

**EFFECTS OF HYPOXIA AND EXERCISE ON *IN VIVO*
LACTATE KINETICS AND EXPRESSION OF
MONOCARBOXYLATE TRANSPORTERS IN
RAINBOW TROUT**

TEYE D. OMLIN

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Ottawa-Carleton Institute of Biology, Faculty of Sciences, University of Ottawa

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Title: Effects of Hypoxia and Exercise on *In Vivo* Lactate Kinetics and Expression of Monocarboxylate Transporters in Rainbow Trout

Author: Teye D. Omlin, B.Sc. (Biochemistry)

Thesis advisor: Jean-Michel Weber, Ph.D., Department of Biology, University of Ottawa

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TRANSPORTERS IN RAINBOW TROUT**

SUMMARY

The current understanding of lactate metabolism in fish is based almost entirely on interpretation of concentration measurements that cannot be used to infer changes in flux. Moreover, the transporters regulating these fluxes have never been characterized in rainbow trout. My goals were: (1) to quantify lactate fluxes in rainbow trout under normoxic resting conditions, during acute hypoxia, and exercise by *continuous infusion* of [U-¹⁴C] lactate; (2) to determine lactate uptake capacity of trout tissues by infusing exogenous lactate in fish rest and during graded exercise, and (3) to clone monocarboxylate transporters (MCTs) and determine the effects of exhausting exercise on their expression. Such information could prove important to understand the mechanisms underlying the classic “*lactate retention*” seen in trout white muscle after intense exercise. In normoxic resting fish, the rates of appearance (R_a) and disappearance (R_d) of lactate were always matched (~ 18 to $13 \mu\text{mol kg}^{-1} \text{min}^{-1}$), thereby maintaining a low baseline blood lactate concentration ($\sim 0.8 \text{ mM}$). In hypoxic fish, R_a lactate increased from baseline to $36.5 \mu\text{mol kg}^{-1} \text{min}^{-1}$, and was accompanied by an unexpected 52% increase in R_d reaching $30.3 \mu\text{mol kg}^{-1} \text{min}^{-1}$, accounting for a rise in blood lactate to 8.9 mM . In exercising fish, lactate flux was stimulated > 2.4 body lengths per second (BL s^{-1}). As the fish reached critical swimming speed (U_{crit}), R_a lactate was more stimulated (+67% to $40.4 \mu\text{mol kg}^{-1} \text{min}^{-1}$) than R_d (+41% to $34.7 \mu\text{mol kg}^{-1} \text{min}^{-1}$), causing an increase in blood lactate to 5.1 mM . Fish infused with exogenous lactate stimulated R_d lactate by 300% (14 to $56 \mu\text{mol kg}^{-1} \text{min}^{-1}$) during graded exercise, whereas the R_a in resting fish increased by only 90% (21 to $40 \mu\text{mol kg}^{-1} \text{min}^{-1}$). Four MCT isoforms were partially cloned and characterized in rainbow trout: MCT1b was the most abundant in heart, and red muscle, but poorly expressed in gill and brain where

MCT1a and MCT2 were prevalent. MCT4 was more expressed in the heart. Transcript levels of MCT2 (+260%; brain), MCT1a (+90%; heart) and MCT1b (+50%; heart) were stimulated by exhausting exercise. This study shows that: (i) the increase in R_d lactate plays a strategic role in reducing the lactate load imposed on the circulation. Without this response, blood lactate accumulation would double; (ii) a high capacity for lactate disposal in rainbow trout tissues is elicited by the increased blood-to-tissue lactate gradient when extra lactate is administered; and (iii) rainbow trout may be unable to release large lactate loads rapidly from white muscle after exhausting exercise (*lactate retention*) because they poorly express MCT4 in white muscle and fail to upregulate its expression during exercise.

RÉSUMÉ

Les connaissances du métabolisme du lactate chez les poissons sont presque entièrement basées sur des interprétations de concentration qui ne peuvent pas expliquer les changements dans le flux glycolytique. De plus, le mouvement transmembranaire du lactate est assuré par les transporteurs de monocarboxyle (MCT), cependant ces protéines n'ont jamais été caractérisées chez la truite arc-en-ciel. Les buts de cette thèse étaient de : (1) quantifier les flux de lactate de base sous des conditions normoxiques, durant l'hypoxie, et l'exercice par *infusion continue* de [U-¹⁴C] lactate; (2) déterminer l'effet de la perfusion de lactate sur la cinétique du lactate dans ces poissons au repos et durant un exercice graduel; (3) cloner les MCTs afin de déterminer leur distribution dans les tissus, et quantifier l'effet de la nage intense sur l'expression de ces transporteurs. De telles informations seraient nécessaires à la compréhension des mécanismes sous-jacents au phénomène classique de la '*rétenion du lactate*' le muscle blanc de la truite arc-en-ciel après un exercice intense. Dans les poissons normoxiques, un changement identique du taux d'apparition (R_a) et de disparition (R_d) du lactate (de 18 à 13 $\mu\text{mol kg}^{-1} \text{min}^{-1}$) maintiennent la concentration de base ($\sim 0,8 \text{ mM}$). Dans les poissons en hypoxie, R_a fut stimulé de 18,4 à 36,5 $\mu\text{mol kg}^{-1} \text{min}^{-1}$. Cette augmentation de R_a fut accompagnée d'une stimulation inattendue de 52% de R_d (de 19,9 à 30,3 $\mu\text{mol kg}^{-1} \text{min}^{-1}$), causant ainsi une augmentation du lactate sanguin de 0,8 à 8,9 mM. Chez les poissons en exercice, les flux de lactate fut stimulés aux vitesses de $> 2,4$ longueur de corps par seconde (BLs^{-1}). Le R_a du lactate (+67%; 40,4 $\mu\text{mol kg}^{-1} \text{min}^{-1}$) fut beaucoup plus stimulé que R_d (+41%; 34,7 $\mu\text{mol kg}^{-1} \text{min}^{-1}$) à la vitesse maximal, causant une augmentation du lactate sanguin à 5.1 mM. Chez les poissons perfusés, le R_d était stimulé de 300% (de 14 à 56 μmol

$\text{kg}^{-1} \text{min}^{-1}$) durant l'exercice, par comparaison aux poissons au repos (+90%; de 21 à 40 $\mu\text{mol kg}^{-1} \text{min}^{-1}$). Quatre isoformes de MCT ont été identifiées et partiellement caractérisées chez la truite : MCT1b était plus abondant dans le cœur et le muscle rouge, que dans les branchies et le cerveau, où MCT1a and MCT2 étaient dominants. L'expression des MCTs était stimulée dans le cerveau (MCT2 :+260%) et le cœur (MCT1a; +90% et MCT1b; +50%) durant l'exercice intense. Cette étude démontre : (i) que l'augmentation de R_d joue un rôle stratégique en réduisant charge de lactate imposée sur la circulation. Sans cette réponse, la concentration du lactate sanguin doublerait; (ii) une haute capacité d'utilisation du lactate chez la truite arc-en-ciel dû à l'augmentation du gradient la perfusion; et (iii) que ces poissons sont incapables de relâcher rapidement de leurs muscles blancs parce que les MCT (particulièrement MCT4) y sont peu exprimés, et l'exercice ne stimule pas l'expression des MCTs.

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CHAPTER 1: General introduction

INTRODUCTION

Animal Energetics

Animals require energy to maintain a basal metabolism and to perform various activities essential for survival (e.g. reproduction, foraging, migration, and evading predators). Therefore, the ability to supply working tissues with fuel is imperative. The energy molecule of most cells is adenosine 5'-triphosphate (ATP). When hydrolyzed or transferred to another molecule, the three phosphate groups release a large amount of free energy that can be utilized by the cell. Unfortunately, ATP can only be stored in minimal quantities within the cell, consequently, animals must store their energy reserve in different forms (i.e. lipids, carbohydrates, and proteins) to ensure adequate ATP supply to working tissues.

Lipid is the most abundant fuel reserve in animals (~85% of total energy reserve), the most concentrated (biochemical form of energy), and the lightest (requires no water for storage) (Weber, 2011). It is mainly stored as triacylglycerol in adipose tissue, liver and muscle, and is the energy substrate of choice for sustained ATP production. When ATP is needed very quickly (during intense exercise), **carbohydrates** are used because they can support much higher maximal rates of ATP production than lipids ($30 \mu\text{mol ATP g}^{-1} \text{min}^{-1}$ vs. $20 \mu\text{mol ATP g}^{-1} \text{min}^{-1}$) (Weber, 2011). Furthermore, carbohydrates are the only substrate that can produce ATP in the absence of oxygen. They are mainly stored as glycogen in liver and muscle. Unlike lipids, carbohydrates require water for storage which makes them a heavy energy substrate to carry, consequently, only small amounts are present in animals (~1% of total energy reserve). Under normal circumstances, **proteins** are not used as energy substrate because: (i) they play very important structural (e.g. contractile

proteins) and regulatory roles for the cell (e.g. enzymes and ion pumps); (ii) their oxidation yields toxic ammonia that must be eliminated or detoxified (Weber, 2011). Proteins are only used under extreme circumstances such as extended starvation or during long migrations, often shortly before death, when the other fuel sources are depleted.

Oxygen is essential for oxidation of metabolic fuels. Acetyl groups derived from fatty acids (lipids), carbohydrates, and amino acids (proteins) enter the citric acid cycle, where they are completely oxidized to carbon dioxide and water via the electron transport chain. However, oxygen availability in the environment or in the cell can be strongly reduced (e.g. hypobaric hypoxia or intense exercise). In recent times, aquatic systems are becoming much more susceptible to hypoxia because of increased human impact on the environment (pollution and global warming).

Aquatic hypoxia

Aquatic hypoxia can result from natural events or human activities. In regions subjected to extreme cold temperatures during winter, there is often formation of ice on the surface of bodies of water. This ice layer prevents normal oxygen exchange between the atmosphere and the water. Consequently, the oxygen becomes depleted by organisms trapped below the ice (Heath, 1995). During summers or in tropical regions experiencing hot temperatures, thermal stratification occurs in many water bodies causing mixing constraints. As a result, there is limited oxygen exchange between the layers causing hypoxia in the hypolimnion (bottom layer) (Heath, 1995; Nilsson and Ostlund-Nilsson, 2008). With increased industrialization, anthropogenic factors such as dumping of chemical

wastes and nutrient rich water runoffs from agricultural lands into rivers and lakes promote algal bloom and proliferation of photosynthetic microorganisms. In the daytime these organisms generate oxygen by photosynthesis, however at night they consume most of the oxygen available thus creating a hypoxic environment which deplete the oxygen available (Heath, 1995).

Physiological hypoxia.

High intensity burst-type exercise generates a physiological hypoxia. Although not sustainable for long periods, burst swimming is utilized by highly athletic fish (i.e. salmonids) to cross rapids and waterfalls during migration, to acquire food, and to evade predators. High intensity exercise is mainly powered by white muscle and fueled by glycolysis (Lenz et al., 1998; Milligan, 1996). High velocity swimming imposes oxygen limitations to the fish, and ATP production cannot meet the increased demand at the cellular level, therefore, anaerobic glycolysis becomes the only way to produce ATP rapidly.

Effects of hypoxia on fish

The most noticeable physiological response to hypoxia is hyperventilation. It is reflected by an increased opercula movement to increase irrigation of the gills (ventilation volume) to continue extracting enough oxygen. For instance, hypoxia causes a 1.6-fold increase in the ventilation frequency of rainbow trout and sea bass (from 60 to 100 and from 27 to 45 breaths min^{-1} , respectively) (Thomas and Hughes, 1982a; Thomas and Hughes, 1982b). Concomitant to hyperventilation, other less noticeable physiological changes take

place in concert to increase oxygen delivery to tissues: (i) ***Bradycardia***: It is the reduction of heart rate which is thought to improve oxygen transfer across the gills to the blood stream and delivery to tissues; (ii) ***Increased blood oxygen carrying capacity***: It allows the fish to increase oxygen binding capacity of the blood in the gills for delivery to tissues; (iii) ***Redistribution of the blood flow***: Blood is redirected to highly aerobic tissues for oxygen delivery (Lai et al., 2006; Randall and Shelton, 1963; Richards et al., 2009) .

Along with physiological adjustments, a biochemical change in energy metabolism takes place whereby anaerobic glycolysis becomes the major pathway for ATP production. Although less efficient than oxidative phosphorylation (2 ATP vs. 36 ATP per glucose), this pathway is important because it can rapidly produce ATP with no oxygen when energy demand is high (Weber, 2001). Glucose enters successive catabolic reactions involving several glycolytic enzymes to produce pyruvate as intermediate substrate (see Fig 1.1). Pyruvate is subsequently metabolized via two alternate routes: (i) homolactic fermentation with production of ***lactate*** as end product (majority of vertebrates) or (ii) alcoholic fermentation through production of ethanol via acetaldehyde as intermediate (goldfish, crucian carp) (Moyes and Schulte, 2008; Shoubridge and Hochachka, 1980; Voet et al., 1999).

Lactate

Activation of anaerobic metabolism is evidenced by increased blood and tissue lactate concentrations (Richards et al., 2009). In rainbow trout, exposure to acute hypoxia (1-3 hours) causes an increase in blood lactate concentration from normoxic values of ~1 to

15 mM, and tissue lactate concentration from ~ 1 to $11 \mu\text{mol g}^{-1}$ of wet tissue (Dunn and Hochachka, 1986; Thomas et al., 1992; van Raaij et al., 1996). High intensity swimming (30-45 min) causes an increase in blood lactate up to 20 mM (Milligan and Girard, 1993), and 30-40 mM in white muscle (Turner et al., 1983). Over the past 200 years, lactate was perceived by the general public as a “**waste**” metabolite, however, recent research now shows that it is not the case. In fact, lactate is an indicator of glycolytic flux, a precursor for glucose and glycogen re-synthesis through the Cori cycle, an oxidative fuel, a metabolic signal (pseudo-hormone) to specific tissues, a regulator of cell redox state (NAD^+/NADH), a stimulator of vasodilation during exercise, and even a stimulus for wound repair (Philp et al., 2005). Given the physiological importance of lactate, suitable mechanisms are needed to facilitate its inter-tissue transport.

Lactate transport across cell membranes is facilitated by proton-linked transport involving a family of monocarboxylate transport (MCT) proteins (Garcia et al., 1994; Roth and Brooks, 1990). MCTs have been well documented in mammals; they are non-glycosylated proteins with 12 transmembrane regions flanked by a cytoplasmic N- and C-terminal ends (Halestrap and Meredith, 2004). Of the known 14 isoforms, **MCT1**, **MCT2**, **MCT3**, and **MCT4** have been reported to facilitate lactate movement (Baba et al., 2008; Halestrap and Price, 1999). MCT1, the first isoform, is ubiquitous in animal tissues and facilitates lactate uptake and efflux (K_m of 4-7 mM) (Brooks et al., 1999; Dubouchaud et al., 2000; Garcia et al., 1994; McClelland and Brooks, 2002). MCT2 is specialized for lactate uptake, has a high affinity for lactate ($K_m = 0.7 \text{ mM}$) and is found predominantly in liver, brain and kidney (Bonnen et al., 2006; Halestrap and Meredith, 2004; McClelland et al., 2003). MCT3 is specialized for lactate transport in the basal lateral membrane of retinal

epithelium (Yoon and Philp, 1998). MCT4 is located in sarcolemmal membranes of fast-twitch fibers, it is mainly responsible for lactate efflux from white muscle (Hashimoto et al., 2005).

MCTs facilitate changes in the rates of appearance (R_a) and disappearance (R_d) of lactate, and changes in these fluxes can cause diverse effects on concentration. For example, an increase in lactate concentration could be due to: (i) increase in R_a caused by stimulation of anaerobic glycolysis and unchanged R_d . But it could also occur if R_a remained unchanged and R_d is decreased because of reduction in lactate utilization. The only reliable information about fluxes provided by concentration measurements are that R_a and R_d remain equal when concentration stays constant. However, this is also true when R_a and R_d undergo identical, parallel changes.

Changes in lactate flux have been investigated in mammals during exercise and hypoxia. In exercising rats R_d lactate can increase from a baseline value of 204 to 518 $\mu\text{mol kg}^{-1} \text{min}^{-1}$ (Donovan and Brooks, 1983). In exercising dogs, R_a lactate ranged from a baseline value of 22 to 108 $\mu\text{mol kg}^{-1} \text{min}^{-1}$, whereas R_d lactate ranged from 11 to 77 $\mu\text{mol kg}^{-1} \text{min}^{-1}$ accounting for an increase in concentration from 0.6 mM to 2 mM (Issekutz et al., 1976). In resting humans, R_a and R_d lactate are $\sim 15 \mu\text{mol kg}^{-1} \text{min}^{-1}$, accounting for an arterial lactate concentration of 0.7 mM. R_a lactate is stimulated to 309 $\mu\text{mol kg}^{-1} \text{min}^{-1}$ during graded exercise, and to 169 $\mu\text{mol kg}^{-1} \text{min}^{-1}$ during hypobaric hypoxia (at 4300 m altitude), accounting for an increase in lactate concentration up to 9 mM (Brooks et al., 1991; Stanley et al., 1985).

MOTIVATION OF THE STUDY

Lactate flux determination by continuous infusion: To date, the direct measurement of lactate flux has only been attempted in 3 fish studies investigating endurance swimming (Weber, 1991), recovery from exhaustive exercise (Milligan and McDonald, 1988), and exposure to hypoxia (Dunn and Hochachka, 1987). They reveal that lactate is produced and utilized rapidly, even in normoxic fish at rest ($\sim 1-4 \mu\text{mol min}^{-1} \text{kg}^{-1}$), and that swimming and hypoxia stimulate lactate turnover by approximately 3-10 fold. However, these early estimates of flux were all obtained by ***bolus injection***, an obsolete method with significant limitations (Wolfe, 1992), instead of the more modern and accurate ***continuous tracer infusion method*** widely used for mammals.

Bolus tracer injection vs. continuous tracer infusion: The *Bolus injection* method requires implantation of only a single catheter in the dorsal aorta of the fish that allows to inject the tracer and quickly draw a minimum of 6–10 blood samples to plot a specific activity decay curve. Flux calculation are based on estimates of surface areas under the decay curves (flux = dose injected / surface area under the specific activity decay curve). Unfortunately, estimates under the surface area of the decay curve is difficult to determine precisely because the curve fitting for early (left hand side of the curve) and later sampling points (right hand side of the curve) is extremely inaccurate. Furthermore, each experiment only yields one value of flux (turnover rate; R_t), preventing the measurement of a time course change in the rate of appearance (R_a) and disappearance (R_d) independently. Finally, this method assumes that the animal is in steady-state condition, which is often not the case (Haman and Weber, 1996). In contrast, the *continuous infusion* method has none of the above limitations, although it requires surgical implantation of double catheters in the dorsal

aorta. On catheter line is to infuse the labeled lactate at a constant rate and the other is for blood sampling at specific time point through the experiment (see Fig. 1.2). Determination of lactate concentration and activity of the isotope in each sample allows to calculate the specific activity of lactate. The rate of appearance (R_a lactate) and of disappearance (R_d lactate) are determined using the non-steady-state equation of Steele as follows (Steele, 1959).

$$R_a = [F - (V \times \bar{LA} \times \Delta SA / \Delta t)] / \bar{SA}$$

$$R_d = R_a - (V \times \Delta LA / \Delta t)$$

Where R_a is the rate of appearance and R_d is rate of disappearance ($\mu\text{mol kg}^{-1} \text{min}^{-1}$), F is the infusion rate ($\text{DPM kg}^{-1} \text{min}^{-1}$), V is the distribution volume for lactate (100 ml kg^{-1}), \bar{LA} is the mean of two consecutive lactate concentration, ΔSA is difference between two consecutive lactate specific activities, Δt is times corresponding to LA and ΔSA , and \bar{SA} is the mean of two consecutive lactate specific activities. More information can be obtained from a single experiment because R_a and R_d can be calculated independently. Flux measurements can be determined over time under steady-state and non-steady-state conditions. In fish, the continuous infusion technique has been used successfully to investigate the kinetics of glucose (Haman et al., 1997b; Shanghavi and Weber, 1999; Weber and Shanghavi, 2000), glycerol (Bernard et al., 1999; Magnoni et al., 2008b), fatty acids (Weber et al., 2002), and triacylglycerol (Magnoni et al., 2008a).

Expression of monocarboxylate transporters (MCTs) in rainbow trout: Partial or complete MCT sequences are now known for zebrafish (*Danio rerio*), cod (*Gadus morhua*), fugu (*Takifugu rubripes*), killifish (*Oryzias latipes*), and stickleback (*Gasterosteus*

aculeatus) (Umezawa et al., 2012). However, MCTs have not been characterized in rainbow trout, even though several studies show that this common model species uses a proton-linked symporter to shuttle lactate between tissues. In 1997, Wang and collaborators studied lactate kinetics in postexercised isolated-perfused tail-trunk of rainbow trout. They showed that lactate efflux from white muscle (from the intracellular fluid to the extracellular fluid) occurred through a lactate-proton symporter and the free diffusion of lactate. Alternatively, they demonstrated a concurrent lactate uptake by white muscle through a lactate-chloride bicarbonate exchanger, supporting the “*lactate retention*” capacity of fish white muscle as previously observed (Turner et al., 1983; Wardle, 1978). This interesting phenomenon prolongs rainbow trout blood lactate clearance of over 12h (*vs.* 1h in mammals) (van Raaij et al., 1996; Wang et al., 1997). Characterizing the expression of (putative) MCT isoforms in rainbow trout and their tissue distribution could prove important molecular evidence to explain the physiological basis for *lactate retention*.

