising salmonids. The main objectives were to measure glycerol kinetics during graded exercise in rainbow trout and fatty acid mobilization during prolonged endurance exercise in a different species known to undergo long migrations in nature; the Atlantic salmon. I hypothesized that salmonids stimulate lipid mobilization during swimming, with peak rates of lipolysis and fatty acid mobilization occurring during moderate intensity exercise when lipid use is at its highest, as is classically observed in mammals.

The work I have presented in this thesis has revealed that salmonids mobilize lipids in constant excess of the requirements for energy metabolism. This conclusion is made on the basis that rainbow trout maintain an elevated rate of lipolysis (chapter 2, Fig. 2.5) at all times (irrespective of exercise intensity; chapter 2, Fig. 2.4) which has the capacity to meet all of the needs for oxidative fuel during locomotion up to 2 BL s^{-1} (~60% U_{crit}; chapter 2, Fig. 2.6) and likely remains excessive at higher exercise intensities due to reduced reliance on lipids as a substrate for ATP production (Lauff and Wood, 1996; Richards et al., 2002b). While R_a palmitate appears to decrease in Atlantic salmon during prolonged endurance exercise (chapter 3, Fig 3.5), the rate of NEFA release still exceeds the energetic requirements of locomotion because only 56% of mean R_a NEFA during swimming would make enough fatty acids available to accommodate 100% of fuel demand for aerobic energy metabolism. This is a very different response from mammals that must stimulate lipolysis several-fold above baseline (Romijn et al., 1993b; Weber et al., 1993; Klein et al., 1994; Klein et al., 1996) and reduce the rate of *in situ* fatty acid reesterification (Newsholme and Crabtree, 1976; Wolfe et al., 1990; Campbell et al., 1992) to increase fatty acid mobilization and make more of this fuel available to energy metabolism. Catecholamines are known to modulate lipolysis differently in salmonids than in

mammals (Magnoni et al., 2008a) and may be essential regulators of this response, though further investigations into the effects of circulating catecholamines on *in vivo* fuel kinetics during exercise will be required. Together, these results provide further evidence in support of previous claims that salmonids sustain high rates of lipid turnover at all times as a strategy allowing for rapid reorganization of membrane phospholipids in response to changing environmental conditions (Bernard et al., 1999; Magnoni et al., 2008b).

Surprisingly and contrary to what has been reported in rainbow trout (Bernard et al., 1999), a decrease in R_a palmitate from baseline was observed during prolonged endurance exercise in Atlantic salmon. It is as of yet unclear what mechanism is responsible for this decrease in fatty acid mobilization. The influence of sample size and handling stress early in the experiments cannot be entirely discounted, but it is speculated that Atlantic salmon may partially inhibit lipolysis during swimming to minimize the energetic burden of TAG:FA cycling. This may be useful to optimize fuel budgets during locomotion over long distances and would be a particularly useful adaptation for species that stop eating during migration. In the future, measuring glycerol kinetics in swimming salmon will allow to determine whether this inhibition of lipolysis indeed occurs.

Salmonids are a diverse family of fishes with species expressing vastly different life history traits (McCormick et al., 1998; Quinn and Myers, 2004; Spares et al., 2015). Barring the effects of diet and training (Anttila et al., 2010), there may be inherent differences in life history and metabolic phenotype between species which could be in part responsible for the differences in lipid mobilization observed during endurance exercise between trout (see Bernard et al., 1999) and salmon. Atlantic salmon are more adaptable in the marine environment and express anadromy to a greater extent than rainbow trout (including steelhead; the name commonly given

to anadromous and migratory rainbow trout) (Quinn and Myers, 2004; Spares et al., 2015). They typically spend more time residing at sea, perform greater vertical migrations in their marine habitats and are more tolerant to varying environmental conditions (broader thermohaline limits) than rainbow trout (Spares et al., 2015). Moreover, in comparison with data from Teulier et al. (2013) in non-cannulated rainbow trout of similar size and age to the salmon used here (U_{crit} experiments, chapter 3, Table 3.2), Atlantic salmon perform better in graded swimming experiments (Fig. 4.1) and have a lower COT at equivalent speeds (Fig. 4.2)(see below).

Fig. 4.1. Critical swim speed of non-cannulated adult rainbow trout (N=6) (data from Teulier et al., 2013) and Atlantic salmon (N=8). Values are means ± s.e.m. * indicates significant difference between species (Mann-Whitney rank sum test; $P<0.05$).

