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REVIEW

Metabolic fuel kinetics in fish: swimming, hypoxia and muscle membranes

Jean-Michel Weber*, Kevin Choi, Alex Gonzalez and Teye Omlin

ABSTRACT

Muscle performance depends on the supply of metabolic fuels and disposal of end-products. Using circulating metabolite concentrations to infer changes in fluxes is highly unreliable because the relationship between these parameters varies greatly with physiological state. Quantifying fuel kinetics directly is therefore crucial to the understanding of muscle metabolism. This review focuses on how carbohydrates, lipids and amino acids are provided to fish muscles during hypoxia and swimming. Both stresses force white muscle to produce lactate at higher rates than it can be processed by aerobic tissues. However, lactate accumulation is minimized because disposal is also strongly stimulated. Exogenous supply shows that trout have a much higher capacity to metabolize lactate than observed during hypoxia or intense swimming. The low density of monocarboxylate transporters and their lack of upregulation with exercise explain the phenomenon of white muscle lactate retention. This tissue operates as a quasi-closed system, where glycogen stores act as an 'energy spring' that alternates between explosive power release during swimming and slow recoil from lactate *in situ* during recovery. To cope with exogenous glucose, trout can completely suppress hepatic production and boost glucose disposal. Without these responses, glycemia would increase four times faster and reach dangerous levels. The capacity of salmonids for glucoregulation is therefore much better than presently described in the literature. Instead of albumin-bound fatty acids, fish use lipoproteins to shuttle energy from adipose tissue to working muscles during prolonged exercise. Proteins may play an important role in fueling muscle work in fish, but their exact contribution is yet to be established. The membrane pacemaker theory of metabolism accurately predicts general properties of muscle membranes such as unsaturation, but it does not explain allometric patterns of specific fatty acids. Investigations of metabolic fuel kinetics carried out in fish to date have demonstrated that these ectotherms use several unique strategies to orchestrate energy supply to working muscles and to survive hypoxia.

KEY WORDS: Muscle metabolism, Fish exercise, Metabolite fluxes, Lactate retention, Monocarboxylate transporters, Glucose, Glucoregulation, Lipoproteins, Membrane pacemaker, Continuous tracer infusion

Introduction

Muscle performance depends critically on the adequate supply of metabolic fuels and disposal of end-products. Therefore, knowing how metabolite fluxes are regulated is necessary to understand muscle energetics. The ATP used for contraction can be generated through various pathways of energy metabolism that catabolize carbohydrates, lipids or proteins, and the mechanisms that coordinate fuel selection have been reviewed elsewhere (Weber, 2011). In mammals, muscle metabolism has been particularly well studied, and a detailed account of its complex interactions with various organ systems is beginning to emerge (Hawley et al., 2014; Jensen and Richter, 2012; Jeppesen and Kiens, 2012). Comparatively little is known for ectotherms, but the design of reliable methods to measure substrate fluxes in fish (Haman et al., 1997a; Haman and Weber, 1996) has allowed researchers to start investigating how fish muscles respond to common stresses such as environmental hypoxia and exercise. In the last 15 years, it has also become clear that membranes play a fundamental role in the regulation of muscle metabolism. Transmembrane proteins control metabolite movements in and out of the sarcoplasm, and key processes accounting for most of the energy used by myocytes depend on membrane proteins, primarily electron transport chain enzymes and ion pumps. By associating these essential membrane functions with the fact that the mass-specific metabolic rate and the fatty acid composition of membrane phospholipids vary allometrically (Couture and Hulbert, 1995; Hulbert et al., 2002; Schmidt-Nielsen, 1990), Hulbert and Else (1999) have formulated the membrane pacemaker theory of metabolism. It states that the relative abundance of polyunsaturated fatty acids in membrane phospholipids sets the metabolic rate by influencing the activity of membrane proteins. Multi-species comparisons within mammals or birds provide support for this theory (Hulbert, 2003; Hulbert and Else, 2000, 2005), but it is unclear whether membranes could play such a role in fish.

This review focuses on the energy supply and the efflux of waste products in fish muscle by examining what is currently known about carbohydrate, lipid and amino acid fluxes in this group of vertebrates. Circulating concentrations of metabolic fuels are easy to monitor, but measuring fluxes is technically more difficult. Therefore, we have started to characterize the relationship between concentrations and fluxes of blood metabolites to determine whether changes in concentration could provide useful information about fluxes. The physiological roles played by different membrane components in the regulation of substrate metabolism are also examined, with particular emphasis on transmembrane transport proteins and the fatty acid composition of the phospholipid bilayer.

Lactate fluxes and monocarboxylate transporters

Lactate plays many essential roles as oxidative fuel, glycolytic end-product, gluconeogenic precursor and intracellular signal (Brooks, 2009; Gladden, 2004; Philp et al., 2005). Comparative physiologists have been particularly interested in this metabolite because it behaves differently in ectotherms than in mammals (Gleeson, 1996). After exhausting exercise, for example, lactate efflux from fish white muscle is approximately 10 times slower than from mammalian muscle (Wang et al., 1997). This capacity for lactate retention has puzzled biologists for a long time (Turner and Wood,

Biology Department, University of Ottawa, Ottawa, Ontario, Canada.

*Author for correspondence (jmweber@uOttawa.ca)

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List of symbols and abbreviations

DHA	docosahexaenoate
GLUT	glucose transporter
LPL	lipoprotein lipase
MCT	monocarboxylate transporter
R_a	rate of appearance
R_d	rate of disposal
TAG	triacylglycerol
U_{crit}	critical swimming speed