GOALS OF THE STUDY

This thesis includes: (1) a series of *in vivo* experiments where continuous tracer infusion is used to determine the effects of hypoxia, exercise, and exogenous lactate administration on the lactate kinetics of rainbow trout, and (2) *In vitro* experiments to clone, characterize (tissue distribution), as well as quantify the effects of hypoxia and exhausting exercise on rainbow trout MCT expression.

In chapter 2, my first objective is to measure the baseline lactate turnover rate of rainbow trout using the most reliable tracer method presently available (continuous infusion

technique). Then I will establish how changes in rates of lactate appearance (R_a) and disposal (R_d) account for the increase in blood lactate concentration elicited by hypoxia. Finally I will identify the main tissues responsible for lactate production.

In chapter 3, my first objective is to use the continuous tracer infusion method to measure the effects of swimming on lactate fluxes. Then, I will determine the relationship between exercise intensity and the rates of lactate production and disposal.

In chapter 4, my first objective is to determine the effects of exogenous lactate on endogenous lactate kinetics in resting and exercising fish. Then, I will determine if the lactate supplement can improve critical swimming speed in rainbow trout.

In chapter 5, I will, first, clone rainbow trout MCTs to identify which isoforms are expressed in this species. Secondly, I will determine their tissue-specific distribution, and finally quantify the effects of acute and chronic hypoxia, as well as exhausting exercise on MCT gene expression in key tissues affecting whole-animal lactate kinetics: white muscle (main lactate producer), red muscle, heart, brain, and gills (lactate oxidation), as well as liver (gluconeogenesis).

Figure 1.1. Diagram of the glycolysis pathway. Substrates, intermediate molecules, and enzymes involved in glycolysis are depicted. Diagram adapted from (Voet et al., 1999).

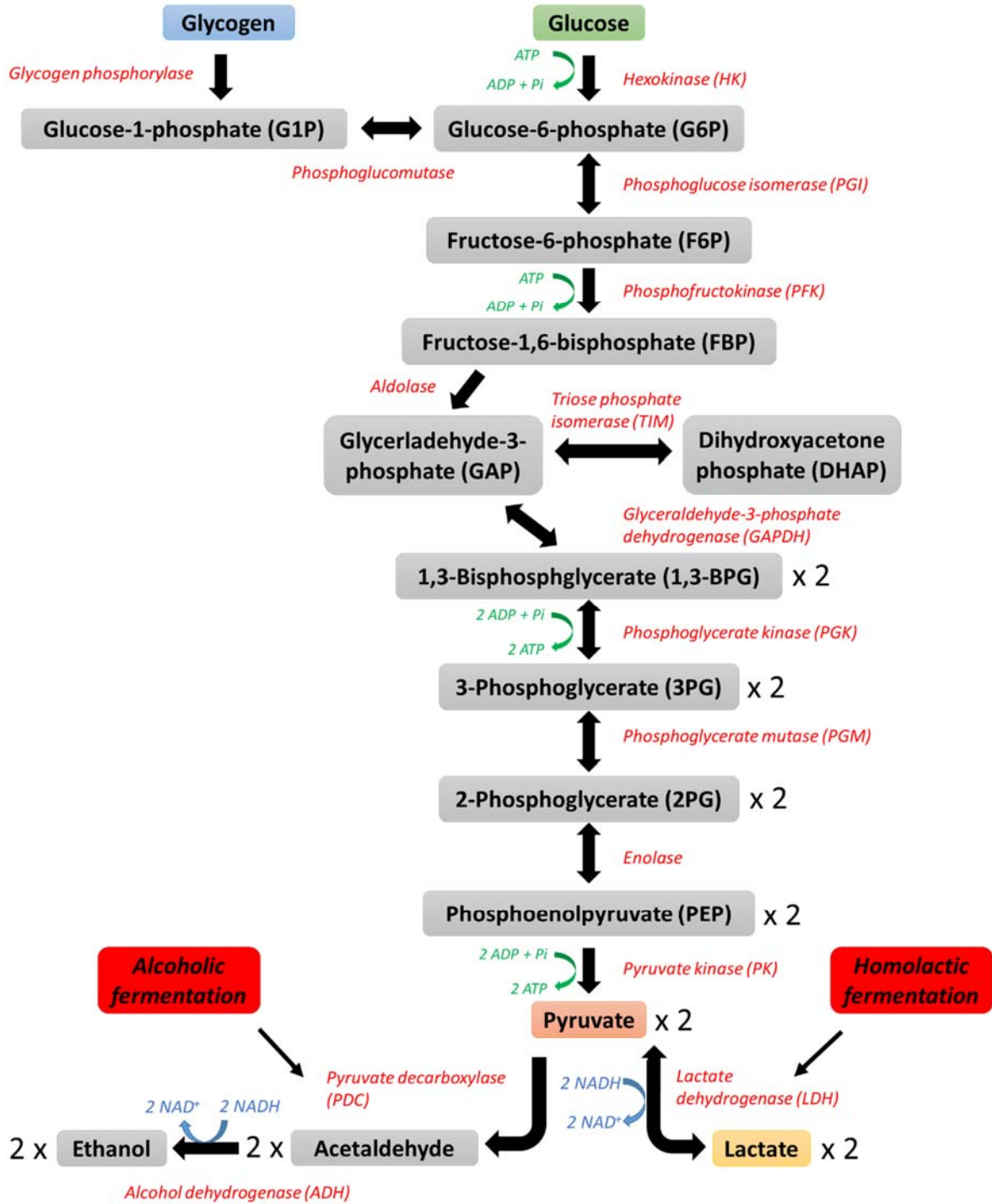
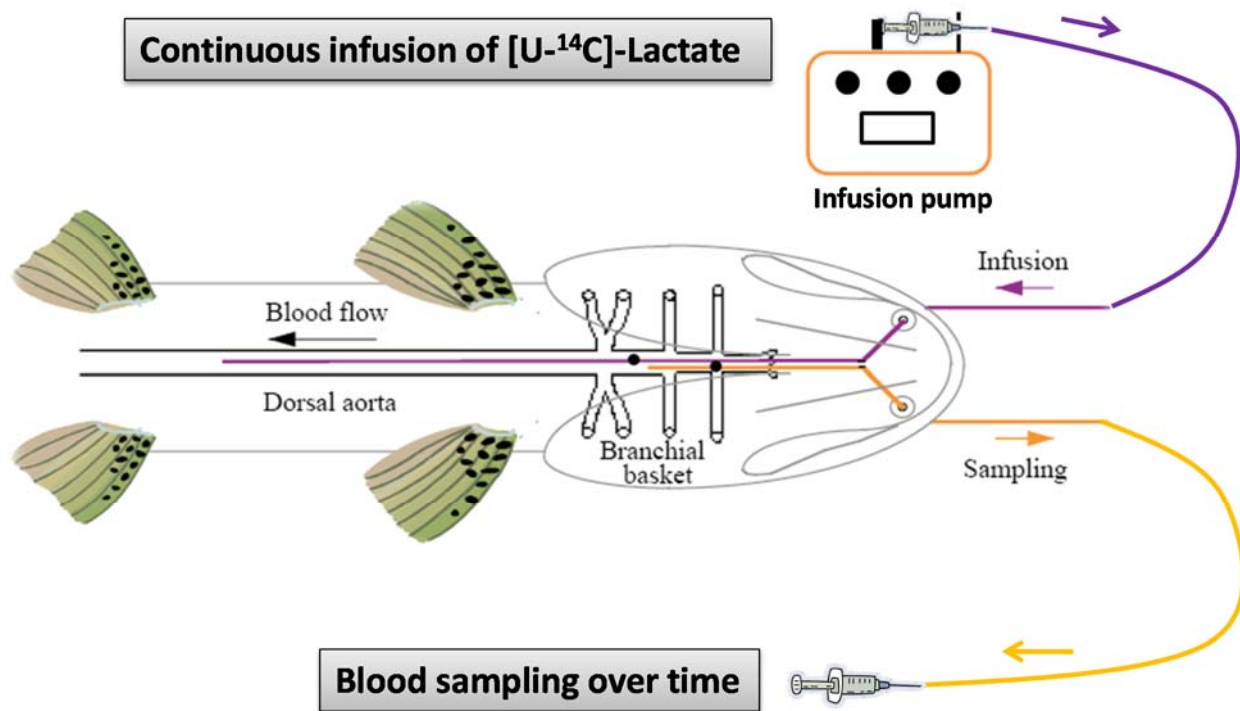


Figure 1.2. Diagram of a doubly cannulated rainbow trout (ventral view). The PE-50 catheters (purple for isotope infusion and orange for blood sampling) are drawn larger than scale to emphasize their exact entry position in the dorsal aorta. Dots (•) represent catheter entry sites in the artery. Figure was modified from (Haman and Weber, 1996)



CHAPTER 2: Hypoxia stimulates lactate disposal in rainbow trout

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Teye Omlin and Jean-Michel Weber

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

INTRODUCTION

Aquatic environments are routinely exposed to hypoxia through natural eutrophication and thermal stratification, but modern human activities causing organic pollution and global warming have amplified this problem (Diaz and Rosenberg, 2008). Reports of mass mortality and fisheries collapse are becoming more common (Wu, 2002), particularly for hypoxia-sensitive organisms like salmonids (Turner et al. 1983). The well characterized response of rainbow trout to hypoxia combines *physiological changes* aimed at trying to maintain normal oxygen supply to tissues (hyperventilation, bradycardia, increased blood O₂-carrying capacity, and redistribution of blood flow) with *metabolic changes* aimed at providing adequate amounts of ATP with less oxygen (stimulation of anaerobic glycolysis) (Richards et al., 2009). Carbohydrates become an essential source of energy because glycolysis can produce ATP in the absence of oxygen. Therefore, hypoxia results in the accumulation of lactate, and the metabolism of this anaerobic end-product has been extensively studied in trout. Exposure to hypoxia for 1-3 hours increases blood lactate concentration from normoxic values of less than 1 mM to 6-15 mM (Dunn and Hochachka, 1986; Thomas et al., 1992; van Raaij et al., 1996). Unfortunately, current understanding of lactate metabolism in fish is based almost entirely on the interpretation of concentration measurements that cannot be used to infer changes in glycolytic flux (Haman et al., 1997b; Stanley et al., 1985). This is because metabolite concentration is determined by dynamic changes in rates of appearance (R_a) and disposal (R_d), and widely different changes in these fluxes can elicit identical effects on concentration. For instance, the same increase in lactate concentration could be due to the stimulation of anaerobic glycolysis (increase in R_a), or to a reduction in the rate of lactate utilization (decrease in R_d). The only reliable clues about

fluxes provided by monitoring concentration are: (1) R_a and R_d always remain equal when concentration stays constant, but this is true when R_a and R_d undergo identical, parallel changes, and (2) R_a and R_d are mismatched when concentration varies.

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

A few studies provide measurements of lactate accumulation in trout tissues in an attempt to characterize the main sources of anaerobic end product during hypoxia (Bernier et al., 1996; Boutilier et al., 1987; Dunn and Hochachka, 1986). Their results support the notion that white muscle is the most important producer of lactate in hypoxic trout, but it is unclear whether this conclusion is true for a 90 min, acute exposure to 25% air saturation at their preferred temperature of 13°C because a variety of conditions have been used in previous studies (exposures of 3-24 h, 13-20% O₂ saturation, and lower temperatures of 4-9°C) (Bernier et al., 1996; Boutilier et al., 1987; Dunn and Hochachka, 1986).

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

MATERIALS AND METHODS

Animals

Male and female rainbow trout *Oncorhynchus mykiss* (Walbaum) (482 ± 17 g, N=24) were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and held in a 1,300-liter flow-through tank in dechlorinated, well-oxygenated water at 13°C under a 12:12 h light-dark photoperiod. Fish were acclimated to these conditions for at least 2

weeks before experiments. They were fed floating fish pellets (Martin Mills, Elmira, Ontario, Canada) 3 times a week until satiation. They were randomly assigned to a control group (normoxia) or treatment groups (hypoxia). All procedures were approved by the Animal Care Protocol Review Committee of the University of Ottawa and adhered to the guidelines established by the Canadian Council on Animal Care for the use of animals in research.

Catheterizations

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

Lactate kinetics

The rates of lactate appearance (R_a) and lactate disposal (R_d) were measured by continuous infusion of [U - ^{14}C] lactate (New England Nuclear, Boston, MA, USA; 4.84 GBq $mmol^{-1}$). Infusates were freshly prepared before each experiment by drying an aliquot of the solution obtained from the supplier under N_2 and resuspending in Cortland saline. Labeled lactate was infused for 4 h in resting animals ($1,500 \pm 25$ Bq $kg^{-1} min^{-1}$, $N=13$), using a calibrated syringe pump (Harvard Apparatus, South Natick, MA, USA; at 1 ml h^{-1}). Lactate (labelled+unlabelled) was infused at rates accounting for $<0.002\%$ of the endogenous R_a lactate measured in resting, normoxic fish.

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

Metabolic rate

Non-cannulated fish ($447 \pm 22\text{g}$, $N=5$) were mildly anaesthetized with ethyl-N-aminobenzoate sulfonic acid (MS-222; 60 mg L^{-1}) in well oxygenated water. They were then transferred into a 13.6-liter respirometry chamber (Loligo Systems, Tjele, Denmark) filled with the same quality water as the holding tank and allowed to recover overnight. Metabolic rate (MO_2) measurements were started 1h before hypoxia exposure the continued during the hypoxia exposure. The water oxygen content was gradually reduced over 90 min by bubbling N_2 through a column containing glass marbles. After reaching 25% saturation ($2.63 \pm 0.01\text{ mg O}_2\text{ L}^{-1}$), the water oxygen was maintained at 25% for 90 min. Metabolic rate measurements were performed by intermittent flow respirometry using galvanic oxygen probes connected to a DAQ-PAC-G4 instrument controlled with AutoResp software (ver. 2) (Loligo Systems, Tjele, Denmark). The oxygen probes were calibrated using N_2 -saturated water (0% O_2) and air-saturated water (100% air) before each measurement.

Tissue sampling

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

Analyses of blood samples

Glucose and lactate concentrations were measured spectrophotometrically (Bergmeyer, 1985) using a Spectra Max plus 384 (Molecular Devices, Sunnyvale, CA, USA). Radioactivity was measured by scintillation counting (Beckman Coulter LS 6500, Fullerton, CA, USA) in Bio-Safe II scintillation fluid (RPI Corp, Mount Prospect, IL, USA). Lactate and glucose were separated using 3 ion exchange columns placed in series to determine specific activities (as in (Katz et al., 1981) with modifications as follows). The 3 columns contained 0.2 ml Dowex 50 (H⁺ form; 100 mesh; to separate amino acids), 0.4 ml Dowex 1 (acetate form; 200 mesh; to separate lactate), and 0.3 ml Dowex 1 (borate form; 200 mesh; to separate glucose). Before passing through the columns, the deproteinized blood samples (150 µl each) were neutralized with 75 µl potassium bicarbonate 1 M, and diluted with 5 ml deionized H₂O. Amino acids were eluted from column 1 with 4 ml ammonium hydroxide 2 M, lactate from column 2 with 4 ml acetic acid 2 M, and glucose from column 3 with 4 ml acetic acid 0.5 M. Preliminary experiments with known amounts of labeled lactate and labeled glucose showed that this procedure separates and recovers >90% of total activity. After column separation, the sum of the activities recovered in the amino acid, lactate and glucose fractions accounted for 84±3% of total plasma activity (N= 13 fish).

Tissue metabolites

Frozen tissues (0.5-1 g) were ground in liquid N₂ with mortar and pestle, before homogenizing for 1 min in perchloric acid (6%; 4:1 v/w) with a Polytron (Kinematica, Luzern, Switzerland). Glucose and lactate concentrations were determined from supernatant as described for blood. Tissue glycogen concentration was measured on subsamples of the homogenates using amyloglucosidase (Fournier and Weber, 1994).

Calculations and statistics

Lactate and glucose specific activities (Bq μmol^{-1}) were calculated as the ratio between activity and concentration. The rates of appearance (R_a lactate) and disposal (R_d lactate) were calculated using the non-steady state equations as described previously (Steele, 1959), with a volume of distribution of 100 ml kg^{-1} (Stanley et al., 1985). To illustrate what would happen to blood lactate concentration if the rate of lactate disposal was not stimulated during hypoxia, hypothetical changes in concentration were computed (see Fig. 2.5). They were calculated for each time using R_a lactate measured during hypoxia and R_d lactate measured during control experiments under normoxic conditions. Statistical comparisons were performed using one- or two-way repeated measures analysis of variance (RM-ANOVA) with Bonferroni *post hoc* test to determine which means were different from baseline. In cases where the assumptions of normality or homoscedasticity were not met, Friedman repeated measures ANOVA on ranks was used with Dunn's test, or the data was normalized by logarithmic transformation before parametric analysis. All values presented are means \pm s.e.m. and a level of significance of $P < 0.05$ was used in all tests.

RESULTS

Water oxygen content

Changes in the oxygen concentration of the water during the measurement of lactate kinetics in control and hypoxic fish are presented in Fig. 2.1A. Control fish were kept in normoxic water averaging $9.5 \text{ mg O}_2 \text{ l}^{-1}$ and this concentration did not vary over time ($P>0.05$). For the hypoxic fish, oxygen content was progressively reduced from $9.4 \text{ mg O}_2 \text{ l}^{-1}$ (at time 0) to $2.6 \text{ mg O}_2 \text{ l}^{-1}$ over 1.5 h and maintained at that level for the remainder of the experiments. Oxygen concentrations of the hypoxia group were significantly lower than normoxic values starting 15 min after the onset of the transition phase ($P<0.001$).

Lactate concentration and specific activity

The time course of changes in blood lactate concentration is shown in Fig. 2.1B. Control fish maintained a low, baseline lactate concentration averaging 0.8 mM throughout the experiments ($P>0.05$). In contrast, the treatment group showed a progressive increase from 1.0 to 8.9 mM during hypoxia ($P<0.001$). Blood lactate specific activity during the measurement of lactate kinetics is presented in Fig. 2.2. Hypoxic fish showed a significant decrease ($P<0.001$; Fig. 2.2A), whereas a small increase was observed in control fish ($P<0.001$; Fig. 2.2B). Dunn's *post-hoc* test only identified the last 2 points of the control group as different from baseline ($P<0.05$).

Effects of hypoxia on lactate fluxes

Changes in the rates of lactate appearance and disposal are shown in Fig. 2.3. Hypoxia caused a large increase in R_a and in R_d lactate ($P < 0.001$; Fig. 2.3A). In fish exposed to hypoxia, R_a lactate went from a baseline value of 18.4 to $36.5 \mu\text{mol kg}^{-1} \text{min}^{-1}$, and R_d lactate from 19.9 to $30.3 \mu\text{mol kg}^{-1} \text{min}^{-1}$ throughout the experiments. In control fish, R_a and R_d lactate were 18.2 and $19.0 \mu\text{mol kg}^{-1} \text{min}^{-1}$ at time 0. After 3 h, these fluxes showed a slight decrease to 13.1 and $12.8 \mu\text{mol kg}^{-1} \text{min}^{-1}$, respectively ($P < 0.001$; Fig. 2.3B). Dunn's *post-hoc* test only identified the last 3 R_a and R_d values of the control group as different from baseline ($P < 0.05$). Initial (baseline) and final values (after exposure to normoxia or hypoxia) for blood lactate concentration, specific activity and lactate fluxes are summarized in Table 2.1.

Because changes in lactate concentration depend on the balance between R_a and R_d lactate, their difference is presented in Fig. 2.4. The $R_a - R_d$ difference increased from -0.5 ± 0.5 to a maximum of $8.3 \pm 1.7 \mu\text{mol kg}^{-1} \text{min}^{-1}$ in fish exposed to hypoxia ($P < 0.001$; Fig. 2.4A), but remained at baseline ($0.02 \pm 0.10 \mu\text{mol kg}^{-1} \text{min}^{-1}$) in control fish ($P = 0.12$; Fig. 2.4B). In the treatment group, all $R_a - R_d$ values were significantly different from 0 after 70 min ($P < 0.05$). Hypoxia caused an unexpected increase in the rate of lactate disposal (Fig. 2.3A), and to evaluate its impact, we have calculated hypothetical changes in blood lactate concentration if this response did not happen. These calculated values are presented in Fig. 2.5 with the concentrations observed in control and hypoxic fish for comparison. The closed triangles show that blood lactate concentration would increase to 18.0 mM if R_d lactate was not stimulated during hypoxia (instead of the maximum of 8.9 mM observed in real fish whose R_d lactate was strongly stimulated; Fig. 2.5).