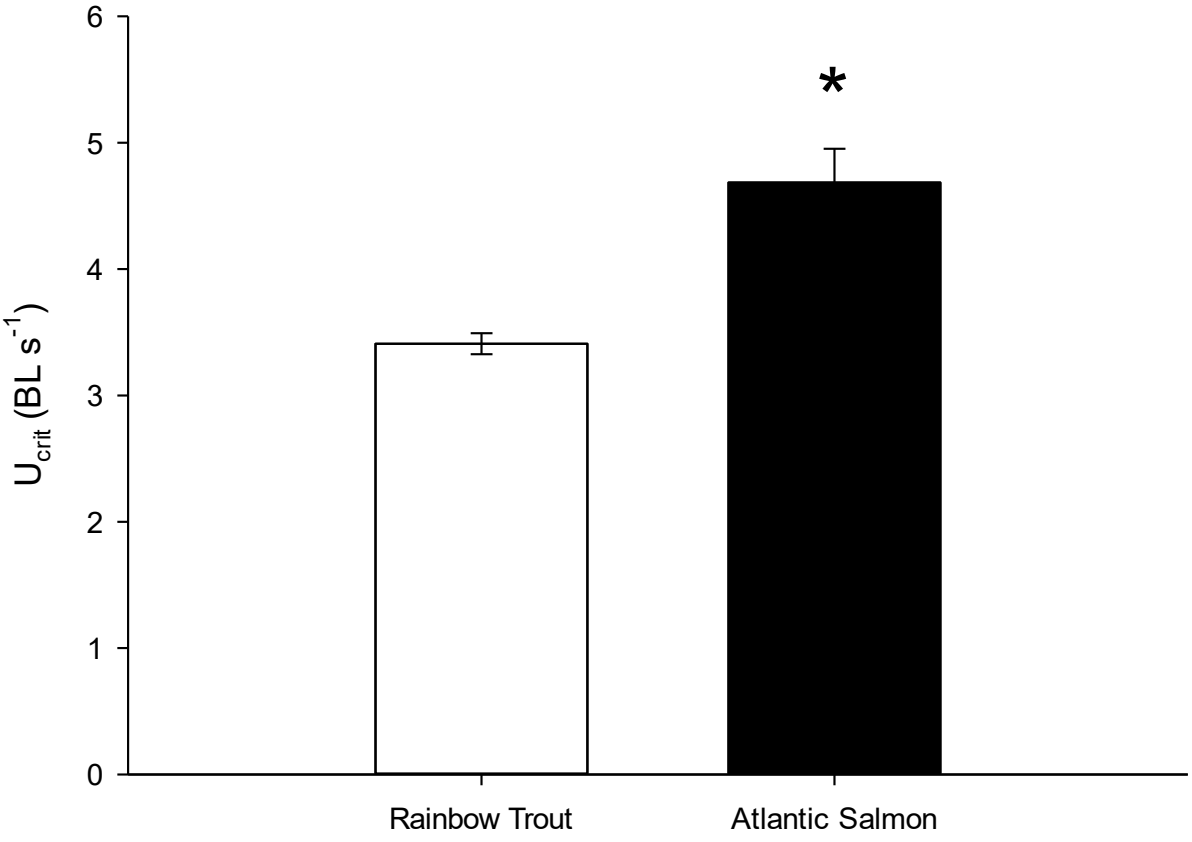
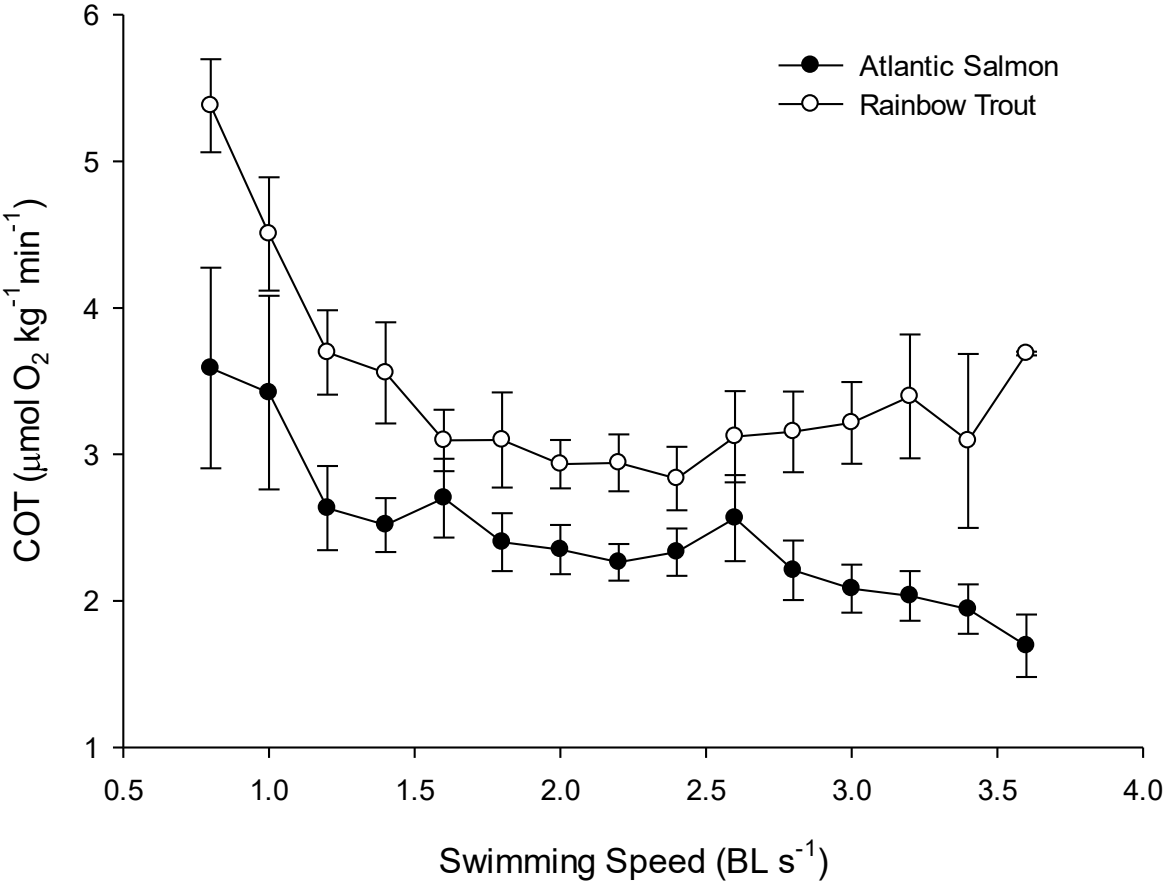