1983; Wardle, 1978). Earlier studies investigated potential mechanisms explaining lactate retention and suggested that fish white muscle may only rely on simple diffusion for transmembrane lactate transport (Laberee and Milligan, 1999; Sharpe and Milligan, 2003; Wang et al., 1997). Monocarboxylate transporters (MCTs) are normally known to facilitate lactate movements across membranes, and they have been particularly well characterized in mammals (Halestrap and Wilson, 2012). Much less information is available on fish MCTs (Liu et al., 2008; Ngan and Wang, 2009; Umezawa et al., 2012), but the presence of four isoforms from this family of proteins was recently demonstrated in rainbow trout (Omlin and Weber, 2013). This study shows that post-exercise lactate retention can be explained as follows: (1) MCT4, the main lactate exporter isoform of mammalian glycolytic muscles, is extremely poorly expressed in trout; (2) the combined expression of all MCT isoforms is much lower in white muscle than in all other trout tissues; and (3) exhausting exercise fails to upregulate the expression of white muscle MCTs. Therefore, white muscle operates as a virtually closed system with regard to carbohydrate metabolism: local glycogen stores fuel intense exercise and are subsequently replenished *in situ* from lactate. Although glycogen synthesis from lactate has been demonstrated in frog muscle (Fournier and Guderley, 1992), it is unclear how recovery is achieved in trout white muscle. Frogs cannot rely on the Cori cycle to synthesize muscle glycogen from accumulated lactate because their liver is unable to use lactate as a gluconeogenic substrate (Fournier and Guderley, 1992). Similarly, the Cori cycle cannot play a significant role in trout because lactate retention prevents export of the end-product to the liver. Frog and trout muscles do not show significant activities of phosphoenolpyruvate carboxykinase and may therefore rely on the reversal of pyruvate kinase for *in situ* re-synthesis of glycogen from lactate during recovery (Gleeson, 1996).

The first estimates of lactate fluxes in fish were obtained by bolus injection of labeled lactate in skipjack tuna (*Katsuwonus pelamis*) (Weber et al., 1986), coho salmon (*Oncorhynchus kisutch*), starry flounder (*Platichthys stellatus*) (Milligan and McDonald, 1988), channel catfish (*Ictalurus punctatus*) (Cameron and Cech, 1990) and rainbow trout (*Oncorhynchus mykiss*) (Weber, 1991). These studies showed that fish maintain high baseline rates of lactate turnover that are stimulated during low-intensity swimming and recovery from strenuous exercise. More recently, the lactate kinetics of rainbow trout have been characterized in more detail using continuous tracer infusion, a method that allows quantification of the rates of appearance (R_a) in the circulation and disappearance from it (R_d) separately. The effects of environmental hypoxia (Omlin and Weber, 2010), graded exercise (Teulier et al., 2013) and exogenous lactate supply (Omlin et al., 2014) on endogenous R_a and R_d lactate have been measured (Figs 1, 2). After 90 min of hypoxia (at 25% air saturation), lactate accumulates to 9 mmol l^{-1} in the circulation because of a mismatch in fluxes resulting from the more rapid rise in R_a (+98%) than in R_d (+52%) (Fig. 1A). This large increase in lactate disposal is rather unexpected for a hypoxia-sensitive animal lacking oxygen, but it plays a strategic role in reducing the lactate load on the circulation. Without this response, blood lactate accumulation would actually double (Omlin and Weber, 2010). Similarly, graded exercise up to critical swimming speed (U_{crit}) causes the stimulation of R_a (+67%) as well as R_d lactate (+41%) (Fig. 1B). Again, the increase in R_d reduces the lactate load on the circulation by half (Teulier et al., 2013). These responses to environmental and functional hypoxia suggest that lactate accumulates because it cannot be processed as rapidly by oxidative tissues (red muscle, heart, gills and brain) as it is produced by anaerobic glycolysis in white muscle (Fig. 1). Therefore, we have used exogenous lactate to assess: (1) whether R_d lactate has an upper limit that constrains capacity for disposal, and (2) whether the high baseline R_a lactate normally seen in resting, normoxic trout is obligatory or whether it can be suppressed. These experiments demonstrate that the metabolic capacity of oxidative tissues for lactate disposal is not responsible for limiting R_d lactate to the highest levels normally seen in hypoxia or during intense swimming because this ceiling can be lifted by 40% when exogenous lactate is provided (Fig. 2). This extra increase in disposal is made possible by elevated circulating lactate levels that accelerate MCT-mediated transport into oxidative tissues such as red muscle and heart (Omlin et al., 2014). MCT protein levels and activity may also be upregulated by exercise or exogenous lactate, but such mechanisms have never been investigated in fish.

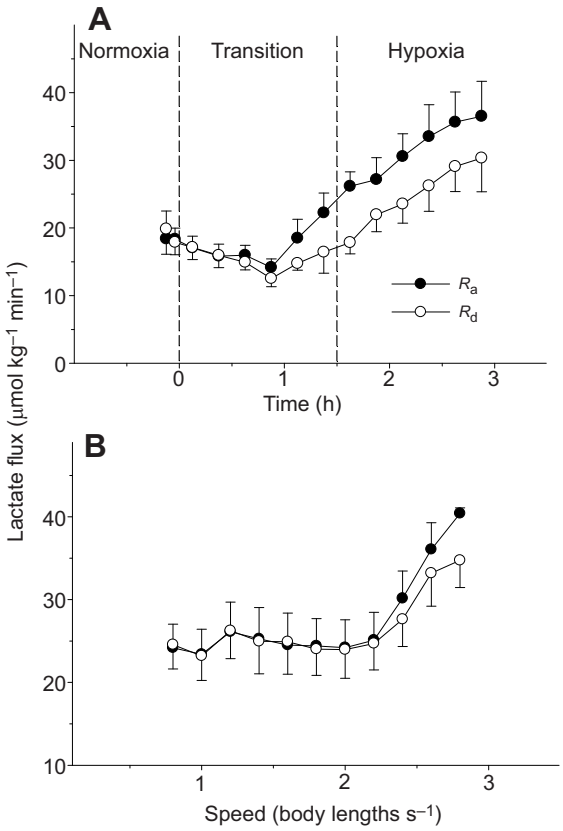

Fig. 1. Effects of physiological stresses on the lactate kinetics of rainbow trout. The rates of lactate appearance (R_a) and disposal (R_d) are both stimulated by (A) hypoxia and (B) graded swimming, but prolonged hypoxia and intense exercise cause their divergence (and lactate accumulation in the circulation) because R_a increases faster than R_d. Error bars indicate ±s.e.m. Data are from Omlin and Weber (2010) and Teulier et al. (2013).