Glucose metabolism

Changes in blood glucose concentration are presented in Fig. 2.6. Treatment fish increased glycemia from 4.9 to 6.1 mM during exposure to hypoxia ($P < 0.001$; Fig. 2.6A), whereas control fish maintained baseline values (4.92 ± 0.02 mM) throughout the experiments ($P > 0.05$; Fig. 2.6B). Bonferroni *post-hoc* test reveals that all the values measured during hypoxia were hyperglycemic (Fig. 2.6A). Figure 2.8 shows changes in blood glucose specific activity during continuous infusion of labeled lactate. Some lactate was used as a precursor for glucose synthesis. Therefore, the specific activity of glucose increased in fish exposed to hypoxia ($P < 0.001$; Fig. 2.7A) as well as in control fish kept at normoxia ($P < 0.001$; Fig. 2.7B).

Tissue carbohydrates

The effects of hypoxia on the concentrations of the main carbohydrates in rainbow trout tissues are shown in Fig. 2.8. Changes in lactate, glucose, and glycogen concentrations were observed. All the tissues accumulated lactate during hypoxia (control vs. hypoxia: $P < 0.001$), with white muscle showing the highest increase from 3.6 to 19.2 $\mu\text{mol g}^{-1}$ (Fig. 2.8A). Comparisons among hypoxia values reveal that lactate concentration was higher in white muscle than in all other tissues ($P < 0.01$; statistics not shown on graph). Increases in glucose concentration were observed in liver, red muscle and white muscle, but no change was observed in the other tissues (Fig. 2.8B). Hypoxia caused significant glycogen depletion in the liver from 299 to 158 $\mu\text{mol glucosyl units g}^{-1}$ ($P < 0.001$), but had no effect in other tissues ($P > 0.05$) (Fig. 2.8C).

Water oxygen content and metabolic rate

Figure 2.9B shows the metabolic rate of fish exposed to hypoxia. The normoxic resting MO_2 was $37.2 \mu\text{mol O}_2 \text{ kg}^{-1} \text{ min}^{-1}$, and did not change significantly during exposure to hypoxia ($P>0.05$).

DISCUSSION

Our study shows that rainbow trout acutely exposed to hypoxia do not only increase lactate production (R_a), but also strongly stimulate lactate disposal (R_d). We report the first measurements of non-steady state lactate kinetics in an ectotherm. The classic increase in blood lactate concentration observed during hypoxia results from a mismatch caused by the more rapid increase in R_a than in R_d (+98% vs. +52%). The increase in lactate production comes as no surprise, but the stimulation of R_d lactate is unexpected in an animal experiencing oxygen deprivation. This metabolic response is essential because it greatly reduces the lactate load imposed on the circulation of hypoxic fish. Simple measurements of changes in concentration could not have shown how fish cope with hypoxic stress and their response could only be characterized through *in vivo* flux measurements. Differential accumulation of lactate in various tissues reveals that white muscle is the principal source of anaerobic end-product.

Stimulation of lactate disposal

The most striking result from this study is the stimulation of R_d lactate elicited by hypoxia (Fig. 2.3 and Table 2.1). The only two pathways available to clear lactate are *oxidation* and *gluconeogenesis*. Intuitively, it would make sense if hypoxia actually decreased R_d lactate because lowering oxidation would spare oxygen and reducing gluconeogenesis would spare energy. Instead, how can lactate oxidation and glucose production from lactate be activated during hypoxia? Overall need for oxidative fuel is the same in normoxic and hypoxic fish because the oxygen deprivation regime used in our experiments does not cause metabolic depression (Fig. 2.9B). The observed increase in R_d lactate could therefore be caused by a change in fuel selection favoring lactate and reducing the use of alternative fuels like glucose and fatty acids. During hypoxia, oxidative tissues probably increase lactate utilization because of the increased blood-to-tissue gradient (Fig. 2.1B). This scenario is consistent with numerous studies on fish and mammals demonstrating that oxidative tissues such as heart, brain and red muscle prefer lactate over other fuels (Bilinski and Jonas, 1972; Drake et al., 1980; Lanctin et al., 1980; Smith et al., 2003). Lactate oxidation has been shown to be stimulated during hypoxia in fish liver, brain and Brockmann bodies (Polakof and Soengas, 2008a; Polakof and Soengas, 2008b) and in mammalian heart (Mazer et al., 1990). However, no direct measurement of lactate oxidation in heart, brain, liver, gut and erythrocytes of hypoxic fish are yet available and all these tissues may play a significant role in stimulating R_d lactate when oxygen supply is limited.

Even though glucose synthesis from lactate costs some ATP, the balance of evidence presently available from the literature suggests that gluconeogenesis is stimulated during hypoxia. The increases in glucose concentration observed here in the blood (Fig. 2.6A) and

in the liver (Fig. 2.8B) are consistent with this idea, but our measurements of glucose specific activity (Fig. 2.7) cannot be used to quantify gluconeogenic flux from lactate. They only provide a qualitative demonstration that trout synthesize glucose from lactate, without revealing whether gluconeogenesis is stimulated during hypoxia. In a previous study on frogs kept under low oxygen for several weeks, the authors concluded that lactate was partly recycled via increased hepatic gluconeogenesis (Donohoe and Boutilier, 1999). Another study on rainbow trout exposed to acute hypoxia also suggests that gluconeogenesis is activated (Wright et al., 1989). Direct measurements of gluconeogenic enzymes in Gulf killifish reveal that fructose-1,6-bisphosphatase activity is stimulated by hypoxia, although phosphoenolpyruvate carboxykinase (PEPCK) was not affected in these experiments (Martinez et al., 2006). Perhaps the most compelling evidence comes from a study investigating how gluconeogenesis is activated by hypoxia in isolated rat hepatocytes. The authors demonstrate that the expression of gluconeogenic enzymes is stimulated when oxygen is lacking, and they characterize the mechanism of PEPCK activation by hypoxia-inducible factor 1 (HIF-1) (Choi et al., 2005). More research will be needed to show whether this same mechanism also activates gluconeogenesis in fish. Finally, it has been demonstrated that high blood lactate concentration (maintained via a lactate clamp) stimulates gluconeogenesis in exercising humans (Roef et al., 2003). Overall, therefore, the increase in R_d lactate observed in trout is probably jointly supported by an increase in lactate oxidation caused by a switch in fuel preference and by the activation of gluconeogenesis that may be required to sustain glucose supply to the nervous system.

Minimizing the lactate load on the circulation

Several previous studies have reported large increases in blood lactate levels for trout acutely exposed to hypoxia. Using the same conditions as in this study (90 min at 25% air saturation), Van Raaij *et al.* reported final blood lactate concentrations of 6 mM (van Raaij *et al.*, 1996). In other studies where different hypoxic stresses were applied, circulating lactate levels reached 7 mM (180 min at 13% O₂ saturation and 4°C; Dunn and Hochachka, 1986) and 15 mM (60 min at 30% O₂ saturation and 10°C; Thomas *et al.*, 1992). Even though the activation of anaerobic glycolysis is clearly responsible for the observed accumulation of lactate, the true extent of this activation could not be established without information on fluxes. Here, we show that R_a lactate is stimulated during hypoxia, but that the accumulation of lactate resulting from this stimulation is strongly attenuated by the simultaneous increase in lactate disposal. The underlying changes in kinetics causing lactate accumulation in the circulation are presented in Fig. 2.4A that shows the difference between the rates of production and disposal. To illustrate the real physiological impact of the hypoxia-induced increase in R_a lactate, we have calculated theoretical values for blood lactate levels if this response was abolished. If the rate of lactate disposal was artificially maintained at baseline levels throughout hypoxia, blood lactate concentration would reach 18 mM or twice the value observed at the end of our experiments (see Fig. 2.5 for comparison). Therefore, the large increase in lactate disposal taking place during hypoxia plays a crucial role in minimizing the lactate load placed on the circulation.

Lactate production and hypoxia

The erroneous notion that anaerobic glycolysis is activated only when O₂ supply fails to meet demand is further put to rest by the fact that normoxic, resting rainbow trout produce lactate at the remarkable rate of 13-18 $\mu\text{mol kg}^{-1} \text{min}^{-1}$ (Fig. 2.3 and Table 2.1). These values obtained by continuous tracer infusion are higher than previous estimates measured by bolus injection (1-4 $\mu\text{mol kg}^{-1} \text{min}^{-1}$) (Dunn and Hochachka, 1987; Milligan and McDonald, 1988; Weber, 1991). Potential causes for this difference include large errors in estimating surface areas under specific activity decay curves (the main reason why bolus injection is now rarely used), label recycling, and, for one of the studies (Dunn and Hochachka, 1987), failure to subtract glucose activity from total activity when estimating lactate specific activity.

In normoxic fish, high rates of lactate production are exactly matched by equally high rates of lactate disposal (Fig. 2.3B and 2.5B) that act in tandem to maintain a low and steady lactate concentration in the circulation (<1 mM; Fig. 2.1B). Work by Richards *et al.* has shown that the pyruvate dehydrogenase complex probably plays a key regulatory role in sustaining lactate production in normoxic fish (Richards *et al.*, 2002). Rainbow trout support higher baseline turnover rates for lactate ($\sim 15 \mu\text{mol kg}^{-1} \text{min}^{-1}$; this study) than for glucose ($\sim 8 \mu\text{mol kg}^{-1} \text{min}^{-1}$) (Haman *et al.*, 1997b; Shanghavi and Weber, 1999; Weber and Shanghavi, 2000), and the same pattern has been observed in humans (Stanley *et al.*, 1985; Weber *et al.*, 1990). Animals appear to maintain high rates of lactate turnover, even under normoxic conditions at rest, because lactate is a particularly mobile intermediate of carbohydrate metabolism involved in numerous fuel shuttles (Gladden, 2004).

The stimulation of glycolytic flux by hypoxia was measured here as a two-fold increase in R_a lactate from 18 to 36 $\mu\text{mol kg}^{-1} \text{min}^{-1}$ (Fig. 2.3A; Table 2.1). The only other study dealing with the effects of acute oxygen deprivation on trout lactate kinetics reported an increase from 3 to 20 $\mu\text{mol kg}^{-1} \text{min}^{-1}$ (Dunn and Hochachka, 1987), but these previous values may not reflect true rates because they were estimated by bolus injection (see limitations above). Nevertheless, hypoxia-induced changes lactate production in trout are consistent with the response observed in mammals. Humans acutely exposed to hypobaric hypoxia show an initial 4-fold increase in lactate production before the response is somewhat attenuated after 3 weeks of acclimation to high altitude (Brooks et al., 1991). Several earlier studies of lactate metabolism in fish had compared proton and lactate movements after exhaustive exercise (e.g. Turner and Wood, 1983; Turner et al., 1983). Rates of proton and lactate flux were estimated from changes in pH and in lactate concentration, either in white muscle or in the circulation. In future investigations of acid-base balance in exercising fish, it could be useful to use measurements of R_a lactate as a tool to estimate rates of proton production indirectly. This approach could be helpful because the stoichiometry of lactate and proton production rarely deviates from a 1:1 ratio (Marcinek et al., 2010) and, to our knowledge, direct measurements of proton flux have not been attempted.

What are the main sites of lactate production during hypoxia? The much higher accumulation of lactate in white muscle (19 $\mu\text{mol g}^{-1}$) than in other tissues (7-10 $\mu\text{mol g}^{-1}$) shows that it is most likely the principal source of lactate in hypoxic trout (Fig. 2.8A). This observation is consistent with previous studies reporting changes in tissue lactate concentrations in rainbow trout (Bernier et al., 1996; Boutilier et al., 1987; Dunn and

Hochachka, 1986) and in Amazonian cichlids (Richards et al., 2007). It is unclear to what extent the lower accumulation of lactate in other tissues than in white muscle is due to endogenous production or to the influx of white muscle-derived lactate via the blood. White muscle lactate is produced from local glycogen, but no significant decline in glycogen concentration could be demonstrated in this tissue (Fig. 2.8C). However, we can calculate that only 8% of the large and variable white muscle glycogen stores would be sufficient to explain the increase in lactate concentration, making the demonstration of a statistically significant decrease very difficult. For this reason, only one previous study of acute hypoxia exposure shows a decrease in white muscle glycogen (Boutilier et al., 1987), whereas two others, like ours, only report non-significant trends (Bernier et al., 1996; Dunn and Hochachka, 1986). During hypoxia, it has been suggested that high rates of lactate production in white muscle may be necessary to maintain cytoplasmic redox state in this tissue (Richards et al., 2007).

CONCLUSION

The accumulation of anaerobic end product in hypoxic trout is caused by the unequal stimulation of lactate production and disposal. This study demonstrates that the doubling of R_a lactate is unexpectedly accompanied by a 52% increase in R_d lactate. This hypoxia-induced increase in lactate disposal plays a strategic role in reducing the lactate load on the circulation. It is probably mutually supported by increasing lactate oxidation through a change in metabolic fuel preference and by stimulating the use of lactate as a gluconeogenic substrate. Resting trout kept in normoxic water produce lactate at higher baseline rates (than previously estimated by bolus injection) that are exactly matched by high rates of lactate

disposal to maintain low and steady blood lactate concentrations (<1mM). During hypoxia, measurements of lactate accumulation in tissues shows that white muscle is the major producer of anaerobic end product. This first *in vivo* study of non-steady state lactate kinetics in fish characterizes fundamental changes in flux that underlie their physiological response to hypoxia.

Figure 2.1. Water oxygen content (A) and blood lactate concentration (B) in rainbow trout during the measurement of lactate kinetics. Filled circles show control fish kept at normoxia. Open circles show treatment fish exposed to hypoxia. For treatment fish, normoxia, transition, and hypoxia phases are separated by dotted lines. Values are means \pm s.e.m (N=8). Differences from baseline (time 0) are indicated by * (P<0.05) or ** (P<0.001).

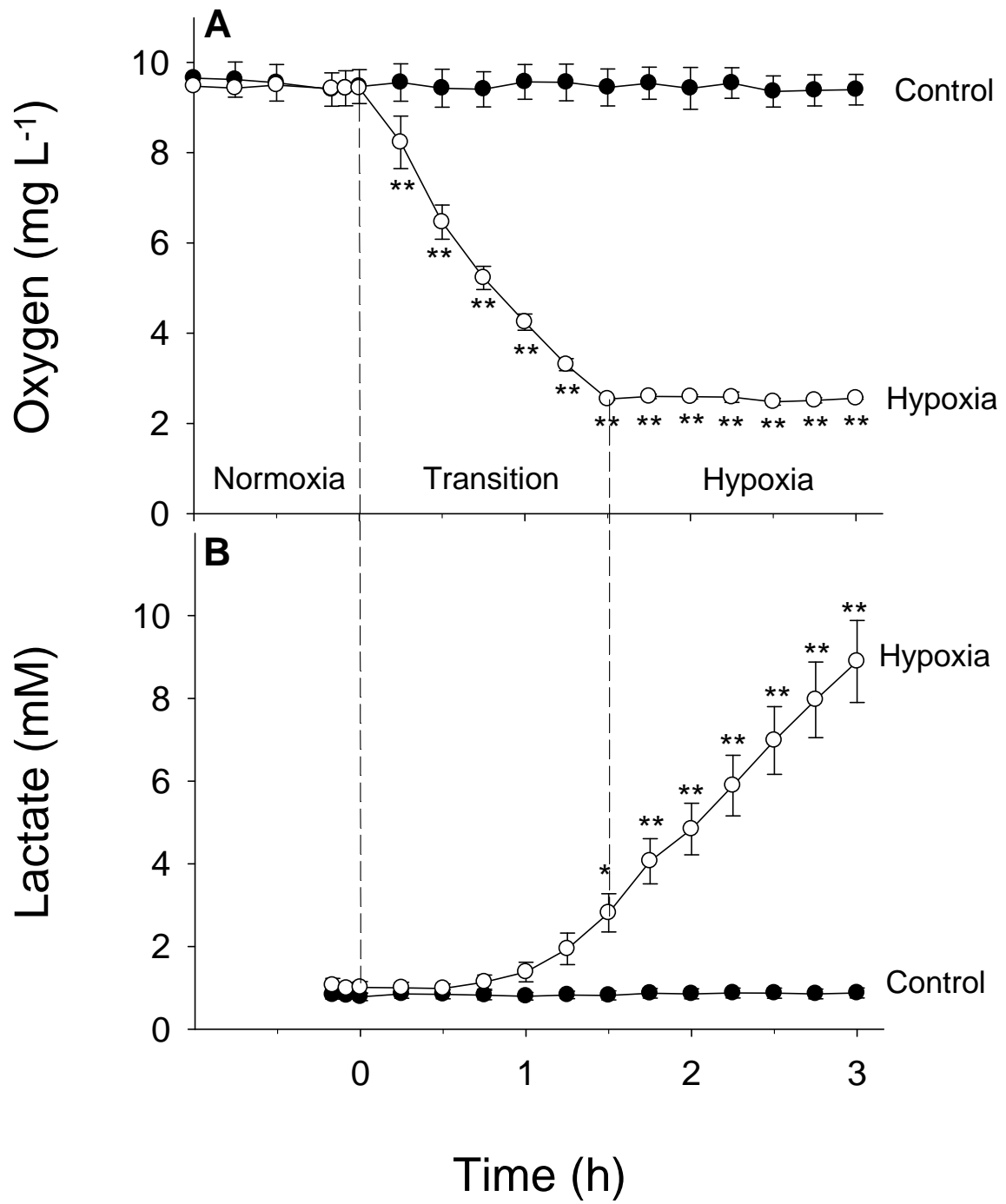


Figure 2.2. Blood lactate specific activity in rainbow trout during continuous infusion of [U-¹⁴C] lactate in hypoxic (A) and control fish (B). Tracer infusions were started 1 h before time 0. In A, normoxia, transition, and hypoxia phases are separated by dotted lines. Values are means \pm s.e.m (N=8 for hypoxia and 5 for control). Differences from baseline (time 0) are indicated by * (P<0.05).

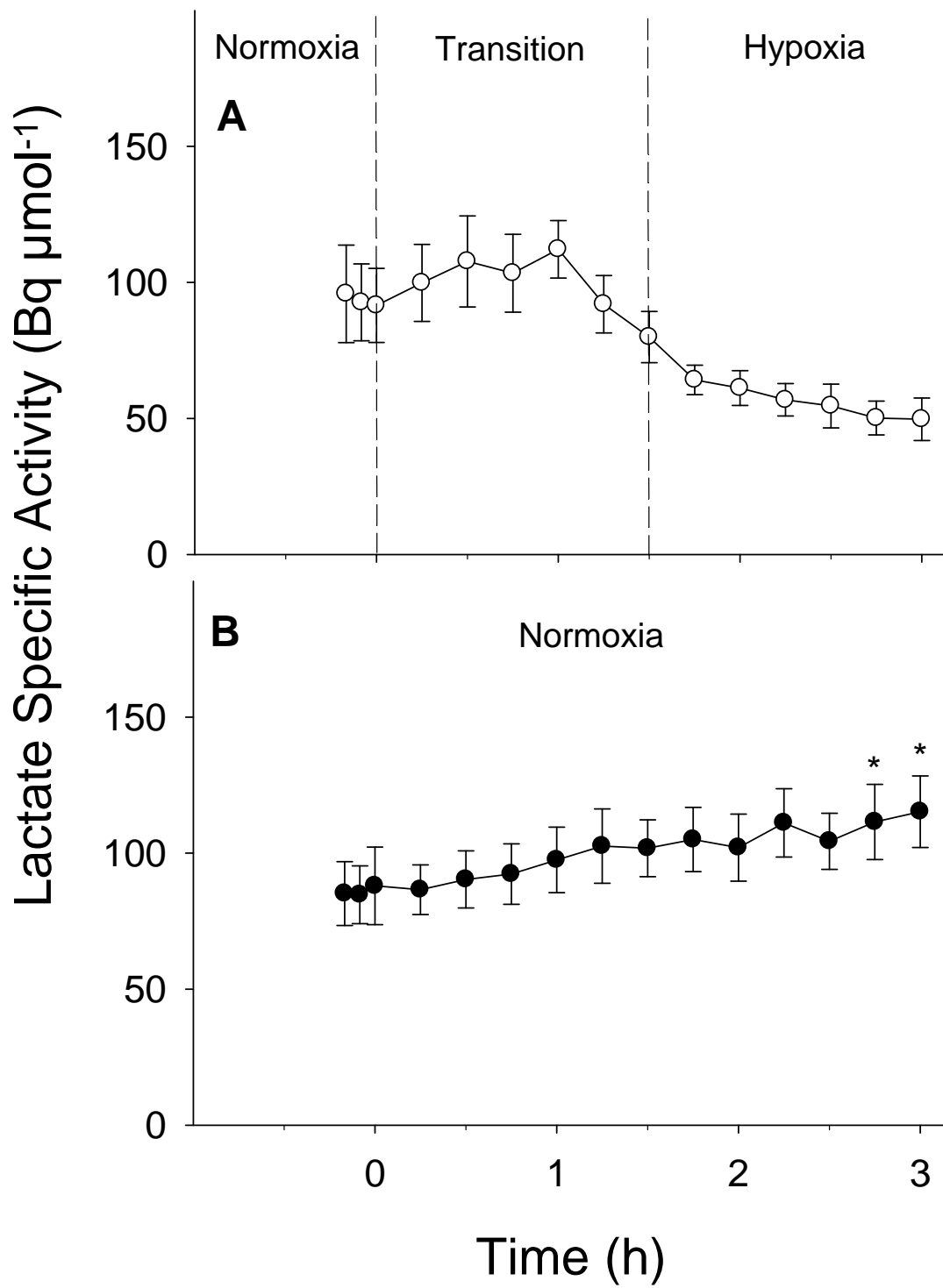


Figure 2.3. Lactate fluxes of rainbow trout exposed to hypoxia (A) or kept at normoxia (B). Filled circles show the rate of appearance of lactate (R_a). Open circles show the rate of lactate disposal (R_d). Tracer infusions were started 1 h before time 0. In A, normoxia, transition, and hypoxia phases are separated by dotted lines. For the control fish (B), most R_d values overlap with R_a (B). Values presented are means and s.e.m (N=8 for hypoxia and 5 for control). Differences from baseline (time 0) are indicated by * ($P<0.05$).