6
5
4
3
2
1
0
U_{crit} (BL s^{-1})
*
Rainbow Trout
Atlantic Salmon

Fig. 4.2. Total cost of transport of swimming non-cannulated rainbow trout (N=6 up to 3.0 BL s^{-1} and decreases progressively to N=2 at 3.6 BL s^{-1}) (data from Teulier et al., 2013) and Atlantic salmon (N=8 up to 3.0 BL s^{-1} and decreases to N=3 at 3.6 BL s^{-1}) throughout graded swimming. Values are means ± s.e.m. (Two-Way RM ANOVA on Ranks; $P<0.05$ for effects of speed and species).


Atlantic Salmon
Rainbow Trout
COT (µmol O2 kg-1min-1)
Swimming Speed (BL s-1)
6
5
4
3
2
1
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0

Differences in population and species-specific thermal optima are known to influence aerobic scope and swimming performance in fish (Lee et al., 2003; Farrell et al., 2008; Brownscombe et al., 2017). However, while trout and salmon possess different thermal optima for swimming performance (Taylor et al., 1996; Booth et al., 1997; Farrell, 2002), all fish used in this thesis were acclimated to an identical intermediate temperature (13°C) which corresponds to neither species' thermal optimum. Therefore, it could still be argued that the Atlantic salmon studied here are more efficient swimmers (lower COT at equivalent speeds; Fig. 4.2) and therefore better athletes (higher U_{crit}; Fig. 4.1) than domesticated rainbow trout. This, coupled with the knowledge that Atlantic salmon tend to reflect a more anadromous phenotype than rainbow trout (Spares et al., 2015), suggests that the results presented here in Atlantic salmon (chapter 3) are more representative of the physiology of true long distance migrant fish. Indeed, long distance migrant fish should be particularly well-adapted to store, mobilize and oxidize lipids efficiently to ensure successful migration (Weber and Haman, 1996; Weber, 2009). The ability to more tightly regulate lipolysis to reduce the energetic burden of TAG:FA cycling while still mobilizing enough fatty acids to support energy metabolism during locomotion may represent an important adaptation for optimizing energy budgets when migrating over long distances.

In summary, this work has demonstrated that lipid mobilization is regulated differently in fish than in mammals in response to exercise. Salmonids mobilize lipids in constant excess of the requirements for energy metabolism, possibly to allow for rapid reorganization of membrane phospholipids as a component of the homeoviscous response to changing environmental conditions. However, more anadromous and migratory phenotypes may rely on a tighter control of lipolysis to minimize the costs of substrate cycling and conserve energy on limited fuel budgets.