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Unfortunately, whole-animal metabolite kinetics only provide information about R_a and R_d (the total rates of entry and exit into and out of the entire circulatory system), but cannot quantify the contributions of individual tissues or organs separately. For example, more *in vitro* experiments on isolated cells or organs will be needed to determine what exact roles red muscle, heart and brain might play in metabolizing the lactate produced by white muscle during hypoxia and intense exercise. When exogenous lactate is supplied to resting fish, endogenous R_a is not suppressed. Trout have an obligatory need to produce lactate at a minimal rate of approximately 10 µmol kg^{-1} min^{-1} (Fig. 2), but the physiological reasons for this continuous production are unclear. Aerobic tissues such as the brain and heart may require constant supply of this preferred oxidative fuel. The numerous lactate shuttles characterized in mammals illustrate the physiological importance of such a mechanism (Brooks, 2002; Gladden, 2004). Another possible explanation might be continuous lactic acid production by the gas gland to release O_2 from hemoglobin into the swim bladder by Root effect and to control buoyancy (Umezawa et al., 2012). Finally, when exogenous lactate is supplied during graded swimming, exercise performance is neither improved nor decreased because U_{crit} remains unchanged (Omlin et al., 2014).

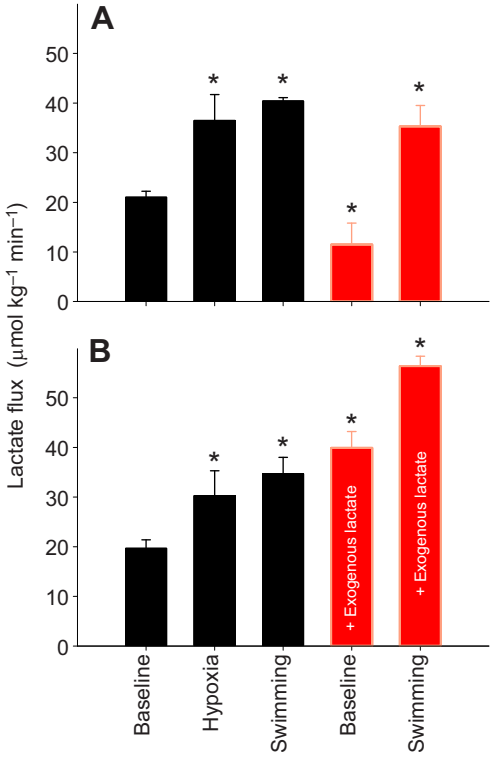

Fig. 2. Comparison of the effects of various physiological stresses on the lactate kinetics of rainbow trout. Red bars indicate experiments where exogenous lactate was supplied at twice the normal baseline rate of endogenous production. (A) The rate of lactate appearance (R_a) is stimulated by hypoxia and intense swimming with or without exogenous lactate administration. R_a is inhibited by exogenous lactate at rest, but it is not completely suppressed. (B) The rate of lactate disposal (R_d) is stimulated to various degrees by all the stresses: exogenous lactate during intense swimming>exogenous lactate at rest>intense swimming alone>hypoxia. Error bars indicate ±s.e.m. Asterisks indicate significant differences from baseline without exogenous lactate (P<0.05). Data are from Omlin and Weber (2010) for hypoxia, Teulier et al. (2013) for swimming, and Omlin et al. (2014) for exogenous lactate at rest and during exercise.

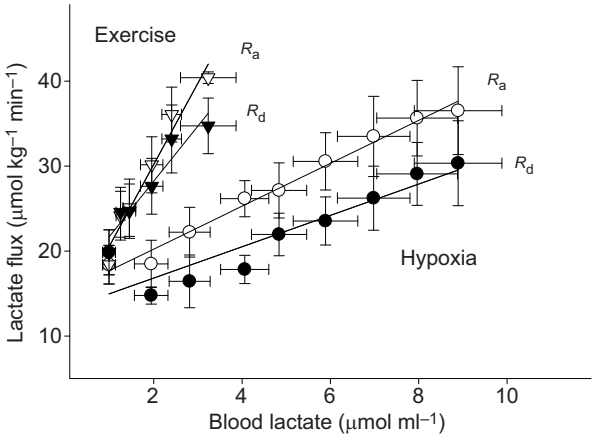

Fig. 3. Relationships between blood lactate concentration and lactate fluxes during hypoxia and during exercise in rainbow trout. These relationships vary between lactate appearance (R_a) and disposal (R_d), and they are very different for hypoxia and for swimming. Slopes are higher for exercise than hypoxia, mainly because of differences in cardiac output. Lines were fitted by linear regression: for hypoxia, R_a lactate=2.52[lactate]+10.18 and R_d lactate=1.84[lactate]+13.14; for exercise, R_a lactate=9.56[lactate] +11.07 and R_d lactate=6.49[lactate]+15.25. Error bars indicate ±s.e.m. Data are from Omlin and Weber (2010) and Teulier et al. (2013).

Is it possible to determine how lactate fluxes vary by simple monitoring of changes in blood lactate concentration? Even though changes in concentration are routinely used to draw conclusions about fluxes, this practice can be greatly misleading (Haman et al., 1997b). To investigate what factors could affect the relationship between concentration and flux, we have plotted how R_a and R_d lactate change with concentration during hypoxia, exercise and exogenous lactate supply (at rest or during swimming). The concentration–flux relationship is different for R_a and R_d, and it varies greatly with the actual physiological stress that causes lactate accumulation (Figs 3, 4). It is linear for hypoxia, exercise and exercise+exogenous lactate, but it is not for exogenous lactate at rest (Fig. 4). Most notably, the slope for graded exercise is steeper than for hypoxia, and this difference can be explained by at least two mechanisms: (1) differences in blood flow, and (2) differences in the metabolic rate of the tissues that metabolize lactate. Both factors influence the blood to tissue lactate gradient. The elevated cardiac output of exercise allows higher lactate fluxes (at the same concentration) to be reached than the lower cardiac output of hypoxia, when the slope of the relationship is strongly reduced (Fig. 3). This effect of perfusion had been suggested previously (Weber et al., 1987) and it is obvious here for the R_d lactate of trout (Fig. 4). At concentrations below 5 mmol l^{-1}, much higher fluxes are reached during exercise than during exogenous lactate infusion (e.g. 5 mmol l^{-1} is reached at higher cardiac output with exercise than with exogenous lactate at rest). It is also interesting to note that R_d reaches a plateau above 8 mmol l^{-1} when exogenous lactate is given to resting fish. Increasing the concentration above this value does not lead to higher fluxes at rest, but it does during swimming as cardiac output keeps increasing towards maximal values. Combining exogenous lactate and graded exercise shows that R_d can be stimulated to impressive levels of 56 µmol kg^{-1} min^{-1}. By cumulating the effects of higher blood flow, higher circulating lactate concentration and higher metabolic rate in lactate-oxidizing tissues, a larger lactate gradient between the blood and tissues such as red muscle and heart can be sustained. Therefore, R_d lactate can be further increased, even in the absence of MCT upregulation. This