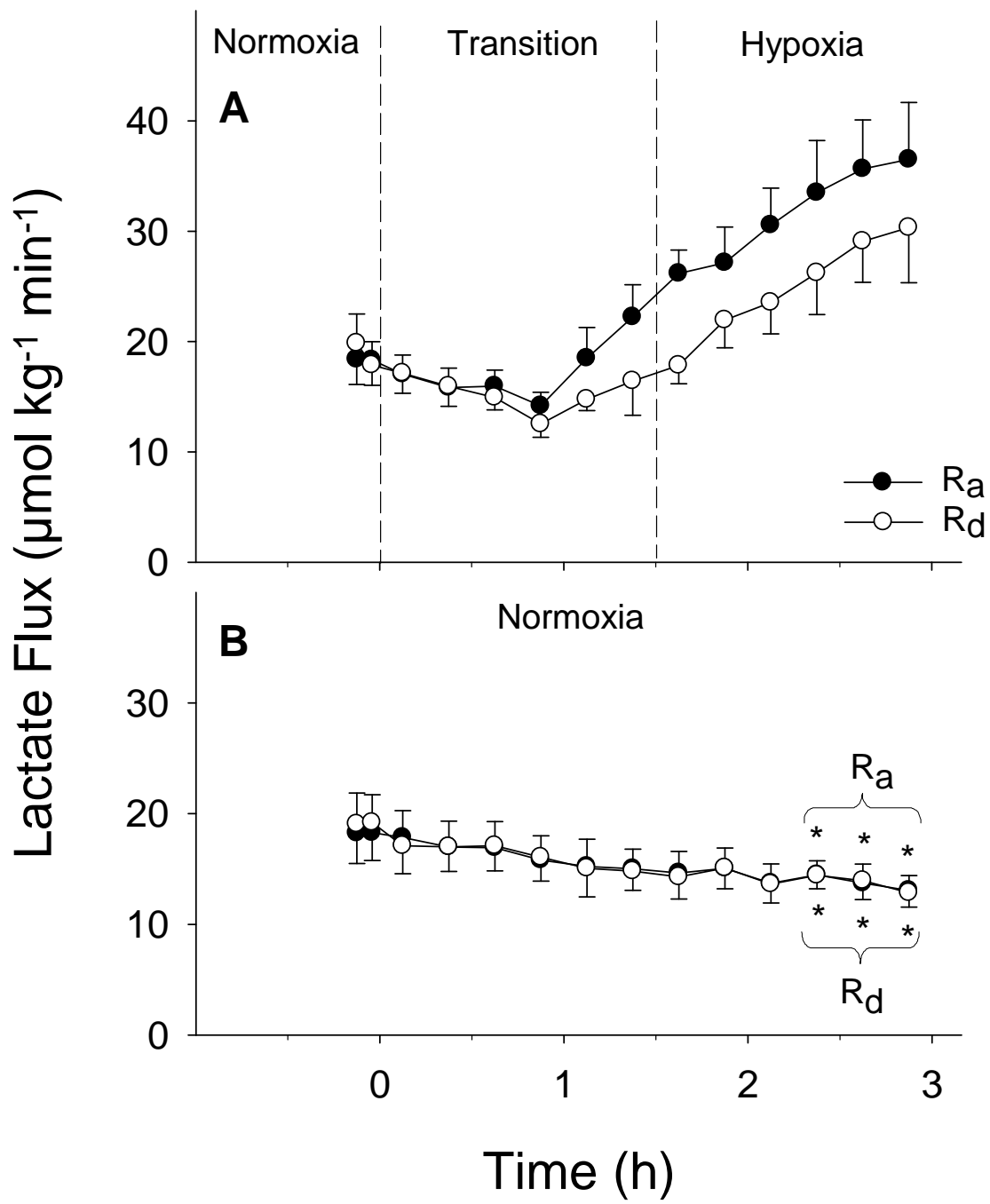


Figure 2.4. Changes in the difference between the rates of lactate appearance and lactate disposal (R_a-R_d) in rainbow trout exposed to hypoxia (A) or kept at normoxia (B). Tracer infusions were started 1 h before time 0. In A, normoxia, transition, and hypoxia phases are separated by dotted lines. Values are means and s.e.m (N=8 for hypoxia and 5 for control). Differences from baseline are indicated by * ($P<0.05$).

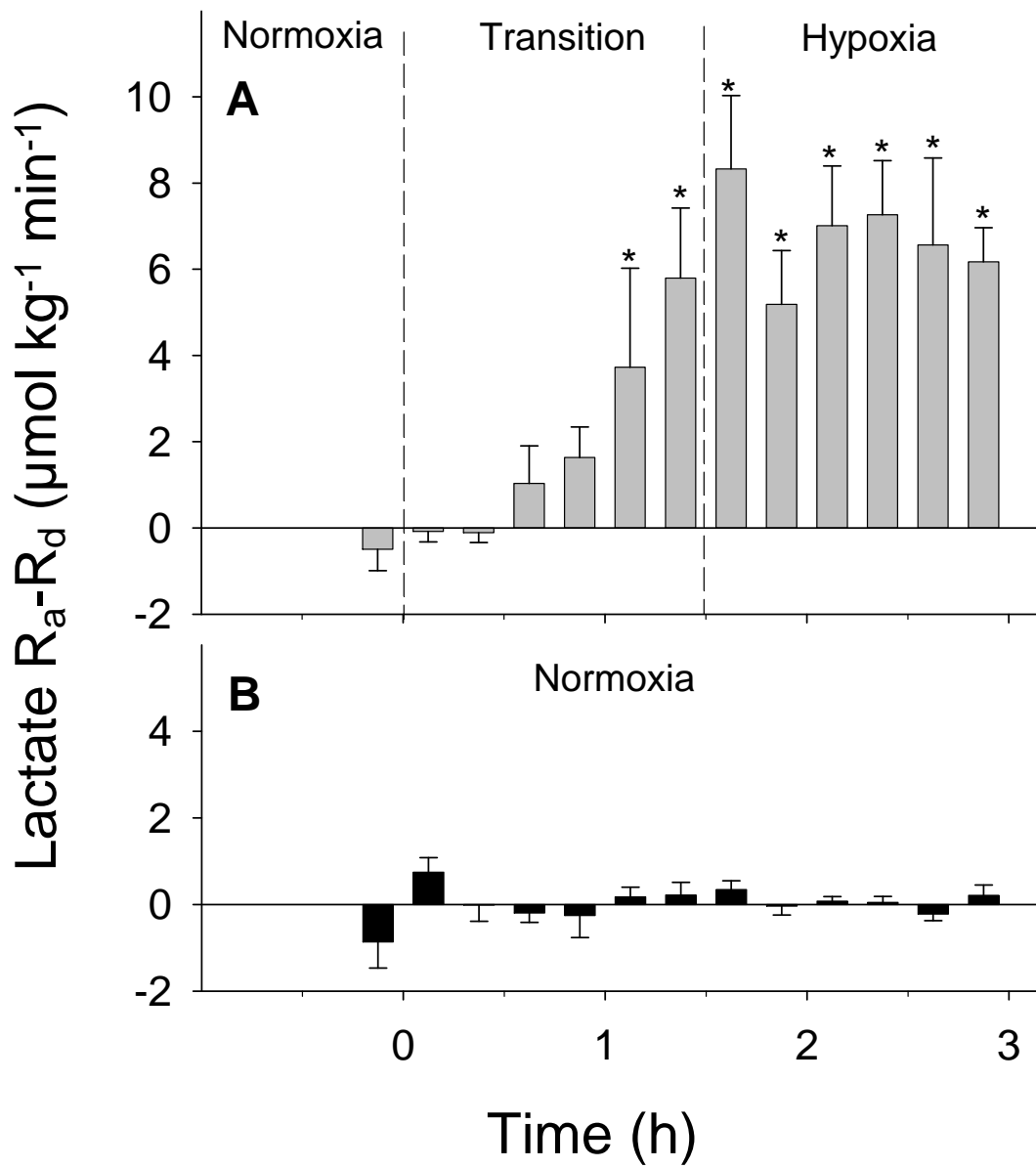


Figure 2.5. Theoretical increase in blood lactate concentration (triangles) calculated for rainbow trout exposed to hypoxia if lactate disposal stayed at baseline, normoxic values. Real blood lactate concentrations measured in trout exposed to hypoxia (open circles) or kept at normoxia (filled circles) are also indicated for comparison.

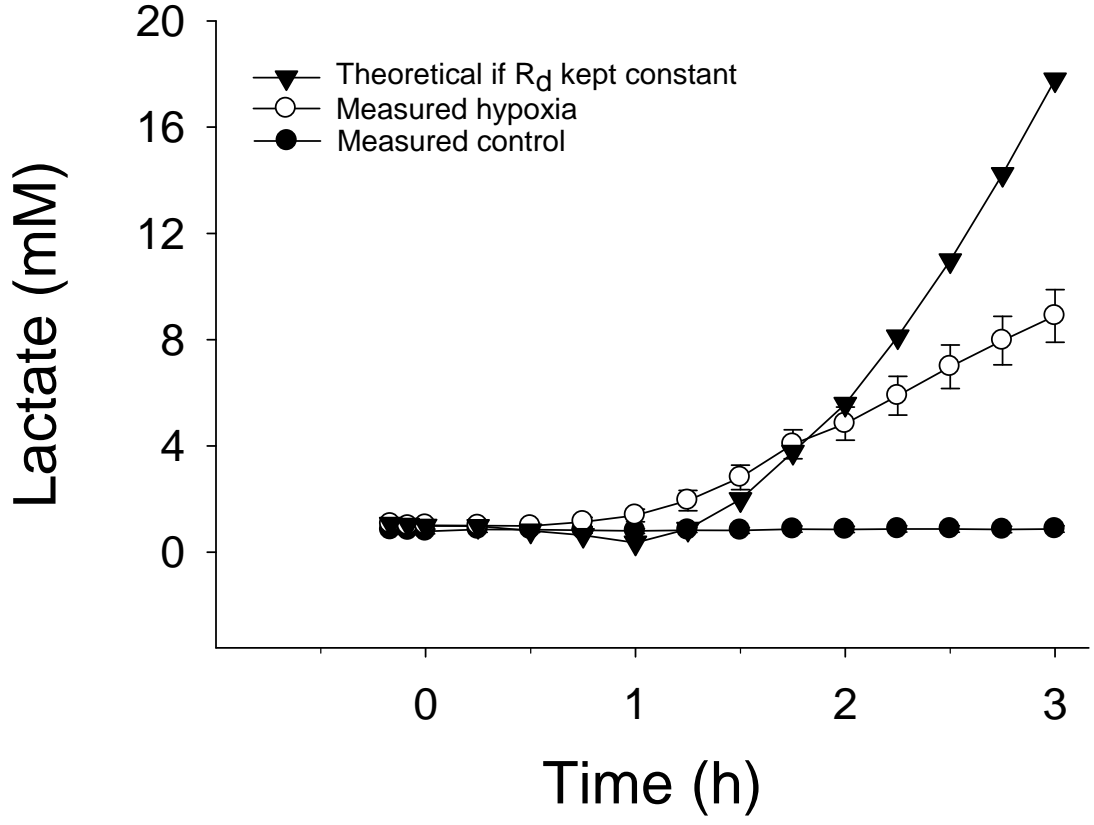


Figure 2.6. Blood glucose concentration in rainbow trout exposed to hypoxia (A) or kept at normoxia (B). In A, normoxia, transition, and hypoxia phases are separated by dotted lines. Values are means \pm s.e.m (N=8). Differences from baseline (time 0) are indicated by * (P<0.05).

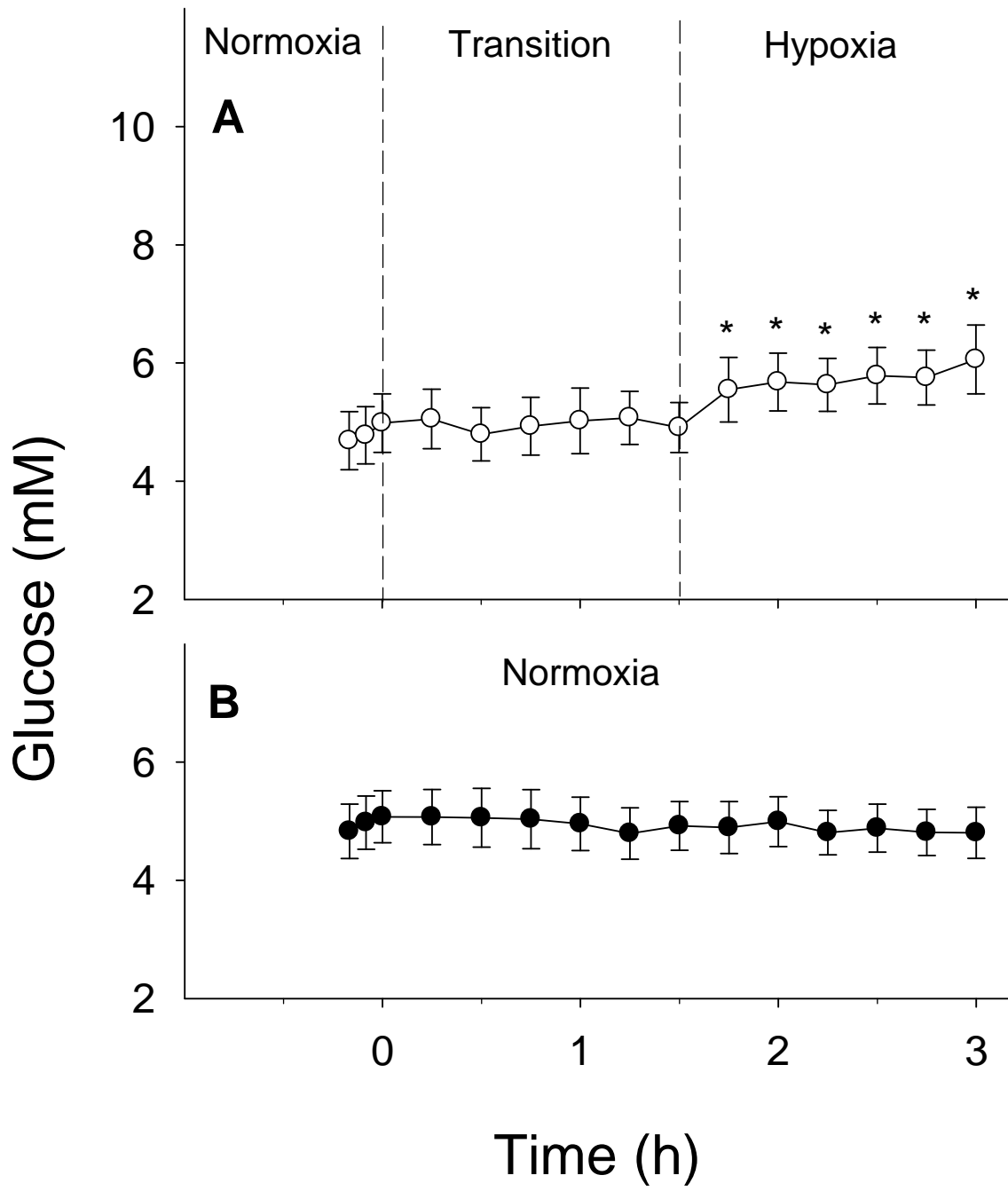


Figure 2.7. Glucose specific activity in the blood of rainbow trout exposed to hypoxia (A) or kept at normoxia (B). Tracer infusions were started 1 h before time 0. In A, normoxia, transition, and hypoxia phases are separated by dotted lines. Values are means \pm s.e.m (N=8 for hypoxia and 4 for control). Differences from baseline (time 0) are indicated by * (P<0.05).

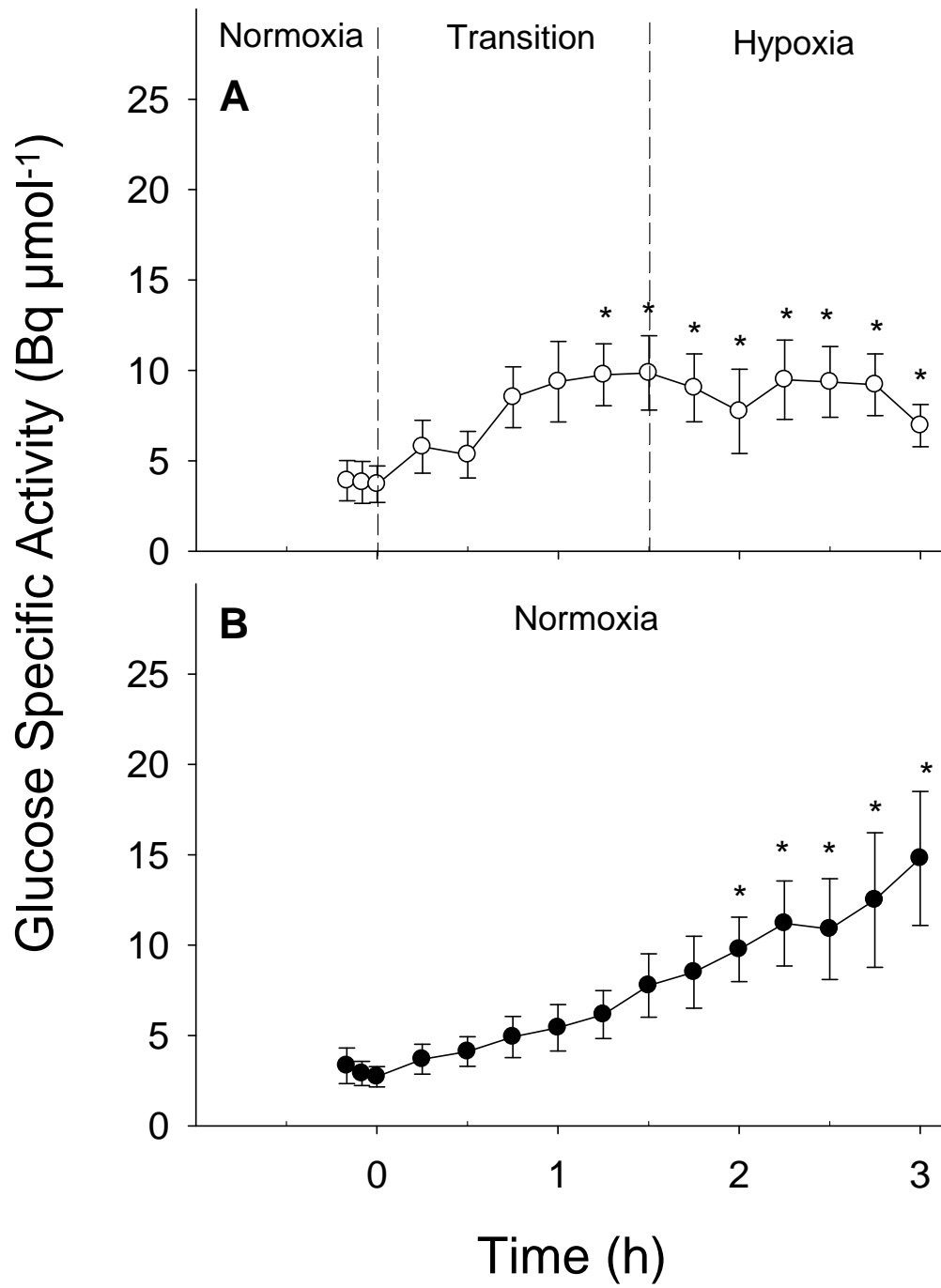


Figure 2.8. Tissue concentrations of lactate (A), glucose (B), and glycogen (C) in rainbow trout kept at normoxia (filled bars) or exposed to hypoxia (grey bars). Values are means + s.e.m (N=6) expressed per gram wet tissue. Significant effects of hypoxia within each tissue are indicated by * (P<0.05), ** (P<0.01), or *** (P<0.001). Comparison of hypoxia values for lactate concentration (A) shows that white muscle is higher than all other tissues (P<0.01; statistics not shown on graph).