APPENDIX

Appendix 1

Ion-Exchange Resin Preparation

- NOTE: Resins are not compatible with oxidizing agents. Do not use with nitric acid or other strong acids other than those listed below. Resins emit odorous and potentially toxic fumes. Working in a well-ventilated area or in a fume hood is recommended. Consult the corresponding MSDS for more details. Never use a stir bar

Components and Equipment

- DOWEX 50Wx8 (50-100 mesh)
- DOWEX 1x8 (100-200 mesh)

} Sigma-Aldrich, St Louis, MO, USA

- 250-500 ml beaker or Erlenmeyer flask
- Large beaker for collecting decanted water
- Litmus paper as a pH indicator

Solutions Needed

- Deionized water
- 1-2M HCl
- 1-2M NaOH
- 1M Formic Acid

Resin Wash

- Transfer 50g of each resin into separate flasks
- Add sufficient deionized water to cover the resin bed by several inches
- Stir or swirl the resin gently for approximately one minute
- Let stand for a minimum of 15-20 minutes or until all fine particles appear to have settled
- Carefully decant the water while trying to retain as much resin as possible
- Replace with fresh deionized water and repeat the above process 3 times, waiting 5-10 minutes each time before decanting

Preparing Hydrogen (H^+) form resin

- Into the flask containing the DOWEX 50Wx8 resin, carefully add enough 1-2M HCl to cover the resin bed by 1-2 inches
- Stir or swirl gently for approximately one minute to ensure adequate mixing
- Let stand 5-10 minutes to allow particles to settle
- Decant as much acid as possible while being careful to retain the resin
- Repeat the process above twice more with fresh acid

- Rinse at least 10 times with deionized water or until pH > 5 is reached. Check the pH by collecting a small drop of decanted water and applying it to a piece of litmus paper.

Preparing Formate form resin

- Into the flask containing the DOWEX 1x8 resin, carefully add enough 1-2M NaOH to cover the resin bed by 1-2 inches
- Using NaOH instead of HCl, follow the same procedure as for the Hydrogen form resin. Finish by rinsing with deionized water until pH < 9 is reached
- Once an appropriate pH is reached, add enough 1M Formic Acid to cover the resin bed by 1-2 inches. Repeat above process and rinse until pH > 5 is reached

Storing resins

- All resins should be kept in a tightly sealed container. They can be refrigerated for storage but should never be frozen
- As a bacteriostat, rinsing with 20% v/v ethanol (in deionized water) can be done prior to storage if desired

*NOTE: If methanol or other miscible solvents are to be used in the elution of components from the resin, the resin should be washed in that solvent as an additional processing run prior to use

Using resins for removal of ^{3}H-Glucose from plasma extracts

- Incubate the aliquot of the sample to be used in scintillation counting to measure glycerol activity with ATP and Hexokinase in an appropriate buffer solution to phosphorylate any glucose present in the sample
- In the desired elution column, insert an appropriately-sized porous polyethylene frit (prevents resin from escaping the column)
- Add Formate form resin, followed by another frit (ensuring it is inserted flush with the surface of the resin) and finally the Hydrogen form resin
- Wash the elution column by filling it completely with deionized water
- When the water is done dripping, place a vial below the column, add the entire hexokinase/sample mixture to the column and elute with deionized water (volume of water will depend on volume of the sample used. For < 100 µl sample in approximately 500 µl hexokinase/buffer solution, 4-5 ml deionized water is sufficient).
- Dry the resulting eluate to remove ^{3}H-H_2O in the sample. Resuspend in deionized water prior to quantifying activity.

Appendix 2

Calculations for steady-state metabolite kinetics (based on Steele, 1959)

$$\text{Infusion rate (Bq kg}^{-1}\text{min}^{-1}) = \frac{\text{Infusate activity (Bq ml}^{-1}) \times \text{Infusion speed (ml min}^{-1})}{\text{body mass (kg)}}$$

$$\text{Specific activity (Bq µmol}^{-1}) = \frac{\text{Background corrected activity (Bq ml}^{-1})}{\text{Concentration (µmol ml}^{-1})}$$

$$\text{Rate of appearance}^{*} \text{ (µmol kg}^{-1}\text{min}^{-1}) = \frac{\text{Infusion rate (Bq kg}^{-1}\text{min}^{-1})}{\text{Specific activity (Bq µmol}^{-1})}$$

* Under steady-state conditions, the rates of appearance and disposal (R_a and R_d) are the same.

Calculating the total oxygen cost of transport (total cost of transport, COT)

$$\text{Total cost of transport (µmol O}_2\text{ kg}^{-1}\text{m}^{-1}) = \frac{\text{Metabolic rate (µmol O}_2\text{ kg}^{-1}\text{min}^{-1})}{\text{Swimming speed (m min}^{-1})}$$

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