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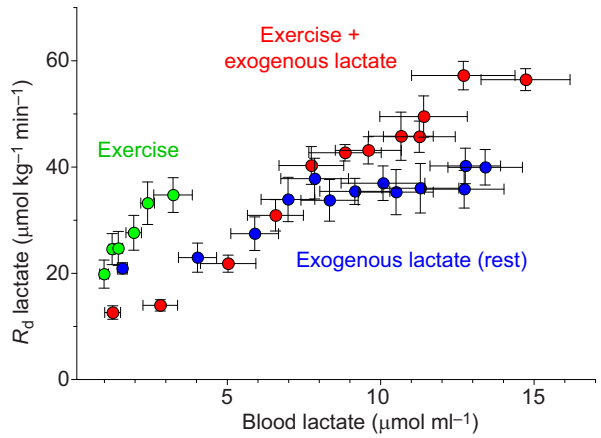

Fig. 4. Comparison of the relationships between blood lactate concentration and lactate fluxes. Data are shown for graded exercise (green), exogenous lactate at rest (blue) and exogenous lactate during graded exercise (red) (Omlin et al., 2014; Teulier et al., 2013). Error bars indicate ±s.e.m.

examination of the relationship between concentration and flux clearly shows that blood lactate concentration cannot be used to obtain reliable information about lactate fluxes.

Glucose kinetics and glucoregulation

Glucose can contribute to energy metabolism in working muscles and is an essential fuel for the nervous system. To ensure the adequate supply of this substrate, mammals and birds need to achieve blood glucose homeostasis to avoid metabolic dysfunction associated with hypoglycemia or the toxic effects of hyperglycemia (Wasserman, 2009). Rainbow trout are classically considered as poor glucoregulators because they normalize glycemia very slowly in glucose tolerance tests and show limited sensitivity to insulin (Polakof et al., 2012). Such differences with endotherms were originally associated with a deficiency in glucose transporters (GLUTs) because early attempts to characterize these proteins in fish were unsuccessful (Wright et al., 1998). However, the existence of at least four isoforms (GLUT1-4) has now been clearly established in a number of teleosts, including trout (Marín-Juez et al., 2014; Polakof et al., 2011, 2010). Fish GLUT4 has received particular attention because it is mainly expressed in skeletal muscle and this large tissue mass may play a significant role in glucose disposal (Capilla et al., 2002; Díaz et al., 2007; Hall et al., 2006; Planas et al., 2000). As in mammals, the density of GLUT4 in the sarcolemma can be regulated through the insulin-dependent translocation of the transporter between intracellular vesicles and the cell membrane. After a glucose challenge, the very different response times between fish and mammals may be directly related to differences in the translocation mechanisms for GLUT4 (Marín-Juez et al., 2014). The lower affinity of fish GLUT4 for glucose could also be responsible for this difference (Capilla et al., 2002).

The true capacity of fish for glucoregulation could be better assessed by quantifying the plasticity of glucose kinetics rather than by relying on concentration changes during glucose tolerance tests. The first glucose fluxes reported for fish were measured by bolus injection of tracer in kelp bass (*Paralabrax clathratus*) (Bever et al., 1977, 1981), skipjack tuna (*Katsuwonus pelamis*) (Weber et al., 1986), seabass (*Dicentrarchus labrax*) (Garin et al., 1987), wolf fish (*Hoplias malabaricus*) (Machado et al., 1989) and brown trout (*Salmo trutta*) (Blasco et al., 1996). These early studies showed that glucose turnover rate can be strongly reduced by prolonged fasting and that resting glucose fluxes vary with the basal metabolic rate of individual species. A double catheterization method was developed to measure *in vivo* metabolite fluxes by continuous tracer infusion and to monitor R_a and R_d under non-steady-state conditions (Haman and Weber, 1996). This tracer technique was specifically tested for glucose kinetics by surgically removing the natural source of endogenous glucose (via hepatectomy), and replacing it with a pump infusing unlabelled glucose to mimic various rates of hepatic production. The accuracy of this method, based on continuous infusion of [6-^{3}H]glucose to quantify *in vivo* glucose fluxes in fish, was therefore validated (Haman et al., 1997a). The effects of environmental hypoxia, acute changes in water temperature, prolonged low-intensity swimming, epinephrine and propranolol were quantified (Fig. 5). Together, these studies show that rainbow trout have some capacity to modulate glucose fluxes. The onset of hypoxia causes a small transient increase in R_a, but low oxygen has no lasting effect (Haman et al., 1997b). A rapid drop in water temperature from 15 to 6°C slows glucose turnover rate by 47%, but this response is a direct consequence of the concomitant decrease in metabolic rate (Haman et al., 1997b). Unexpectedly, prolonged swimming at 1.5 body lengths s^{-1} causes a decline in R_a and R_d glucose, suggesting that circulating glucose is not an important oxidative fuel for trout muscle (Shanghavi and Weber, 1999). This response is particularly surprising because all mammals subjected to an exercise bout of equivalent duration and intensity show a twofold to fourfold increase in glucose fluxes. Unfortunately, the regulation of glucose kinetics by insulin and glucagon has not been characterized in trout. However, catecholamines play a significant role in modulating *in vivo* hepatic glucose production that can be doubled by epinephrine and inhibited by propranolol (Weber and Shanghavi, 2000).