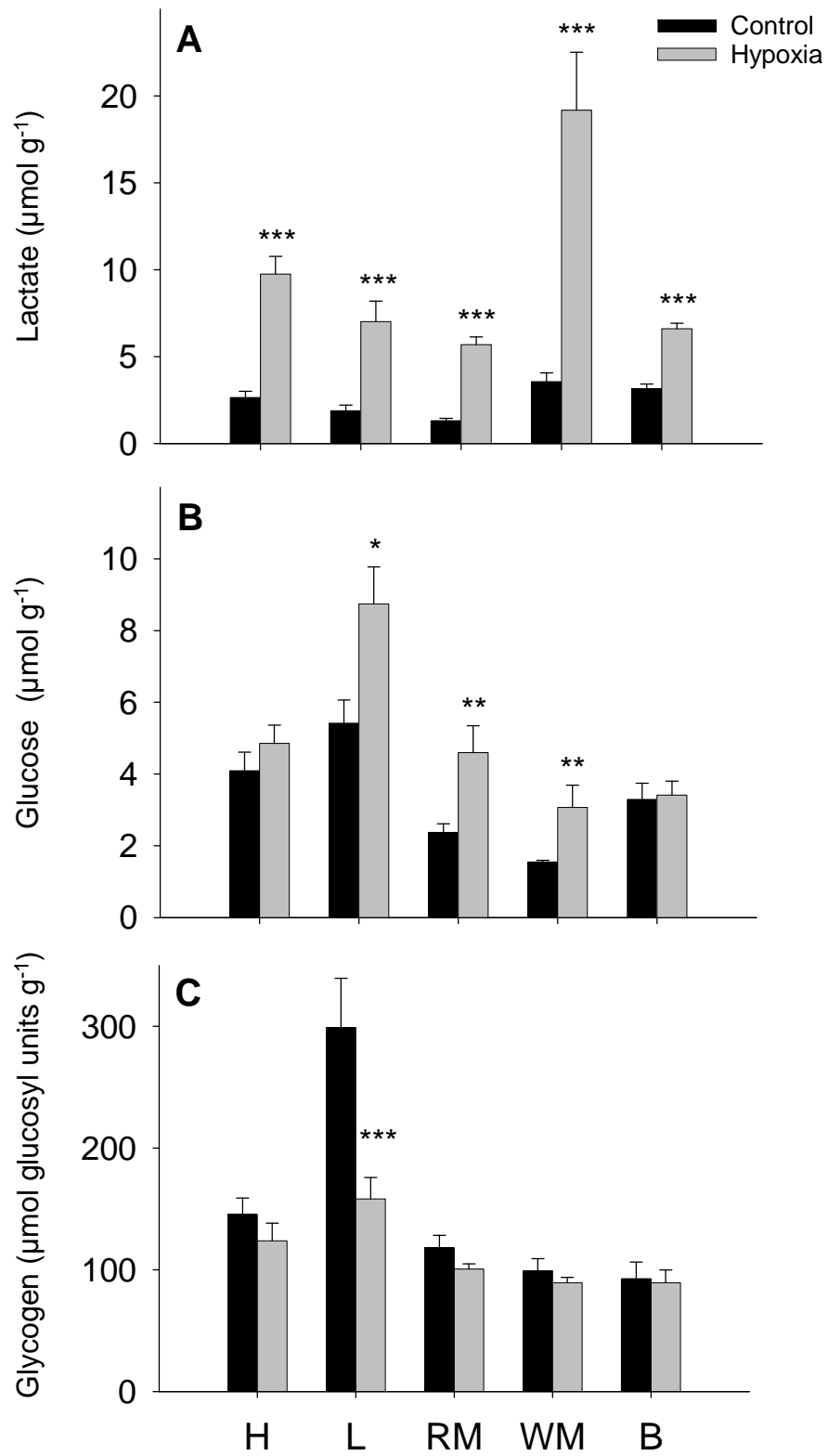


Figure 2.9. Water oxygen content (A) and metabolic rate (B) of rainbow trout during exposure to hypoxia. Normoxia, transition, and hypoxia phases are separated by dotted lines. Values are means \pm s.e.m (N=5). Differences from baseline (time 0) are indicated by * (P<0.05).

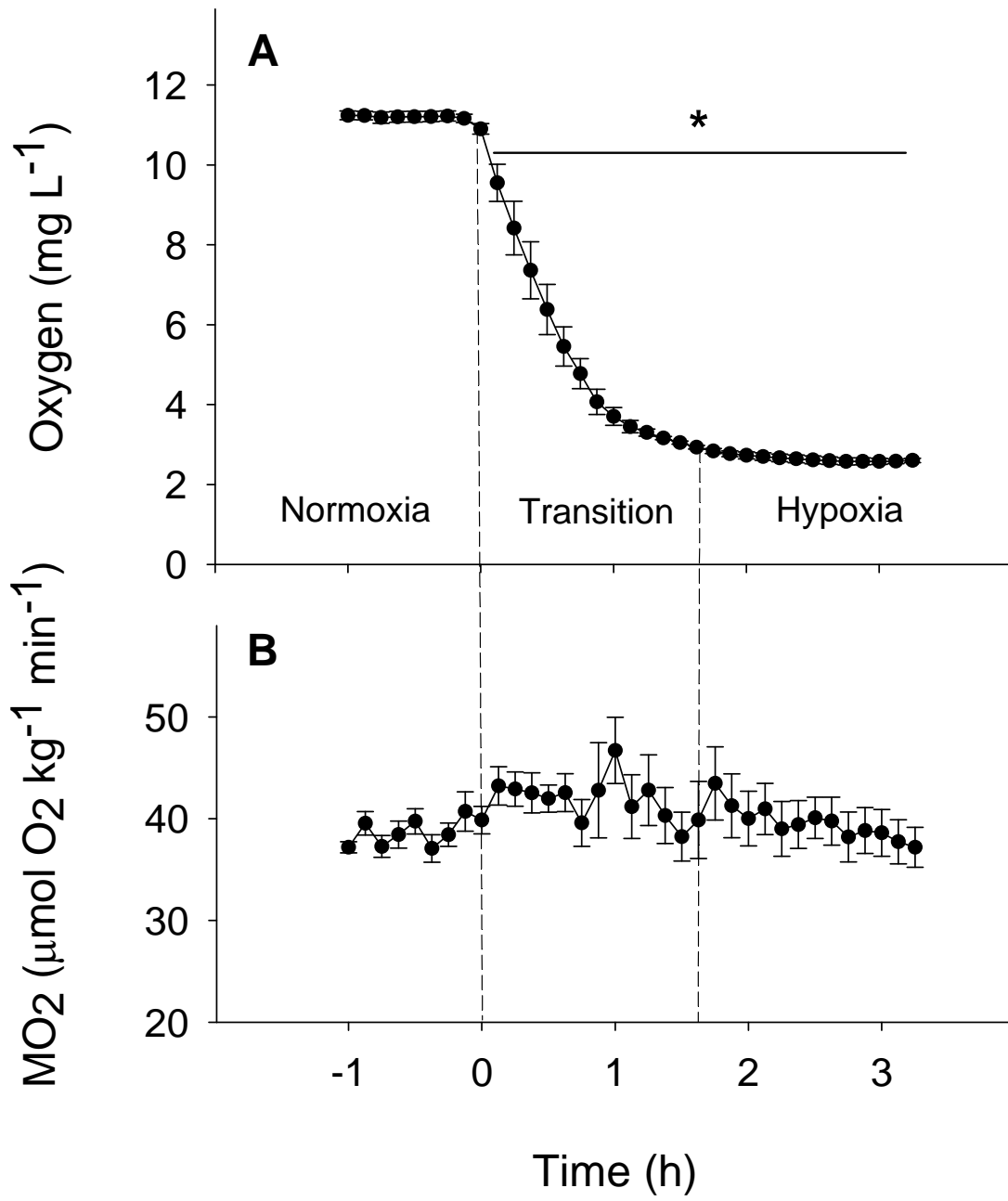


Table 2.1. Blood metabolite concentrations and specific activities before and after normoxia (control) or hypoxia in rainbow trout during continuous infusion of [U-14C]-lactate. Rates of appearance (R_a) and disposal of lactate (R_d) are also indicated.

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

Values are means ± s.e.m. with sample size in parentheses. Initial values were measured at time 0 and final values at 3 h. S.A., specific activity; R_a , rate of appearance; R_d , rate of disposal. Statistical differences are indicated as * ($P < 0.05$) or ** ($P < 0.001$).

CHAPTER 3: Exercise lactate kinetics in rainbow trout: steady-state vs. graded exercise

Partially based on:

Loïc Teulier ^{1,2}, Teye Omlin ¹ and Jean-Michel Weber ¹

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¹ Biology, University of Ottawa, Ottawa, Ontario, Canada K1N 6N5

² Laboratoire d'Ecologie des Hydrosystèmes Naturels et Anthropisés, UMR 5023, CNRS,

Université de Lyon 1, Lyon, France

INTRODUCTION

As glycolytic end-product, lactate is one of the most dynamic intermediates of cell metabolism because it can be used as oxidative fuel and gluconeogenic substrate (Brooks, 1991; Gladden, 2004; Philp et al., 2005). Animals process lactate at high rates even under resting, normoxic conditions and, in mammals, many studies show that inherently high baseline lactate fluxes are strongly stimulated during exercise (Bergman et al., 1999; Donovan and Brooks, 1983; Issekutz et al., 1976; Stanley et al., 1985; van Hall et al., 2003; Weber et al., 1987a). Little is known for fish because adequate methods to quantify lactate fluxes accurately under controlled exercise conditions have only become available recently.

Previous studies of fish lactate kinetics report turnover rates that were estimated by bolus injection (Cameron and Cech Jr., 1990; Milligan and McDonald, 1988; Weber, 1991; Weber et al., 1986): an obsolete method with significant limitations (Omlin and Weber, 2010; Wolfe, 1992). They show that the lactate turnover rate of rainbow trout doubles during prolonged, low-intensity swimming (Weber, 1991). Although no measurements have been made at higher swimming speeds, a 3 to 10-fold increase over baseline has been observed during recovery from exhausting exercise for channel catfish (*Ictalurus punctatus*), coho salmon (*Oncorhynchus kisutch*), and starry flounder (*Platichthys stellatus*) (Cameron and Cech Jr., 1990; Milligan and McDonald, 1988). Continuous tracer infusion techniques have now been adapted and validated for fish (Haman and Weber, 1996; Haman et al., 1997b). They have been used to quantify the rates of metabolite appearance (R_a) and disposal (R_d) accurately, under non-steady state conditions. This technique has allowed to measure glucose and lipid kinetics in fish during exercise (Bernard et al., 1999; Magnoni et al., 2008a; Shanghavi and Weber, 1999). However, these measurements only determined

steady-state, low-intensity swimming. Consequently, the effects of exercise on the rates of lactate production and disposal during exercise are unknown. Therefore, the goals of this study were: (1) to apply continuous tracer infusion methods to measure the effects of swimming on lactate fluxes, and (2) to determine the relationship between exercise intensity and the rates of lactate production and disposal.

MATERIALS AND METHODS

Animals

Male and female rainbow trout (*Oncorhynchus mykiss*, Walbaum) (428.3 ± 10.7 g, N=15) were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). They were held in a 1300-liter flow-through tank containing dechlorinated, well-oxygenated water at 13°C for at least 2 weeks before experiments. Fish were kept under a 12h:12h light-dark photoperiod and fed commercial floating pellets (Martin Mills, Elmira, Ontario, Canada) 3 times a week until satiation. The effects of exercise were either measured during prolonged, steady-state swimming or during graded swimming (U_{crit} protocol). All procedures were approved by the animal care committee of the University of Ottawa and adhered to the guidelines established by the Canadian Council on Animal Care for the use of animals in research.

Catheterizations

Fish were doubly cannulated in the dorsal aorta using PE-50 catheters (Intramedic, Clay-Adams, Sparks, MD, USA), as detailed in (Haman and Weber, 1996). Briefly, they were fasted for at least 24 h before surgery. They were anesthetized with ethyl-N-

aminobenzoate sulfonic acid (MS-222; 60 mg L⁻¹) in well oxygenated water, the catheters inserted in the dorsal aorta before they were transferred into the swim tunnel to recover overnight and for acclimation to the experimental setup. During this period, water velocity was kept at 0.5 BL s⁻¹, a low speed requiring no swimming but enabling the fish to rest at the bottom of the respirometer. The catheters were kept patent by flushing with Cortland saline (Wolf, 1963) containing 50 U ml⁻¹ heparin (Sigma-Aldrich, St-Louis, MO, USA). Only animals with a hematocrit >20% after recovery from surgery were used in tracer experiments.

Swim tunnel respirometry

All experiments were carried out at 13°C in a 90 l swim tunnel respirometer (Loligo Systems, Tjele, Denmark) filled with the same quality water as the holding tank. A “honeycomb” grid was placed before the swimming chamber to promote laminar flow. The fish always swam in the anterior part of the chamber (kept dark) to avoid the posterior part (brightly lit). The swim tunnel was calibrated with a flow probe (Global Water Geotech, Denver, CO, USA) to establish the linear relationship between water velocity (in cm/s) and motor speed (in RPM). Swimming speeds were corrected for solid blocking as in (Claireaux et al., 2006). Metabolic rate (MO₂) was measured by intermittent flow respirometry using galvanic oxygen probes connected to a DAQ-PAC-G1 instrument controlled with AutoRespTM software (ver. 2) (Loligo Systems). The oxygen probes were calibrated before each measurement using N₂-saturated water (0% O₂) and air-saturated water (100% air). For the steady-state swimming group, the speed was gradually increased (over 30 min) to reach 1.7 BL s⁻¹ (Shanghavi and Weber, 1999) and subsequently maintained for 90 min. For

the graded swimming group, critical swimming speed (U_{crit}), the effects of exercise on MO_2 , and lactate kinetics were quantified using a stepwise U_{crit} protocol (Jain et al., 1997) with velocity increments of 0.2 BL s^{-1} every 20 min. Graded swimming experiments were terminated at exhaustion when the fish was unable to remove itself from the rear grid.

Lactate kinetics

The catheters were made accessible through the swim tunnel lid by channeling them through a water-tight port. The rates of lactate appearance (R_a) and lactate disposal (R_d) were measured by continuous infusion of [U - ^{14}C] lactate (New England Nuclear, Boston, MA, USA; $4.84 \text{ GBq mmol}^{-1}$). 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. Labeled lactate was infused for 1 h in resting fish to quantify baseline lactate kinetics and continued for 2 h during steady-state swimming or 4-5 h during graded swimming. It was administered at a rate of $2029 \pm 227 \text{ Bq kg}^{-1} \text{ min}^{-1}$ ($N=15$) using a calibrated syringe pump (Harvard Apparatus, South Natick, MA, USA) at 1 ml h^{-1} . For the steady-state swimming fish, blood samples ($100 \mu\text{l}$ each) were drawn 50, 55, and 60 min after the start of infusion to ensure that isotopic steady-state had been reached and to quantify baseline lactate fluxes. Additional samples were then taken every 10 min. For the graded swimming group, only one blood sample was drawn at the end of the resting period. We deemed that under these conditions, isotopic steady-state is reached in <50 min. The additional samples were drawn at regular intervals (5 min before each stepwise velocity increment). The water was kept normoxic throughout the measurements ($10.64 \pm 0.07 \text{ mg O}_2 \text{ l}^{-1}$). Lactate (labelled+unlabelled) was infused at rates accounting for $<0.002\%$ of the

endogenous R_a lactate measured in resting fish. The total amount of blood sampled from each fish accounted for <10% of blood volume. Blood samples were immediately deproteinized in 200 μ l perchloric acid (6% w/w) and centrifuged for 5 min at 16,000 G (Eppendorf 5415C, Brinkmann, Rexdale, Canada). Supernatants were kept frozen at -20°C until analyses.

Sample analyses

Blood lactate concentration was measured spectrophotometrically (Bergmeyer, 1985) using a Spectra Max plus 384 (Molecular Devices, Sunnyvale, CA, USA). To measure activity, lactate was separated using ion exchange columns as described in (Omlin and Weber, 2010). Before passing through the columns, each deproteinized blood sample was neutralized with 1 M potassium bicarbonate and diluted with 5 ml deionized H₂O.

Preliminary experiments with known amounts of labeled lactate showed that 70% of total activity was recovered, and measured lactate activities were corrected accordingly.

Radioactivity was measured by scintillation counting (Beckman Coulter LS 6500, Fullerton, CA, USA) in Bio-Safe II scintillation fluid (RPI Corp, Mount Prospect, IL, USA).

Calculations and statistics

Lactate specific activity (Bq μ mol⁻¹) were calculated as the ratio between activity and concentration. The rates of appearance (R_a lactate) and disposal (R_d lactate) were calculated using the non-steady state equations of Steele (Steele, 1959), with a volume of distribution of 100 ml kg⁻¹ (Stanley et al., 1985). Statistical comparisons were performed using one- or two-way repeated measures analysis of variance (RM ANOVA) with Dunnett's post-hoc test

to determine which means were different from baseline, or Holm-Sidak test to compare treatments (SigmaPlot v.12, Systat Software, Inc., San Jose, CA, USA). When the assumptions of normality (Shapiro-Wilk test) or homoscedasticity (Levene test) were not met, Friedman RM ANOVA on ranks was used or the data were normalized by \log_{10} transformation before parametric analysis. All values presented are means \pm s.e.m and $P < 0.05$ was used as level of significance in all tests.

RESULTS

MO₂ and lactate concentration during steady-state swimming

Swimming speed, metabolic rate, and lactate concentration during the steady-state exercise experiments are presented in Fig. 3.1. The first 60 min were monitored at rest to quantify baseline lactate kinetics. The transition from rest to steady-state swimming was made progressively over 30 min before maintaining 1.7 BL s^{-1} constant for 90 min (Fig. 3.1A). Metabolic rate increased from resting levels of $\sim 80 \mu\text{mol O}_2 \text{ kg}^{-1} \text{ min}^{-1}$ to a maximum of $126.8 \pm 10.6 \mu\text{mol O}_2 \text{ kg}^{-1} \text{ min}^{-1}$ after 40 min of exercise. MO_2 was maintained above resting values between 40 and 80 min ($P < 0.05$) before declining to $99.9 \mu\text{mol O}_2 \text{ kg}^{-1} \text{ min}^{-1}$ over the last 30 min (Fig 3.1B). Lactate concentration increased from a resting value of $0.8 \pm 0.1 \text{ mM}$ to $\sim 1.4 \text{ mM}$ during the first 30 min of exercise, and stayed at that level until the end of the experiment ($P < 0.05$; Fig. 3.1C).

MO₂ and lactate concentration during graded swimming

Metabolic rate and lactate concentration during graded exercise experiments are presented in Fig. 3.3. MO₂ increased progressively with swimming speed and was higher than resting metabolic rate at all speeds ($p < 0.001$; Fig 3.3A). Lactate concentration increased from a baseline value of 1.3 mM to a maximum of 5.1 mM with exercise intensity ($P < 0.001$; Fig. 3.3B). Mean blood lactate concentrations for speeds above 2.0 BL/s were higher than baseline ($P < 0.05$).

Effects of exercise on lactate flux

Changes in blood lactate specific activity and in lactate fluxes during steady swimming at 1.7 BL/s are shown in Fig. 3.2. Blood lactate specific activity showed a significant decrease at the end of the 30 min transition phase and returned to near baseline levels after 90 min ($P < 0.05$; Fig. 3.2A). Both, R_a and R_d lactate increased over time ($P < 0.001$) from baseline values of $22.4 \pm 4.0 \mu\text{mol kg}^{-1} \text{min}^{-1}$ (R_a) and 23.7 ± 4.5 (R_d) to maximal levels of 30.9 ± 5.09 (R_a) and 29.8 ± 5.3 (R_d) (Fig. 3.2B). Mean R_a and R_d lactate were higher than baseline between 30 and 50 min of steady-state swimming ($P < 0.05$), but returned to resting values for the last 60 min of exercise ($P > 0.05$).

Changes in lactate specific activity and kinetics during graded exercise are presented in Fig. 3.4. Swimming speed was progressively increased over 4 h following a stepwise increment. There was no statistically significant decrease in lactate specific activity during the increase in exercise intensity ($P > 0.05$; Fig 3.4A), however both R_a lactate ($P < 0.001$) and R_d lactate ($P < 0.01$) were strongly stimulated at higher exercise intensities (Fig. 3.4B). Mean R_a lactate values above 2.4 BL s⁻¹ were higher than baseline ($P < 0.05$). R_a lactate increased

from a baseline level of $24.2 \pm 2.9 \mu\text{mol kg}^{-1} \text{min}^{-1}$ (N=7) to a maximum of $40.4 \pm 0.7 \mu\text{mol kg}^{-1} \text{min}^{-1}$ (N=2). R_d lactate increased from $24.6 \pm 2.9 \mu\text{mol kg}^{-1} \text{min}^{-1}$ (N=7) to a maximum of $34.7 \pm 3.3 \mu\text{mol kg}^{-1} \text{min}^{-1}$ (N=2).