More recently, exogenous glucose infusion (at twice the normal rate of hepatic production) in chronically hyperglycemic animals was used to evaluate the capacity of rainbow trout to cope with a strong glucose challenge (Choi and Weber, 2015). The goal of these

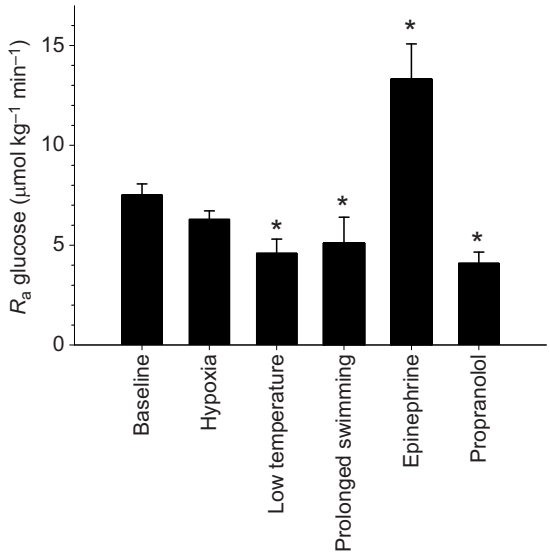

Fig. 5. Effects of various treatments on the rate of hepatic glucose production (R_a) in rainbow trout. Low temperature, prolonged low-intensity swimming and propranolol inhibit R_a glucose, whereas epinephrine stimulates it. Error bars indicate ±s.e.m. Asterisks indicate significant differences from baseline (P<0.05). Data are from Haman et al. (1997b) for hypoxia and low temperature, Shanghavi and Weber (1999) for exercise, and Weber and Shanghavi (2000) for epinephrine and propranolol.

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experiments was to determine whether hyperglycemic fish with intrinsically high baseline glucose fluxes would be able to decrease R_a and/or stimulate R_d in response to exogenous glucose. This experimental model was used to assess glucoregulatory ability under conditions that could push the plasticity of trout glucose kinetics to its physiological limits. In Fig. 6, we have plotted the relationship between blood glucose concentration and glucose fluxes, and it is fairly linear for R_a as well as R_d. Fig. 6A shows that the fish have the same R_a (~10 μmol kg^{-1} min^{-1}) at plasma concentrations of 11 μmol ml^{-1} (baseline) as well as 27 μmol ml^{-1} (when receiving exogenous glucose). As demonstrated for lactate, it should be kept in mind that the concentration–flux relationship for glucose will also most likely vary between resting and swimming fish because of differences in cardiac output. Therefore, glucose concentration cannot be used to draw conclusions about glucose fluxes. Here, the exogenous glucose experiments show that resting fish are not only able to suppress hepatic production completely (Fig. 6A), but to stimulate glucose disposal by 160% (Fig. 6B). Even though glycemia increases 2.5-fold during exogenous infusion, the capacity of rainbow trout to cope with such a massive challenge is remarkable. Without the drastic suppression of R_a and the stimulation of R_d, glycemia would have increased 10-fold to reach 107 mmol l^{-1} over the 4 h experiments (Choi and Weber, 2015). Overall, these measurements of glucose fluxes clearly show that trout have a much better capacity for glucoregulation than generally implied by the literature.

Lipid kinetics: lipoproteins as a fuel for muscle

Lipids are the most crucial substrate for sustained muscle work because they pack more energy per gram than any other fuel (Weber, 2011). Therefore, fish muscle is particularly dependent on fat stores to support endurance swimming. Lipid oxidation also plays an important role after swimming to exhaustion, when it provides most of the ATP for white muscle recovery (Richards et al., 2002). Early work on the lipid kinetics of rainbow trout shows that the mobilization of fat reserves [or lipolysis measured as the rate of appearance of glycerol (R_a glycerol)] and fatty acid fluxes are not stimulated by submaximal exercise like they are in mammals, even when swimming lasts for several days (Fig. 7). Because free fatty acid fluxes fail to respond to prolonged exercise, the possibility that fish might use lipoproteins to shuttle energy between adipose reserves and working muscles was considered. Circulating lipoprotein levels of salmonids are approximately four times higher than in post-absorptive mammals, and they account for over 90% of total circulating lipids. The first indication that fish could use lipoproteins to support muscle work came from sockeye salmon (*Oncorhynchus nerka*). This is because salmon lipoproteins vary dramatically over the course of migration, and in a manner consistent with their use as an oxidative fuel by muscle (Magnoni et al., 2006). However, fish migration includes many challenges other than exercise – such as changes in environmental salinity and egg production – that could also have a large impact on lipoproteins.

Measuring lipoprotein fluxes presents a challenge because there are many classes of lipoproteins that all include different components [triacylglycerol (TAG), phospholipids, apo-proteins and cholesterol]. One of these components must be strategically selected for labelling, and TAG was chosen for this purpose because it is an abundant core element that is least chemically variable between lipoprotein classes. Tracer techniques to quantify the turnover rate of fish TAG have shown that rainbow trout sustain very high baseline lipoprotein fluxes of ~40 μmol TAG kg^{-1} min^{-1} (Magnoni et al., 2008). Even though these fluxes are not further

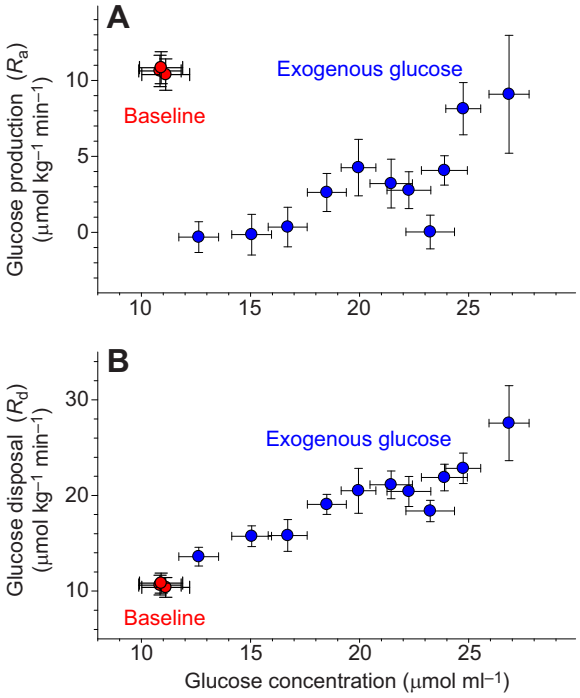

Fig. 6. Relationship between blood glucose concentration and glucose fluxes in resting rainbow trout receiving exogenous glucose at twice their normal rate of hepatic production. To cope with this strong glucose challenge, the fish can (A) completely suppress endogenous hepatic glucose production (R_a), and (B) stimulate glucose disposal (R_d) by 160%. Error bars indicate ±s.e.m. Data are from Choi and Weber (2015).

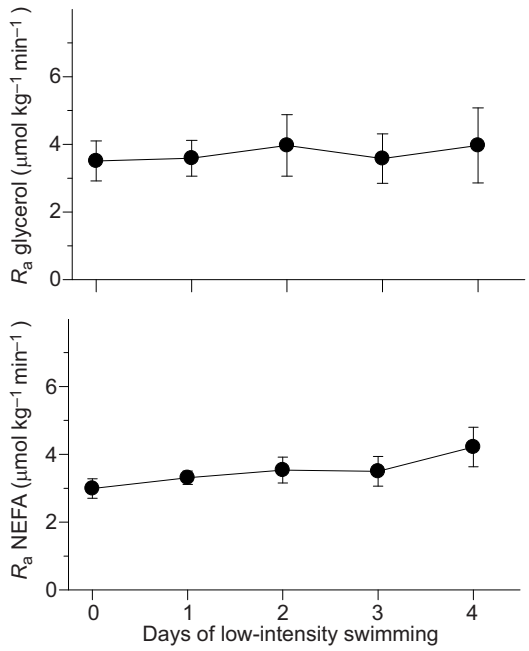

Fig. 7. Effects of prolonged low-intensity swimming on lipolysis measured as the rate of appearance of glycerol (R_a glycerol) and on the rate of appearance of non-esterified fatty acids (R_a NEFA) in rainbow trout. The fish were exercised at 1 body length per second for 4 days and they maintained baseline fluxes for both glycerol and NEFA. Error bars indicate ±s.e.m. Data are from Bernard et al. (1999).

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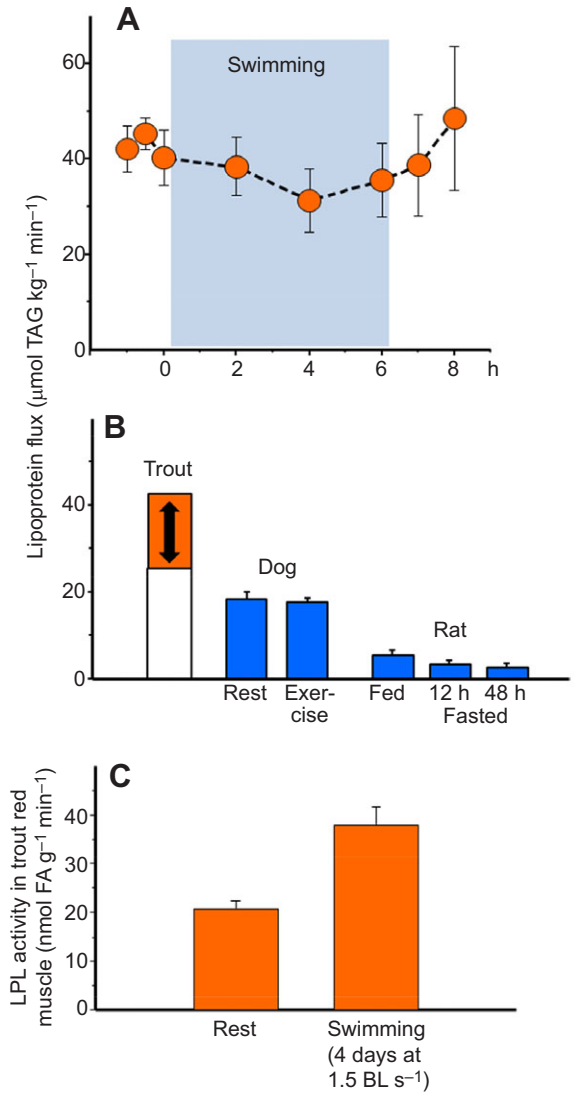

Fig. 8. Use of lipoproteins as an energy shuttle between adipose lipid reserves and working muscles in rainbow trout. (A) Trout lipoprotein fluxes [measured as triacylglycerol (TAG) fluxes] are permanently elevated, but do not respond to prolonged low-intensity swimming. (B) The range of values measured for trout fluxes is indicated by a black arrow and compared with mammalian fluxes measured in dogs (at rest and after endurance exercise) and rats (fed or after fasting for 12 or 48 h). Baseline trout lipoprotein fluxes provide enough energy to support endurance swimming several times. (C) The activity of lipoprotein lipase (LPL) in trout red muscle is stimulated by endurance swimming at 1.5 body lengths per second. Error bars indicate ±s.e.m. Data are from Bagby et al. (1987), Magnoni et al. (2008), Magnoni and Weber (2007), Terjung et al. (1982) and Teusink et al. (2003).

stimulated by endurance exercise (Fig. 8A), they are sufficient to provide several times the energy necessary for sustained swimming and exceed rates measured in mammals (Fig. 8B). More convincing evidence comes from the fact that endurance swimming causes the strong activation of lipoprotein lipase in trout red muscle (Magnoni and Weber, 2007) (Fig. 8C). Together, these studies show that salmonids recruit lipoproteins to transport energy from lipid reserves to working muscles during prolonged exercise. Their strategy differs drastically from that of mammals that use albumin-bound fatty acids for this same purpose.