DISCUSSION

This study is the first to characterize the relationship between exercise intensity and lactate kinetics in an ectotherm (Table 3.1). It shows that the lactate fluxes of rainbow trout are stimulated at speeds greater than 2.4 BL s^{-1} when lactate production starts diverging from lactate disposal. At these high exercise intensities, the change in R_a lactate stops being matched by the increase in R_d lactate, leading to a significant accumulation of glycolytic end product in the circulation. By contrast, steady-state submaximal exercise causes R_a and R_d lactate to increase similarly from ~ 20 to $\sim 30 \mu\text{mol kg}^{-1} \text{min}^{-1}$, with a trivial mismatch between production and disposal that only affects blood concentration minimally (from 0.7 to 1.4 mM). Earlier measurements by bolus injection had underestimated true lactate fluxes, but the same relative effect of low-intensity swimming was observed (Weber, 1991).

Lactate flux during steady-state swimming

The rapid change in swimming speed from 0 to 1.7 BL s^{-1} (within 30 min) increased the metabolic rate of the fish from $79.1 \mu\text{mol O}_2 \text{ kg}^{-1} \text{min}^{-1}$ to $126.8 \mu\text{mol O}_2 \text{ kg}^{-1} \text{min}^{-1}$ (Fig. 3.1B). This led to activation of anaerobic glycolysis to supply ATP rapidly to working muscles, resulting a transient increase in lactate production (+38%) from $22.4 \pm 4.0 \mu\text{mol kg}^{-1} \text{min}^{-1}$ to $30.9 \pm 5.0 \mu\text{mol kg}^{-1} \text{min}^{-1}$ (Fig. 3.2). Lactate disposal was also transiently stimulated (+25%) from a baseline value of 23.7 ± 4.5 to maximal levels of 29.8 ± 5.3

and returned to resting level (Fig. 3.2B). The trivial mismatch between R_a and R_d lactate only slightly increased blood concentration to 1.4 mM (Fig. 3.1C). Interestingly, while lactate concentration remained constant (~ 1.4 mM) for the 90 min steady swimming, R_a and R_d always stayed equal while undergoing a parallel decrease to baseline level. This unexpected flux change could not have been determined by only measuring lactate concentration (Omlin and Weber, 2010). It is unclear why lactate flux returned to baseline during steady-state swimming, however the concomitant decrease in metabolic rate indicates that the fish settled in their swimming speed overtime, exercising more efficiently to reduce energy expenditure.

Lactate production during graded swimming

Below 2.4 BL s^{-1} , swimming has no effect on R_a and R_d lactate of rainbow trout (Fig. 3.4). At higher speeds, glycolysis is sharply stimulated, causing an increase in lactate production from 24 to $40 \mu\text{mol kg}^{-1} \text{ min}^{-1}$ (Fig. 3.4B). This 67% rise in R_a lactate was measured at the highest speed allowing metabolite flux measurements in a swimming fish. Trout may be able to upregulate R_a lactate more strongly than reported here because previous studies suggest several-fold changes for flounder, salmon and catfish between rest and recovery from exhausting exercise (Cameron and Cech Jr., 1990; Milligan and McDonald, 1988). However, these fluxes measured post-exercise were estimated by bolus injection and may need to be confirmed with more reliable methods.

The stimulation of lactate flux is stronger in mammals than in trout because submaximal exercise induces a 6-fold increase in dogs (Issekutz et al., 1976), thoroughbred horses (Weber et al., 1987a), and humans (Bergman et al., 1999). Moreover, humans can

increase lactate production by 22-fold over resting values during a graded exercise protocol similar to what was used here for fish (Stanley et al., 1985). Trout may only be able to show a modest relative increase in flux because their metabolic scope is much smaller than in mammals (Brett, 1972). A greater stimulation of lactate fluxes may not be possible for trout because their baseline levels could already be quite high. This notion is supported by the fact that the R_a lactate/ MO_2 ratios of trout and humans are similar during intense exercise (8.9 for trout vs. 6.4 for humans), but much higher in resting trout (19.5) than in resting humans (only 2.9)(Stanley et al., 1985; this study).

Intense exercise stimulates lactate disposal

Above 2.4 BL s^{-1} , R_d lactate increases by 41% (Fig. 3.4B). Without this response, circulating lactate would reach twice the concentration actually observed at the end of exercise. Therefore, increasing the rate of lactate disposal during intense swimming plays an important role in reducing the lactate load on the circulation, a metabolic strategy previously noticed during exposure to hypoxia (Fig. 2.6, chapter 2). Such a response is rather surprising at a time when energy metabolism depends significantly on anaerobic glycolysis. As the only two pathways available for lactate clearance, how could *gluconeogenesis* and/or *oxidation* contribute to the increase in R_d lactate? The effects of swimming on gluconeogenesis have never been measured directly in fish, but several tracer studies suggest that this pathway is not stimulated by exercise (Moyes and West, 1995). Hepatic glucose production actually decreases during submaximal swimming, but it is unclear whether gluconeogenesis or glycogenolysis are responsible for this decline (Shanghavi and Weber, 1999). Intuitively, stimulating gluconeogenesis during swimming would seem undesirable

because glucose synthesis is energetically costly (6 ATP/glucose (Clark et al., 1974)). Overall, current information suggests that the stimulation of lactate disposal reported here is not accounted for by gluconeogenesis, but by an increase in lactate oxidation. Highly aerobic tissues like heart, red muscle, kidney and brain can readily use lactate as an oxidative fuel (Bilinski and Jonas, 1972; Soengas and Aldegunde, 2002) and they are probably responsible for increasing lactate clearance during exercise. For example, perfused trout heart experiments show that lactate oxidation is stimulated when cardiac work or lactate availability become elevated (Lanctin et al., 1980; Milligan and Farrell, 1991). In addition, important physiological roles for various lactate shuttles have been demonstrated in mammals (Brooks, 1998; Gladden, 2004). Presumably, exercising fish also rely on lactate shuttles to transport the end product from white muscle to aerobic tissues for oxidation. In trout, however, inter-tissue lactate shuttles may be constrained by the minimal expression of monocarboxylate transporters in white muscle (Omlin and Weber, 2013). The spatial separation of fish white and red muscles also precludes the intramuscular lactate shuttle between adjacent glycolytic and oxidative fibers well characterized within mixed mammalian muscles (Brooks, 1998; Van Hall, 2000) .

Previous experiments by bolus injection underestimated lactate fluxes

Continuous tracer infusion is the preferred method to quantify *in vivo* metabolite fluxes accurately in humans and animals (Wolfe, 1992). Its application to fish shows that the lactate fluxes of rainbow trout are ~3 times higher than previously estimated by bolus injection (Weber, 1991). This is because the bolus injection method relies on problematic estimates of surface areas to calculate flux (flux = dose injected / surface area under the

specific activity decay curve). Overestimation of surface area under the decay curve can happen for a number of reasons: (1) curve fitting for early sampling points is extremely inaccurate because specific activity changes very rapidly just after the injection of the bolus, (2) ^{14}C recycling can artificially increase specific activities in the right hand side of the decay curve (later sampling times), and (3) a single catheter is used for bolus injection of the tracer and subsequent blood sampling; therefore, residual bolus activity on the catheter walls can increase specific activity in sampled blood by contamination. Finally, the bolus injection technique assumes steady state conditions, and each experiment only yields a single measurement of flux: two important restrictions that do not apply to continuous tracer infusion. For all these reasons, bolus injection has been virtually abandoned as a practical tool to quantify metabolite kinetics.

CONCLUSION

This study is the first to show how the lactate kinetics of an ectotherm change with exercise intensity and it quantifies the rates of lactate production and disposal in rainbow trout: from rest to U_{crit} . This athletic species maintains high baseline lactate fluxes of $24 \mu\text{mol kg}^{-1} \text{min}^{-1}$ that are only increased at speeds greater than 2.4 BL s^{-1} or $\sim 85\% U_{\text{crit}}$. When the fish accelerates to reach U_{crit} , R_a lactate is more strongly stimulated than R_d lactate (+67% vs. +41%) and this mismatch causes a 4-fold increase in blood lactate concentration. Without this stimulation of R_d , the accumulation of end product would double and impose an extra load on the circulation. Increased lactate oxidation by aerobic tissues (red muscle, heart, kidney and brain) is probably responsible for the higher R_d lactate observed during intense swimming.

Figure 3.1. Changes in metabolic rate (panel A) and blood lactate concentration (panel B) in rainbow trout during steady-state swimming at 1.7 BL s⁻¹. Panel A shows the change in speed from rest, transition (grey area) to reach the steady speed of 1.7 BL s⁻¹. Values are means ± s.e.m. (N=6). * indicates differences from baseline at time 0 (P<0.05).

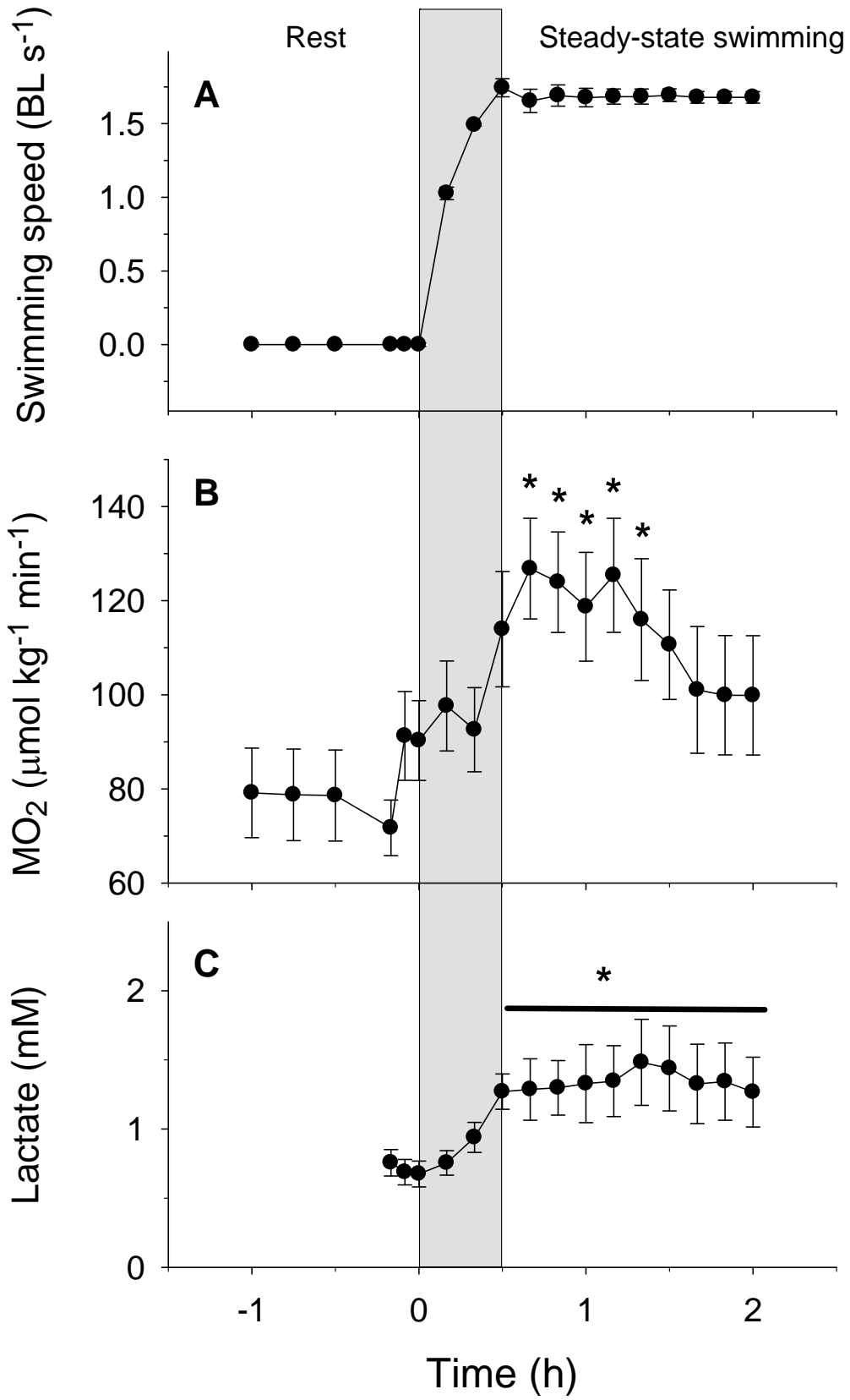


Figure 3.2. Effects of steady-state exercise on lactate metabolism in rainbow trout. Changes in lactate specific activity (panel A) and lactate fluxes (panel B) are indicated. Panel B shows the rates of lactate appearance (R_a lactate; filled circles) and disposal (R_d lactate; empty circles). Exercise was started at time 0 and tracer infusion at -60 min. Values are means \pm s.e.m. (N=6) * indicates differences from first value ($P<0.05$).

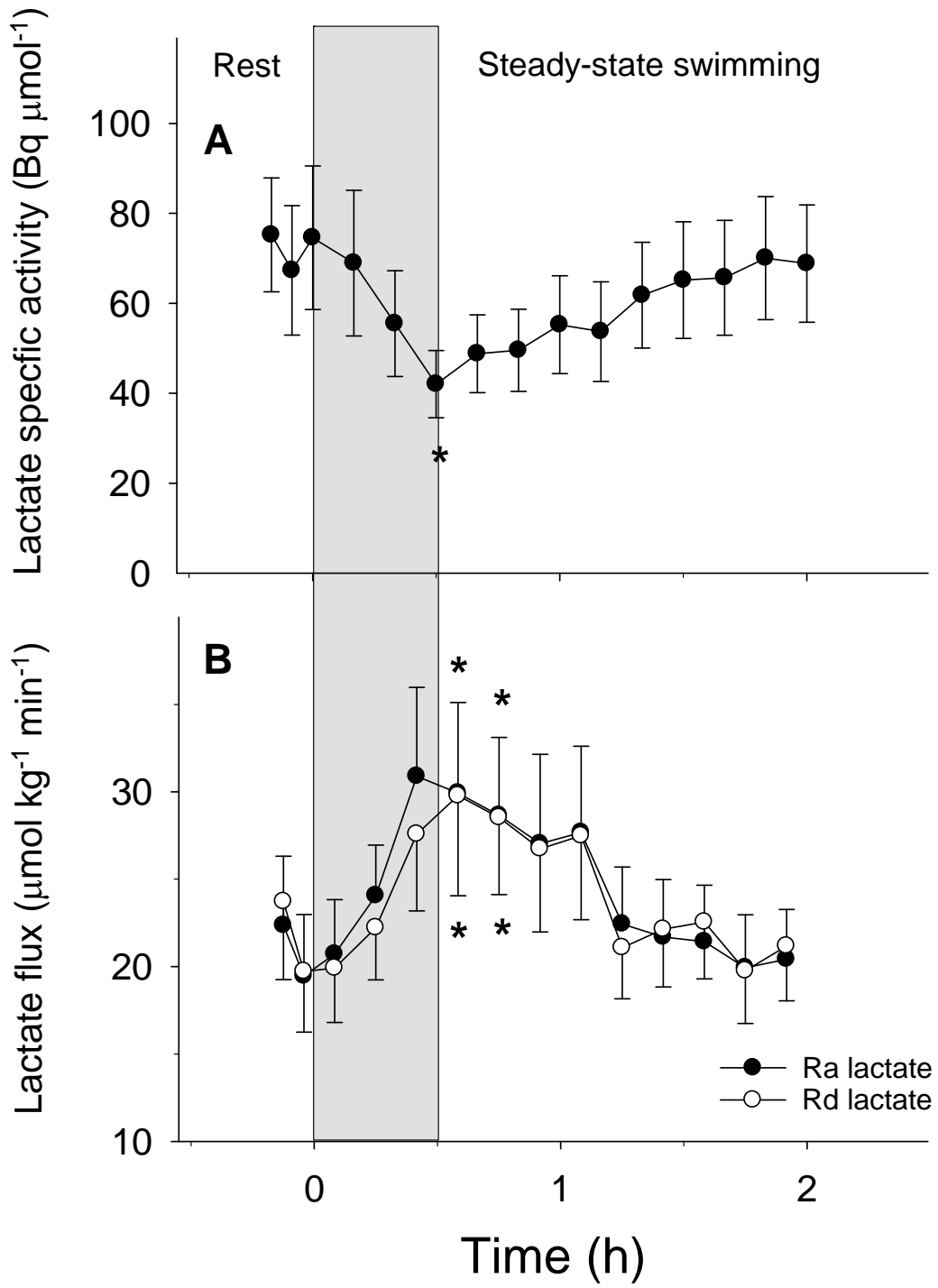


Figure 3.3. Changes in metabolic rate (panel A), and blood lactate concentration (panel B) in rainbow trout during graded swimming (from 0.8 to 2.8 BL s⁻¹. Values are means ± s.e.m. (N=7 for MO₂ and lactate concentration; N=8 for lactate S.A.). * indicates differences from baseline at time 0 (P<0.05).

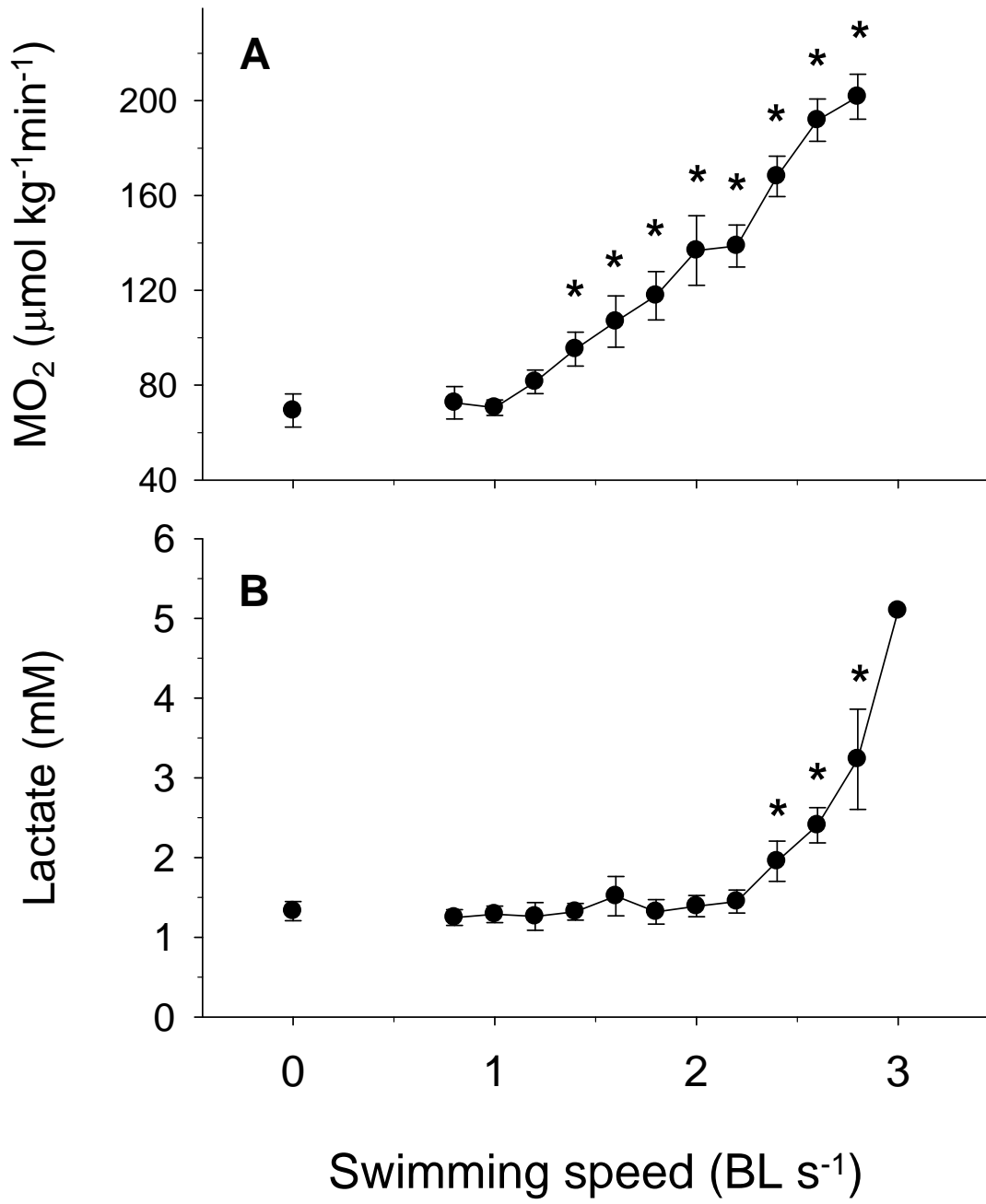


Figure 3.4. Effects of graded exercise on lactate specific activity (A) and lactate fluxes (B).

The rates of lactate appearance (R_a lactate; filled circles) and disposal (R_d lactate; empty circles) are indicated. Exercise was started at time 0 and tracer infusion at -60 min. Values are means \pm s.e.m. (for lactate fluxes, N=7 before 180 min, and N=4 and 2 for the last 2 points). * indicates differences from first value ($P < 0.05$).

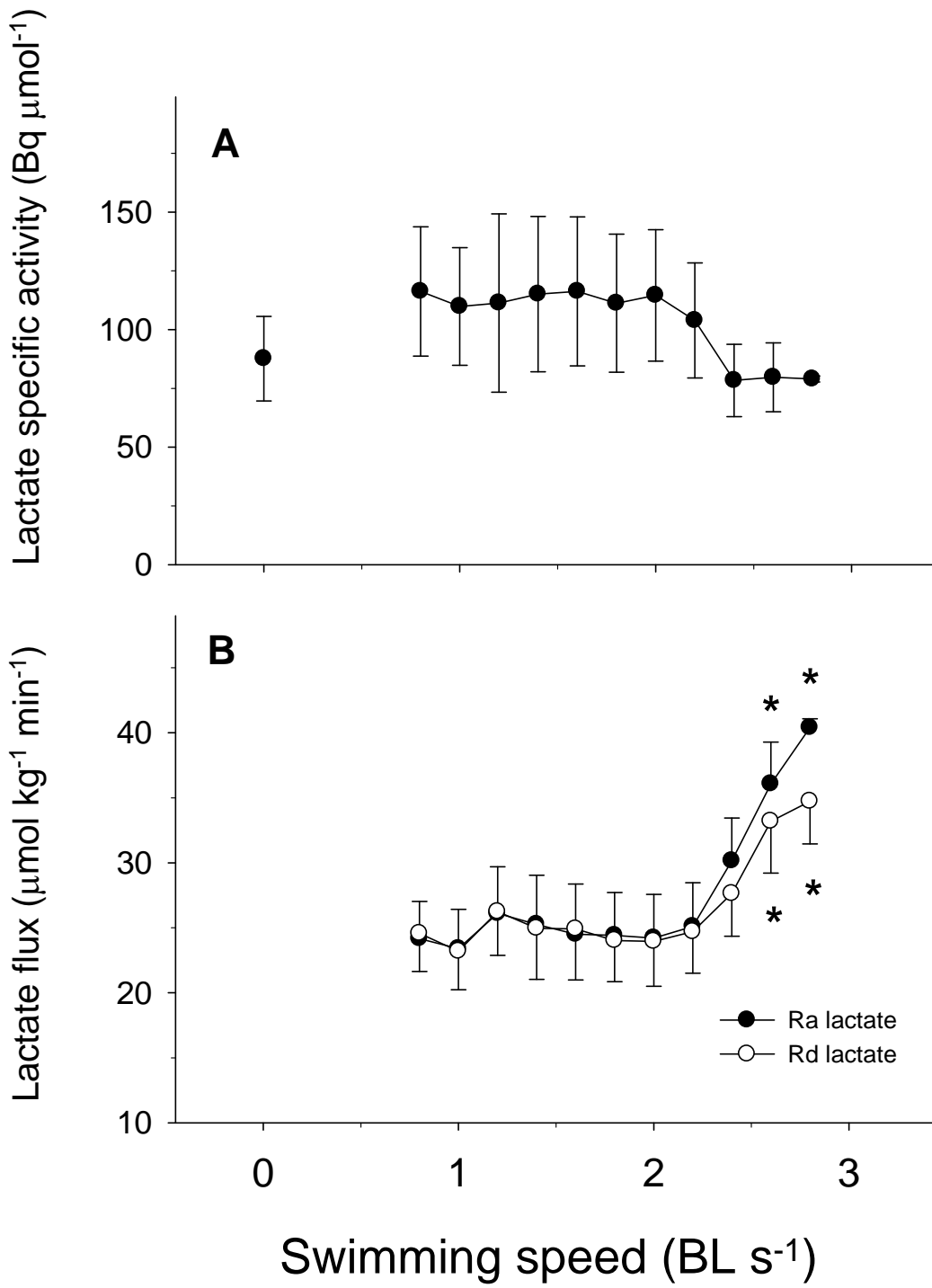


Table 3.1. Blood lactate concentration, fish metabolic rate, lactate specific activity, and lactate flux of exercising fish (steady-state and graded swimming). Table indicates control and highest observed values except for the specific activity were control and the lowest values are indicated.

	Steady-state swimming		Graded swimming	
	Control value	Highest value	Control value	Highest value
Lactate (mM)	0.8 ± 0.1 (7)	1.48 ± 0.3 (7)	1.3 ± 0.1 (7)	5.3 ± 1.0 (7)
MO ₂ (μmol kg ⁻¹ min ⁻¹)	79.1 ± 9.5 (7)	126.82 ± 10.6 (7)	69.3 ± 6.9 (8)	201.6 ± 6.5 (8)
Lactate S.A. (Bq μmol ⁻¹)	75.2 ± 12.6 (7)	42.02 ± 7.4 (7)**	87.6 ± 17.9 (7)	49.7 ± 7.8 (7)**
R _a lactate (μmol kg ⁻¹ min ⁻¹)	22.3 ± 3.96 (7)	30.8 ± 5.0 (7)	24.1 ± 2.8 (7)	40.4 ± 1 (2)
R _d lactate (μmol kg ⁻¹ min ⁻¹)	23.7 ± 4.4 (7)	29.7 ± 5.5 (7)	24.5 ± 2.6 (7)	34.7 ± 3.2 (2)

Values are means ± s.e.m. with sample size in parentheses. S.A., specific activity; R_a, rate of appearance; R_d, rate of disposal. ** indicate the lowest value measured for the specific activity.

**CHAPTER 4: Supplemental lactate does not affect
swimming performance or endogenous lactate
kinetics in rainbow trout.**

Partially based on:

Teye Omlin and Jean-Michel Weber

Manuscript in preparation

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

INTRODUCTION

The flux of circulating lactate is high, even under resting normoxic conditions, and further stimulated as exercise intensity increases (Donovan and Brooks, 1983; Stanley et al., 1985). In rainbow trout, graded exercise stimulated the rates of lactate appearance (R_a) and disappearance (R_d) by 67% and 41%, respectively, and the mismatch between both rates was apparent at a swimming speeds beyond 2.4 body lengths per second (BLs^{-1}) (Chapter 3). A limited capacity for lactate uptake by tissues appears be a probable reason for the lower stimulation of R_d that resulted in 4-fold increase in blood lactate concentration (to 5.1 mM; Chapter 3).

Previous studies on mammalian lactate kinetics have reported enhanced capacity for lactate uptake by tissues when extra lactate is administered (Drury and Wick, 1956; Ferrannini et al., 1993; Haesler et al., 1995; Miller et al., 2002a; Miller et al., 2002b; Searle et al., 1989). Administration of exogenous lactate in exercising humans stimulated oxidation, gluconeogenesis, and the basal metabolic rate of the experimental subjects. Surprisingly, the supplemental lactate did not improve exercise performance (Drury and Wick, 1956; Ferrannini et al., 1993; Haesler et al., 1995; Miller et al., 2002a; Miller et al., 2002b; Searle et al., 1989). Furthermore, two of those studies reported contradicting results for the endogenous lactate production of the experimental subjects: Searle and collaborators demonstrated that infusion of exogenous lactate in humans caused 12-100% inhibition of endogenous lactate production (Searle et al., 1989), whereas, Jenssen and his group found that exogenous lactate infusion had no effect on endogenous lactate production (Jenssen et al., 1993).

Consequently, a number of questions arise regarding the tissue lactate uptake capacity of rainbow trout: Could this capacity be boosted by infusing exogenous lactate during a bout of graded exercise as reported in mammals? Could the extra lactate serve as fuel to enhance swimming performance? Or, would it accumulate in the tissues as unwanted anaerobic end-product and therefore decrease performance? If the major fate of lactate is to fuel the oxidative metabolism, would production of endogenous lactate be depressed or even shut down when supplemental lactate is administered? Would the extra lactate serve as gluconeogenic precursor to replenish glucose and glycogen stores? The goals of this study were, therefore: (i) to determine whether R_d lactate could be stimulated beyond values previously observed (in Chapter 3) by infusing exogenous lactate in rainbow trout during graded exercise; (ii) to determine whether the supplemental lactate could help improve the critical swimming speed (U_{crit}) of the fish; and (iii) to determine the effects exogenous lactate infusion on endogenous lactate production. We anticipate that lactate disposal and gluconeogenesis would be strongly stimulated. Furthermore, the extra lactate would be used as energy to improve U_{crit} , and that endogenous lactate production would be depressed at rest but stimulated during intense exercise.

MATERIALS AND METHODS

Animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum) of both sexes (380 ± 14 g; N=29) were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and held in a 1,300-liter flow-through tank in dechlorinated, well-oxygenated water at 13°C under a 12:12 h light-dark photoperiod. The animals were acclimated to these conditions for at least

2 weeks before experiments. They were fed floating fish pellets (Martin Mills, Elmira, Ontario, Canada) 3 times a week to satiation. They were divided in 2 subgroups to measure the effects of exogenous lactate administration on swimming performance (MO_2 , U_{crit} , $N=17$) or on lactate kinetics at rest and during graded exercise ($N=12$). All procedures were approved by the Animal Care Committee of the University of Ottawa and adhered to the guidelines established by the Canadian Council on Animal Care.

Catheterization

Before surgery, the fish were fasted for 24 h and anesthetized with ethyl-N-aminobenzoate sulfonic acid (MS-222; 60 mg L^{-1}) in well oxygenated water. They were cannulated in the dorsal aorta using BPE-T50 catheters (Instech Laboratories Inc, Plymouth Meeting, PA, USA) following the procedure of Haman and Weber (Haman and Weber, 1996). Animals used to measure swimming performance were fitted with a single catheter for the administration of exogenous saline (controls) or exogenous lactate. The fish used for lactate kinetics were fitted with 2 catheters: one to infuse lactate (labeled tracer and exogenous, unlabelled lactate), the other for blood sampling. Only animals with a hematocrit $>20\%$ after recovery from surgery were used in experiments. The catheters were kept patent by flushing with Cortland saline (Wolf, 1963) containing 50 U ml^{-1} heparin. They were made accessible through water-tight ports in the respirometer lids.

Respirometry, exogenous lactate infusions, and U_{crit} protocol

After surgery, each animal was allowed to recover overnight in a 13.6 liter, cylindrical respirometer (resting experiments), or in a 90 liter swim tunnel respirometer (swimming experiments) from Loligo Systems (Tjele, Denmark). Respirometers were supplied with the same quality water as the holding tank. Metabolic rate (MO_2) was measured by intermittent flow respirometry as previously (chapter 3). The effects of exogenous lactate were assessed by comparing control animals receiving saline infusions with test animals receiving lactate infusions administered with a calibrated syringe pump (Harvard Apparatus, South Natick, MA, USA; 1 ml h^{-1}). Exogenous Na-lactate was infused at a rate of 30 $\mu\text{mol kg}^{-1} \text{min}^{-1}$ or twice the baseline rate of endogenous lactate production measured previously (chapter 2). Control fish were infused with Cortland saline containing matching amounts of sodium. For all swimming experiments, the fish were subjected to a stepwise U_{crit} protocol (Jain et al., 1997) as detailed in chapter 3.

Lactate kinetics

Lactate kinetics were measured for 4-5 h at rest or during graded swimming (U_{crit} test). The rates of lactate appearance (R_a) and lactate disposal (R_d) were measured by continuous infusion of [U - ^{14}C] lactate (New England Nuclear, Boston, MA, USA; 4.84 GBq mmol^{-1}) as previously (chapter 2). The infusates containing labeled lactate were freshly prepared before each experiment and administered at a rate of $1635 \pm 90 \text{ Bq kg}^{-1} \text{min}^{-1}$ ($N=12$) using a calibrated syringe pump (Harvard Apparatus, South Natick, MA, USA; at 1 ml h^{-1}). Blood samples (100 μl each) were drawn 50, 55, and 60 min after the start of infusion to ensure that isotopic steady-state had been reached. Additional samples were taken every 15 min (resting) or 20 min (exercise) until the end of the experiments. The blood sampled from each fish accounted for <10% of total blood volume.

Samples were immediately deproteinized in 200 µl perchloric acid (6% w/w) and centrifuged for 5 min at 16,000 G (Eppendorf 5415C, Brinkmann, Rexdale, Canada). Supernatants were kept frozen at -20°C until analyses.

Sample analyses

Blood lactate and glucose concentrations were measured spectrophotometrically (Bergmeyer, 1985) using a Spectra Max plus 384 (Molecular Devices, Sunnyvale, CA, USA). Lactate and glucose were separated using ion exchange columns as previously (chapter 2). Before passing through the columns, each deproteinized blood sample was neutralized with 1 M potassium bicarbonate. Radioactivity was measured by scintillation counting (PerkinElmer Tricarb 2910TR, Waltham, MA, USA) in Bio-Safe II scintillation fluid (RPI Corp, Mount Prospect, IL, USA), and measured activities of lactate and glucose were corrected for recovery from the ion-exchange column separation.

Calculations and statistics

Critical swimming speed (U_{crit}) (Brett, 1964) was calculated as follows:

$$U_{crit} (\text{BL s}^{-1}) = V_f + [(t_f/t_i) \times V_i]$$

Where U_{crit} , V_f = highest speed at which a full time interval was completed (BL s^{-1}); V_i = speed increment between intervals (0.2 BL s^{-1}); T_f = time spent swimming during the last interval causing exhaustion (**min**); T_i was the full interval (**20 min**). The rates of lactate appearance (total R_a = endogenous R_a + exogenous R_a) and disposal (R_d) were calculated using the non-steady state equations of Steele (Steele, 1959). Endogenous lactate appearance (fish R_a) was determined by subtracting the infusion rate of exogenous lactate from total R_a

lactate. Statistical comparisons were performed using one- or two-way repeated measure analysis of variance (RM-ANOVA) with the Bonferroni *post hoc* test to determine which means were different from controls. In cases where the assumptions of normality or homoscedasticity were not met, the data was normalized by \log_{10} transformation before parametric analysis. Friedman repeated measures ANOVA on ranks was used with Dunn's test when normality or homoscedasticity were not met after transformation. All values presented are means \pm SE and a level of significance of $P < 0.05$ was used in all tests.

RESULTS

1. Exogenous lactate infusion

MO₂, lactate concentration and specific activity

The time course of changes in metabolic rate (MO_2), blood lactate concentration and lactate specific activity in resting fish is shown in Fig. 4.1. The MO_2 remained unchanged throughout the experiment, averaging $44 \mu\text{mol O}_2 \text{ kg}^{-1} \text{ min}^{-1}$ ($P=0.67$; Fig. 4.1A). Lactate concentration increased progressively from 1.5 to 13.4 mM ($P < 0.001$; Fig. 4.1B), whereas the lactate specific activity decreased from 69 to 42 Bq μmol^{-1} ($P < 0.05$; Fig. 4.1C) over the infusion period. Figure 4.4 shows the MO_2 , lactate concentration and specific activity in swimming fish. The MO_2 and lactate concentration increased progressively with speed (from 52 to $162 \mu\text{mol O}_2 \text{ kg}^{-1} \text{ min}^{-1}$ and from 1.2 to 14.7 mM, respectively) ($P < 0.001$; Fig. 4.4A and B). Blood lactate specific activity decreased from 69 to 42 Bq μmol^{-1} . Dunnett's *post-hoc* test only identified 3 of the last 4 points as different from baseline ($P < 0.05$; Fig. 4.4C).

Effects of exogenous lactate infusion on lactate fluxes

Changes in the rates of lactate appearance (total R_a and fish R_a) and disappearance (R_d) for resting and swimming fish are shown in Figs. 4.2 and 4.5. *Total R_a* lactate is the sum of the exogenous and endogenous R_a lactate, whereas *fish R_a* lactate is equal to endogenous R_a lactate only. In resting fish, total R_a lactate increased from a baseline value of 22.5 to 41.9 $\mu\text{mol kg}^{-1} \text{min}^{-1}$, and R_d lactate from 20.9 to 39.9 $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ($P < 0.001$; Fig. 4.2). In swimming fish, total R_a and R_d lactate were also significantly stimulated from 21.9 to 65.3 $\mu\text{mol kg}^{-1} \text{min}^{-1}$ and from 14.0 to 56.4 $\mu\text{mol kg}^{-1} \text{min}^{-1}$, respectively ($P < 0.001$; Fig. 4.5). Total R_a and R_d were more strongly stimulated in swimming fish than in the resting group ($P < 0.02$; Table 4.1). Endogenous lactate production (fish R_a) lactate was calculated as the difference between total R_a and the infusion rate of the exogenous lactate. The values for the first 3 time points of fish R_a lactate after the beginning of the exogenous lactate infusion were omitted. During this period the exogenous lactate infused did not completely mix with the endogenous lactate pool, therefore the fish R_a values calculated for these 3 time points were inaccurate. Fish R_a lactate was not affected at rest ($P = 0.17$; Fig. 4.4), remaining at baseline, but it was increased to 35 $\mu\text{mol kg}^{-1} \text{min}^{-1}$ in swimming fish ($P < 0.05$; Fig. 4.5).

Critical swimming speed (U_{crit})

The effect of exogenous lactate administration on critical swimming speed (U_{crit}) of fish receiving control saline solution or exogenous lactate is shown in Fig. 4.3. Administration of exogenous lactate had no inhibiting or stimulating effect on U_{crit} (2.8 vs. 2.9 BL s^{-1} , for the lactate group; Fig. 4.3).

Glucose metabolism

Changes in blood glucose concentration and specific activity are presented in Fig. 4.6. Both groups maintained baseline values of ~5 mM throughout the experiments ($P>0.05$; Figs. 4.6A and B). The specific activity of glucose increased slightly throughout the experiments in both groups ($P<0.001$; Fig. 4.6A and B), showing that some lactate was used as a precursor for glucose synthesis.

DISCUSSION

This study is the first to investigate the effects of exogenous lactate infusion on lactate kinetics (R_a and R_d lactate) and swimming performance (U_{crit}) in rainbow trout. It shows that rainbow trout tissues have a much higher capacity for lactate uptake (R_d lactate) than previously measured in fish without administration of exogenous lactate (see Chapter 3). In fish supplied with exogenous lactate, R_d lactate was stimulated by 300% during graded exercise. This study also shows that the infusion of exogenous lactate does not cause a reduction in endogenous lactate production (R_a lactate), either at rest or during exercise. Finally, increasing the availability of circulating lactate does not improve or impair swimming performance.

High capacity for lactate disposal in rainbow trout

The most striking result from this study is the high capacity for lactate disposal in rainbow trout. These fish stimulated R_d lactate by 300% (from 14 to 56 $\mu\text{mol kg}^{-1} \text{min}^{-1}$) during graded exercise when they were administered exogenous lactate (Fig. 4.5). This capacity for lactate uptake is even more remarkable when compared to that of fish subjected

to the same exercise regimen but without infusion of exogenous lactate (+300% vs. +41%; Fig. 4.7A) (Chapter 3). In non-lactate infused fish (Chapter 3), the maximum R_d lactate was only $35 \mu\text{mol kg}^{-1} \text{min}^{-1}$, whereas in lactate infused fish R_d lactate reached $56 \mu\text{mol kg}^{-1} \text{min}^{-1}$). The measurement of blood lactate concentration suggests that blood-to-tissue lactate gradient was the limiting factor in non-infused fish (5.1 mM vs. 14 mM). The increase circulating lactate by exogenous administration stimulated uptake by tissues most likely through increased monocarboxylate transporters (MCTs) isoforms 1 and 2 activities (Chapter 5).

Preferential lactate oxidation by heart, red muscle, and brain have been shown in fish and mammals (Bilinski and Jonas, 1972; Drake et al., 1980; Lanctin et al., 1980; Milligan and Farrell, 1991; Smith et al., 2003). This scenario is consistent with the stimulation of R_d lactate and MO_2 observed in rainbow trout (Fig. 4.4A). However, theoretical calculations of lactate oxidation demonstrate that at speeds between 1.6 to 2.8 BL s^{-1} , 15 to 88% of the MO_2 account R_d lactate. Therefore, the remaining lactate may be retained *in situ* by the tissues for eventual oxidization, given that gluconeogenesis plays a minimal role in lactate clearance (negligible incorporation of ^{14}C into glucose; Fig. 4.6). By contrast, when the fish did not receive extra lactate (Chapter 3), 80 to 57% of the MO_2 was required to oxidize all the lactate disposed of at the same speeds (Fig. 4.7B).

Supplemental lactate does not improve U_{crit} in rainbow trout

Infusion of lactate ($30 \mu\text{mol kg}^{-1} \text{min}^{-1}$) did not improve the swimming performance (U_{crit}) of rainbow trout (2.8 BL s^{-1} vs. 2.9 BL s^{-1} ; lactate vs. control groups; Fig. 4.3A). This observation comes as a surprise given the high lactate disposal rate in these animals (Fig.