It is unclear why trout sustain such high TAG fluxes in the resting state, but lipoproteins may also play an important role in membrane restructuring when environmental temperature fluctuates. In fish (and possibly ectotherms in general), they could be used to supply fatty acids of different chain length and degree of unsaturation to membranes undergoing homeoviscous adjustments. Because fish lipoproteins play important physiological roles in muscle energetics, reproduction and possibly membrane plasticity, the slow accumulation of lipid-lowering drugs in aquatic environments has become a concern. For example, gemfibrozil is routinely prescribed to treat humans at risk for heart disease and it is found in ng l^{-1} to μg l^{-1} concentrations in surface waters around the world. Unfortunately, this common fibrate drug can greatly disrupt lipoprotein metabolism in fish (Prindiville et al., 2011).

Producing ATP from proteins

Little is known about the use of proteins as a fuel for muscle work in fish. This scarcity of information stems from the fact that proteins play only a trivial role as an energy source in mammalian muscles, but the situation may be quite different for fish. Evidence from sockeye salmon shows that proteins become the dominant fuel towards the end of migration, when all of the other substrates reach depletion (Mommsen et al., 1980). This conclusion is based on changes in enzyme activities, protein content and free amino acid concentrations in salmon tissues. The only direct measurements of protein catabolism during swimming come from studies where rates of nitrogen excretion were monitored in juvenile trout. They show that protein oxidation accounts for 20 to 45% of metabolic rate during exercise (Lauff and Wood, 1996), but less than 20% after aerobic training (Lauff and Wood, 1997). However, adult animals may have a different degree of reliance on proteins than juvenile trout with extremely high growth rates. In addition, amino acid fluxes have never been measured in exercising fish. Resting turnover rates are available, but they do not provide any insights into the potential contribution of protein oxidation to muscle energetics. One study has examined the roles of glutamate, alanine and aspartate as gluconeogenic precursors in resting kelp bass (Bever et al., 1981). More recently, the fluxes of all amino acids have been measured in resting rainbow trout (Robinson et al., 2011). It is unclear whether the high rates of protein catabolism observed in migrating salmon and in juvenile trout are typical of active fish muscles in general or whether they occur only under exceptional circumstances of extreme exercise or rapid growth. More work on the amino acid kinetics of swimming fish is clearly needed to solve this intriguing problem.

Fish muscle and the membrane pacemaker theory of metabolism

Using data from endotherms, Hulbert and Else formulated the membrane pacemaker theory of metabolism by relating the facts that: (1) mass-specific metabolic rate scales with body size, (2) fatty acid composition of membrane phospholipids also varies allometrically, and (3) most cell processes that consume energy depend on membrane function (Hulbert, 2003; Hulbert and Else, 1999, 2000). The theory proposes that membrane composition sets metabolic rate by modulating the molecular activity of integral proteins. It is based on the fact that the functional properties of membrane proteins such as electron transport chain enzymes, ion pumps and other ATPases can be affected by their local lipid environment (Guo et al., 2005; Leaf et al., 2005; Swanson et al., 1989; Turner et al., 2005). In trout, experiments where dietary fatty acids were used to manipulate membrane composition of red muscle have provided mixed results. Some show that mitochondrial enzyme activities are modulated in accordance with this theory (e.g. Guderley et al., 2008), but others

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fail to support it (e.g. Martin et al., 2013). The concept of the membrane pacemaker has also been criticized because the allometric patterns of membrane composition that provided its basis were not corrected for the effects of phylogeny. After appropriate correction for genetic relatedness and body mass, all the correlations initially characterized between metabolic rate and membrane composition of mammals lose significance (Valencak and Ruf, 2007). Even after correction for phylogeny, however, recent work on orchid bees shows that hovering metabolic rate varies with the fatty acid composition of flight muscle membranes as predicted by the theory (Rodriguez et al., 2015).

Because allometric tests of the membrane pacemaker concept had never been performed in ectotherms, 12 species of cypriniform fish ranging from 4 g minnows to 5.5 kg carp were selected for this purpose (Fig. 9A). They were used to seek potential correlations between body mass, muscle membrane composition and calcium-ATPase activity (Gonzalez et al., 2015). Some of the results support the theory and show that membrane unsaturation decreases with body mass (Fig. 9B). Interestingly, however, fish replace 22:6 with 16:0 when body size increases (Fig. 9C), rather than substituting it with 18:1 like birds and mammals (Couture and Hulbert, 1995; Hulbert et al., 2002). To correct the relationships observed in fish for the effects of phylogeny, a tree was created for cypriniforms (Fig. 9A), but no phylogenetic signal was detected for the double bond index of muscle membranes. Therefore, a general allometric pattern of membrane unsaturation seems to hold across taxa, despite differences in genetic heritage, activity level, diet or habitat, and it is consistent with the concept of the membrane pacemaker. By contrast, the relationships between fish size and relative levels of 16:0 and 22:6 become non-significant when corrected for phylogeny (Gonzalez et al., 2015). This last observation corroborates earlier allometric studies of phylogenetic signals detected in the levels of individual fatty acids for endotherm membranes (Ruf et al., 2006; Turner et al., 2006; Valencak and Ruf, 2007). Therefore, except for orchid bee flight muscles (Rodriguez et al., 2015), relationships between the relative abundance of individual membrane fatty acids and body mass are mostly based on kinship rather than size or metabolic rate.

The membrane pacemaker concept predicts that calcium-ATPase activity of fish muscle should vary with body mass and/or membrane composition. For example, allometric relationships for relative levels of 18:2 and 22:6 should be detectable because these two fatty acids are known to modulate this enzyme in mammals (Giroud et al., 2013). In cypriniforms, however, we found that calcium-ATPase activity is not correlated with body mass, membrane unsaturation or the relative abundance of any specific fatty acid. Overall, current evidence only provides limited support for extending the membrane pacemaker theory of metabolism to ectotherms (Gonzalez et al., 2015). It shows that fish membrane composition depends on multiple factors including body mass and phylogeny, but no effect of membrane composition or body mass on calcium-ATPase activity has been demonstrated in skeletal muscle.

Conclusions and future directions

During hypoxia and intense swimming, fish glycolytic white muscle produces lactate at higher rates than it can be processed by aerobic tissues such as red muscle and heart. However, rainbow trout also respond to these stresses by strongly stimulating lactate disposal, and this strategy plays an important role in reducing end-product accumulation in the circulation when coping with environmental or functional hypoxia. Exogenous lactate experiments show that trout can metabolize this oxidative fuel much faster than at the maximal rates measured during hypoxia or burst swimming. Oxidative tissues have a high capacity for lactate disposal that can reach impressive rates of 56 µmol kg^{-1} min^{-1}, but increasing lactate availability does not affect exercise performance. The low expression, tissue transcript distribution and lack of response to swimming by trout MCTs

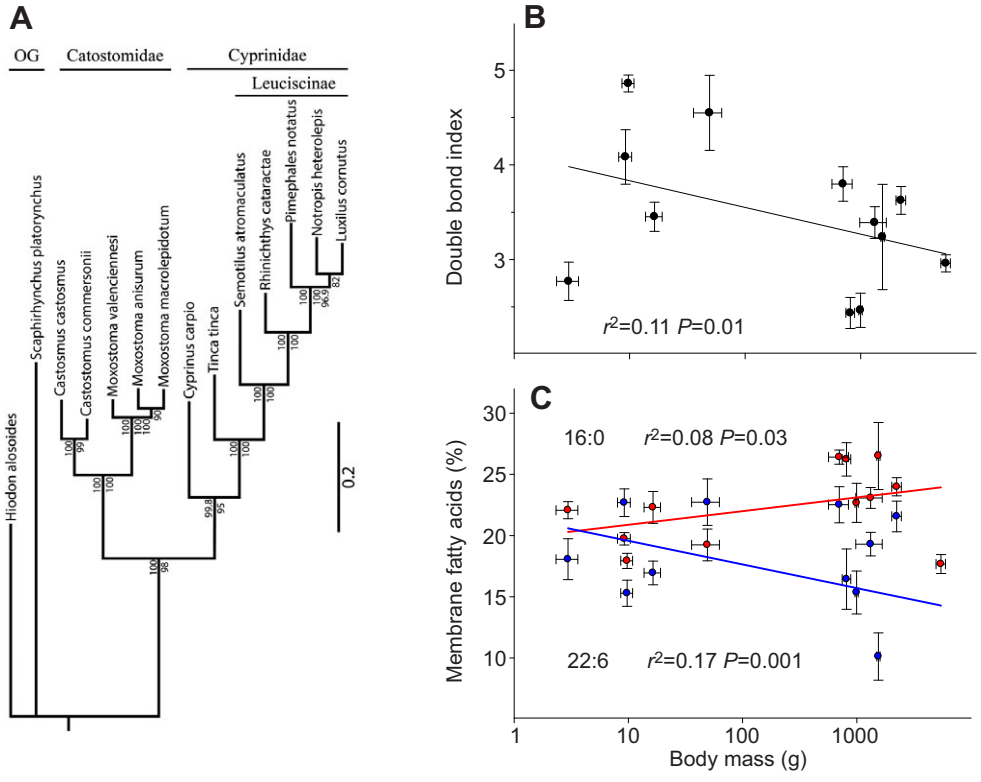

Fig. 9. Test of the membrane pacemaker theory of metabolism in fish muscle. (A) Phylogenetic tree of 12 species of cypriniform fish. (B) The degree of fatty acid unsaturation (measured as double bond index) in muscle membrane phospholipids decreases with body mass, and this relationship remains significant after correction for phylogeny. (C) The relative levels of only two membrane fatty acids change with mass. Percent palmitate (16:0) increases and % docosahexaenoate (DHA or 22:6) decreases with mass. However, these relationships between levels of individual fatty acids and mass are driven by genetic relatedness because they lose significance after correction for phylogeny. Error bars in B and C indicate ±s.e.m. Data are from Gonzalez et al. (2015).

explain the classic phenomenon of post-exercise lactate retention in white muscle. This tissue expresses all MCT isoforms extremely poorly and exhausting exercise has no stimulating effect on their mRNA abundance. Therefore, white muscle operates as a quasi-closed system where glycogen stores act as an 'energy spring' that alternates between explosive power release during intense swimming and slow *in situ* recoil from lactate during prolonged recovery.

The current literature describes rainbow trout as a poor glucoregulator, but large and rapid changes in glucose kinetics elicited by exogenous glucose supply suggest otherwise. Trout can completely suppress hepatic glucose production and boost glucose disposal to cope with a massive glucose challenge. These responses are typical of mammals, but rather unexpected for an ectotherm. Without such changes in flux, trout glycemia would increase four times faster and reach dangerous levels exceeding 100 µmol ml^{-1} within a few hours. The endocrine regulation of glucose fluxes has not been investigated and presents an important area for future work. The exact mechanism for translocation of GLUT4 from intracellular stores to the plasma membrane and its modulation could provide important clues about differences in glucoregulation between fish and mammals. The relationship between the circulating concentration and the flux of metabolic fuels is complex and varies greatly with the physiological state of the animal. Widely different relationships prevail during hypoxia, exercise or the administration of exogenous fuel. This is because fluxes depend on concentration gradients that are sensitive to blood flow, tissue metabolic rate and exogenous supply. Therefore, using metabolite concentration to make inferences about fluxes remains highly unreliable, unless the relationship between the two parameters has been clearly characterized for the specific physiological state under investigation.

Fish use lipoproteins to shuttle energy from adipose tissue reserves to working muscles during prolonged swimming. They show a different tactic than mammals that fuel endurance exercise with fatty acids bound to albumin. The relative importance of proteins as an oxidative fuel for fish muscle has not been established, but current information suggests that they could play a much more important role than in mammals. The membrane pacemaker theory of metabolism accurately predicts general properties of fish membranes such as overall unsaturation, but it does not explain allometric patterns of composition in specific fatty acids. Phylogenetic effects prevent this theory from allowing useful predictions about finer properties of membrane phospholipids. Therefore, evidence to date only provides limited support for an extension of the membrane pacemaker concept to ectotherms. Finally, measurements of metabolite kinetics have shown that fish orchestrate energy supply to their muscles differently than other animals. They have evolved several original strategies to fuel metabolism during exercise and to survive hypoxia.

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Competing interests
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