4.5). Intuitively, utilization of the extra lactate to improve performance was expected, but it is not the case. Alternatively, the excess lactate did not impair swimming performance either. This contradicts the misconception that lactate is a waste product that causes muscle fatigue (Philp et al., 2005). Similarly, mammalian exercise studies show that infusion of exogenous lactate does not improved or impaired performance (Emhoff et al., 2013; Miller et al., 2002a; Miller et al., 2002b). It is unclear why supplemental lactate does not improve exercise performance in mammals and fish, future investigations would be needed to elucidate the underlying reasons.

Exogenous lactate infusion does not affect endogenous lactate production

Endogenous lactate production (fish R_a) was calculated for the infused fish at rest and during exercise. In the resting group, fish R_a lactate was maintained at baseline throughout the experiments (from 22.5 to 11.5 $\mu\text{mol kg}^{-1} \text{min}^{-1}$; $P > 0.05$; Fig. 4.2). In the exercise group, fish R_a lactate was gradually stimulated (from 21.9 to 35.3 $\mu\text{mol kg}^{-1} \text{min}^{-1}$; $P < 0.05$; Fig. 4.5). Our results suggest that baseline lactate production is not to make lactate available as an oxidative fuel, but probably for an obligatory production of ATP by cells that cannot produce ATP by aerobic means. For instance, in the astrocyte, lactate is constantly being produced by Na^+/K^+ -ATPase pumps through glycolysis (Gladden, 2004). Furthermore, it has been shown that teleosts control buoyancy by acidifying the blood close to the gas glands through secretion of lactic acid which caused release of O_2 by the haemoglobin by root effect into the swim bladder (Umezawa et al., 2012). Lactate has also been shown to be metabolic signal (pseudo-hormone), and even a stimulus for wound repair (Philp et al., 2005). Our results are in agreement with findings by (Jenssen et al., 1993)

which showed that exogenous lactate infusion (6 to 40 $\mu\text{mol kg}^{-1} \text{min}^{-1}$ for 3h) had no effects on endogenous lactate production in humans. However, our results are in contradiction with findings from (Searle et al., 1989) which show that, humans downregulate endogenous lactate when extra lactate is administered.

CONCLUSION

This study demonstrates that rainbow trout have a high capacity for blood lactate disposal. R_a lactate was stimulated by +300% when exogenous lactate was administered, as compared to non-infused fish (+41%; Chapter 3). Surprisingly, the exogenous lactate administered did not improve the swimming performance of these fish because U_{crit} of control (saline infused) and lactate-infused fish were virtually identical. Alternatively, the excess lactate did not impair their swimming performance, thus contradicting the notion that lactate is a waste product that causes muscle fatigue. Finally, we showed that endogenous lactate production cannot be downregulated or suppressed even when exogenous lactate is supplied. This demonstrate that the role of baseline lactate production is not to make lactate available as fuel, but as the consequence of obligatory ATP production by cells that only use glucose as fuel.

Figure 4.1: Metabolic rate (panel A), blood lactate concentration (panel B) and blood lactate specific activity (panel C) during continuous infusion of [U-¹⁴C] lactate and unlabeled lactate in rainbow trout at rest. The tracer infusion was started 1 h before time 0 when infusion of the exogenous lactate was initiated. The tracer and the exogenous lactate infusions were continued until the end of experiments. Values are means ± s.e.m (N=7). Differences from baseline (time 0) are indicated by * (P<0.05).

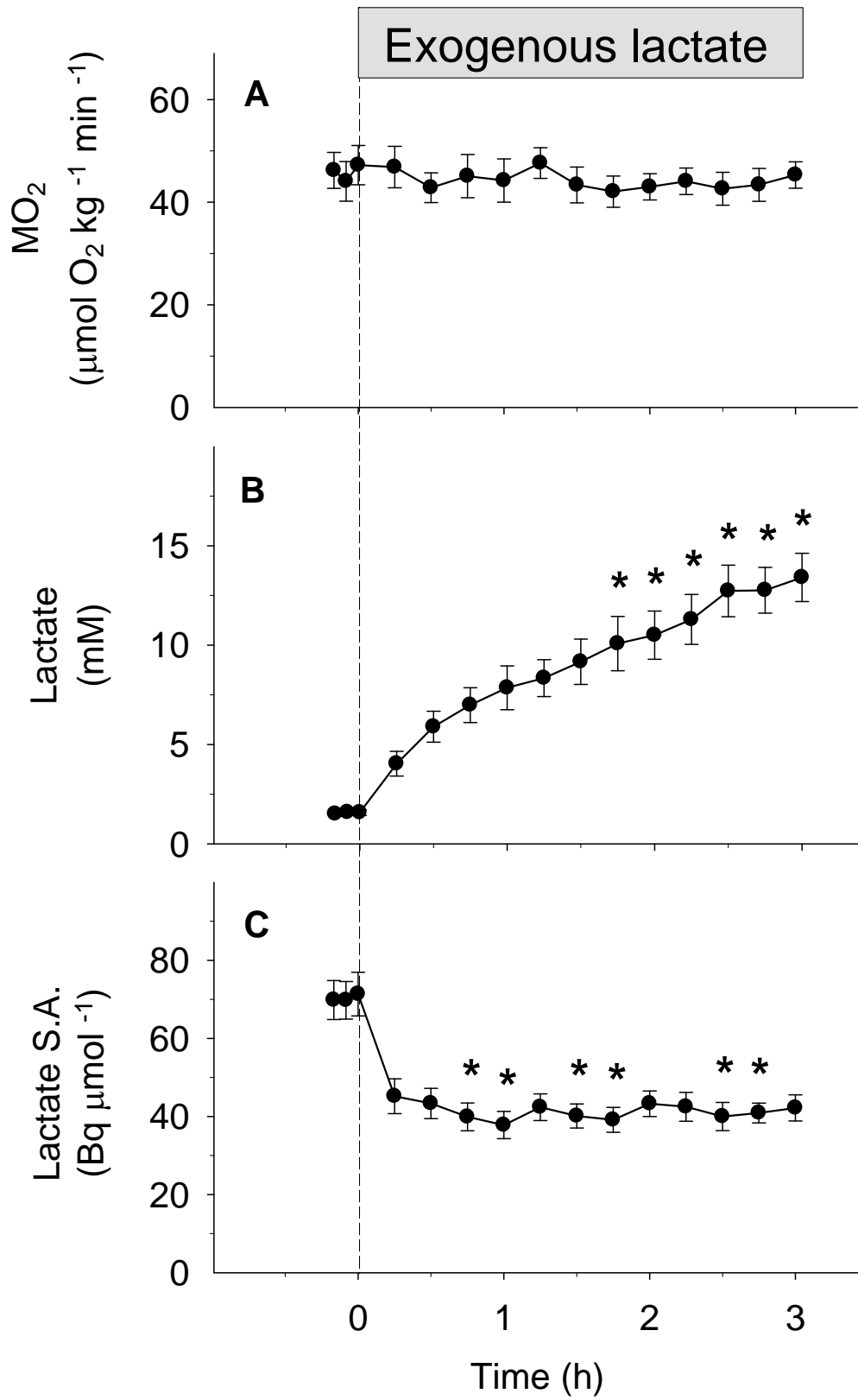


Figure 4.2: Lactate fluxes of resting rainbow trout during infusion of exogenous lactate at a rate of $30 \mu\text{mol kg}^{-1} \text{min}^{-1}$. Filled circles represent the total rate of appearance of lactate (Total R_a lactate), open circles represent the rate of lactate disposal (R_d lactate), and filled triangles represent the endogenous lactate production (fish R_a lactate). Tracer infusion was started 1 h before time 0 when infusion of the exogenous lactate was initiated. The tracer and the exogenous lactate infusions were maintained until the end of experiments. Values are means and s.e.m (N=7). Differences from baseline (time 0) are indicated by * (P<0.05).

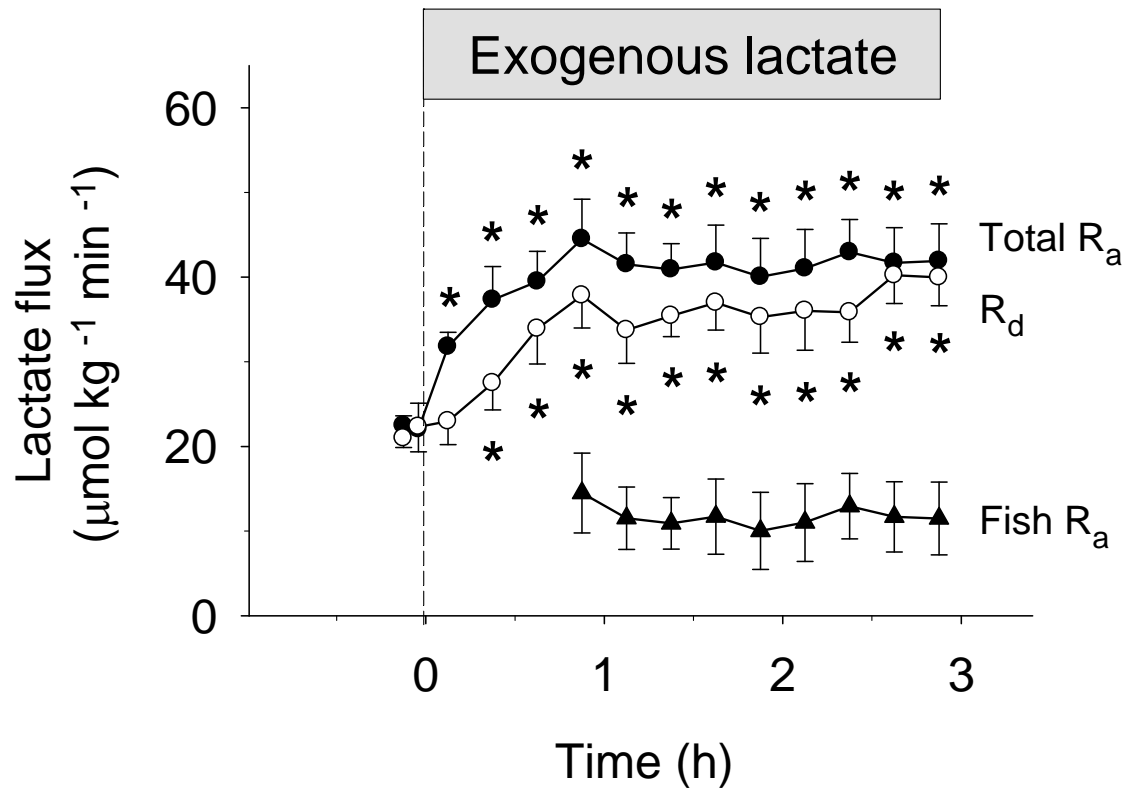


Figure 4.3: Critical swimming speed (U_{crit}) of rainbow trout infused with control saline (open circles) or exogenous lactate (filled circles). Panel A represents changes in MO_2 during the swimming experiments and panel B represents the U_{crit} values. Infusion of exogenous lactate was initiated at time 0 and maintained through the experiment. Values are means and s.e.m (N=8 for lactate, N=9 for controls).

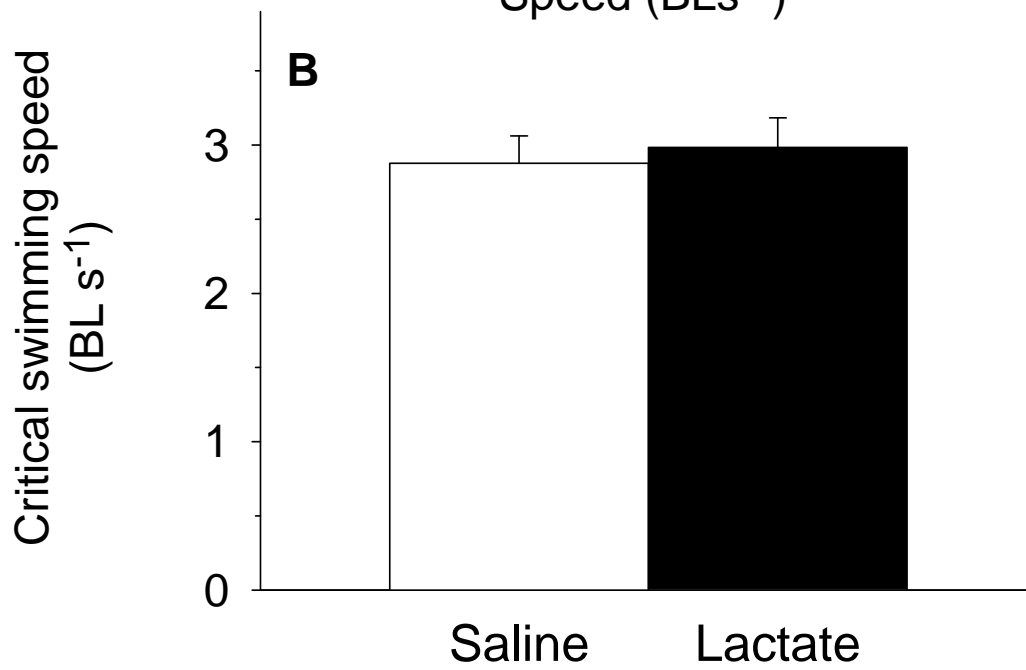
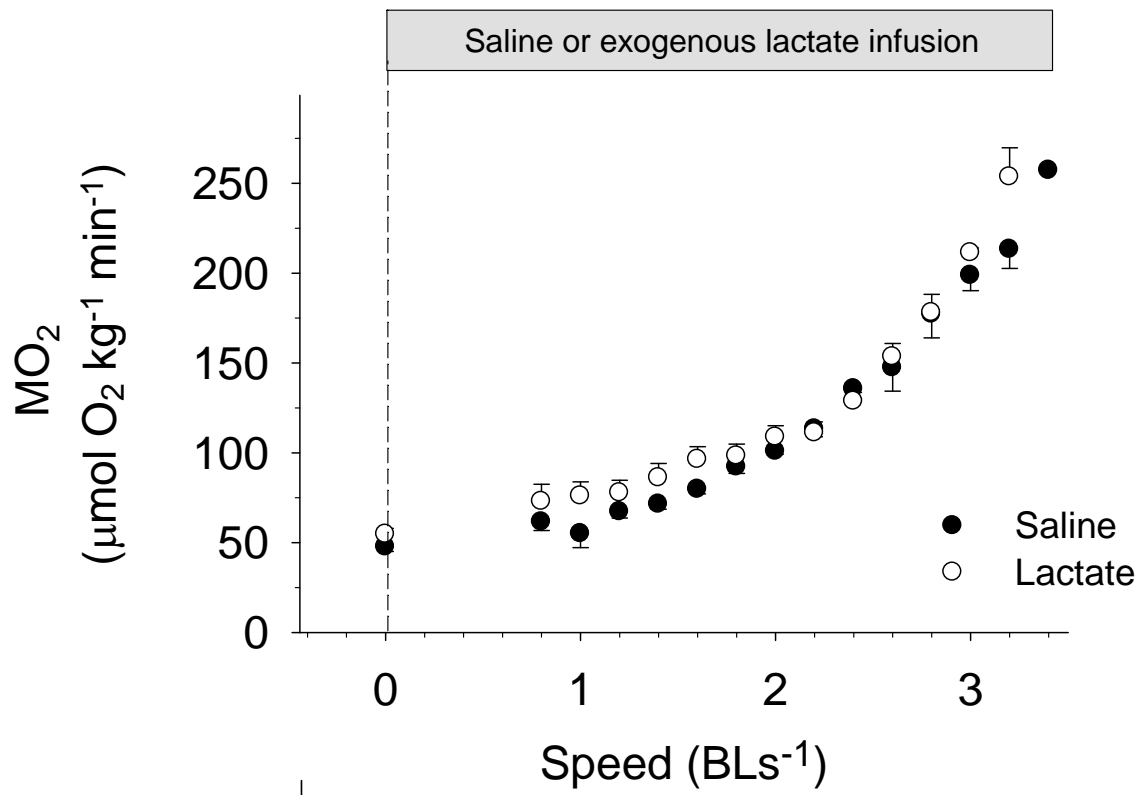


Figure 4.4: Metabolic rate (panel A), blood lactate concentration (panel B) and blood lactate specific activity (panel C) during continuous infusion of [U-¹⁴C] lactate and unlabeled lactate in rainbow trout during graded exercise. The tracer infusion was started 1 h before start of experiments (speed = 0 BLs⁻¹). Infusion of the exogenous lactate was initiated after the initial 1h, then the speed was increased by 0.2 BLs⁻¹ every 20 min. The tracer and the exogenous lactate infusions were maintained throughout the experiments. Values are means ± s.e.m (N=5). Differences from baseline (time 0) are indicated by * (P<0.05).

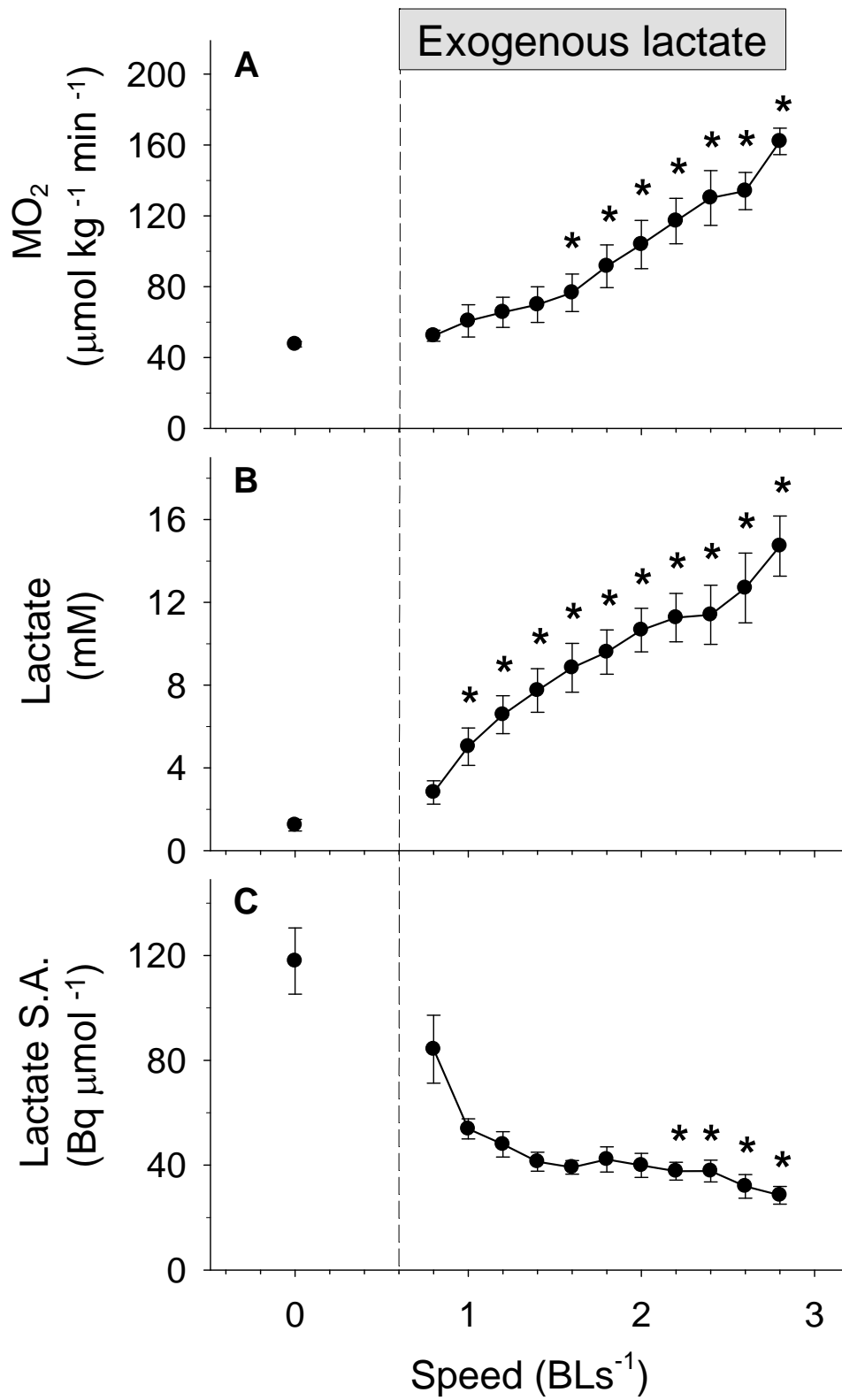


Figure 4.5: Lactate fluxes of exercising rainbow trout during infusion of exogenous lactate. Filled circles represent the total rate of appearance of lactate (Total R_a lactate), open circles represent the rate of lactate disposal (R_d lactate), and filled triangles represent endogenous lactate production (fish R_a lactate). The tracer infusion was started 1 h before start of experiments (speed = 0 BLs^{-1}). Infusion of the exogenous lactate was initiated after the initial 1h, then the speed increased by 0.2 BLs^{-1} every 20 min. The tracer and the exogenous lactate infusions were maintained through experiments. Values are means \pm s.e.m (N=5). Differences from baseline (time 0) are indicated by * ($P < 0.05$).

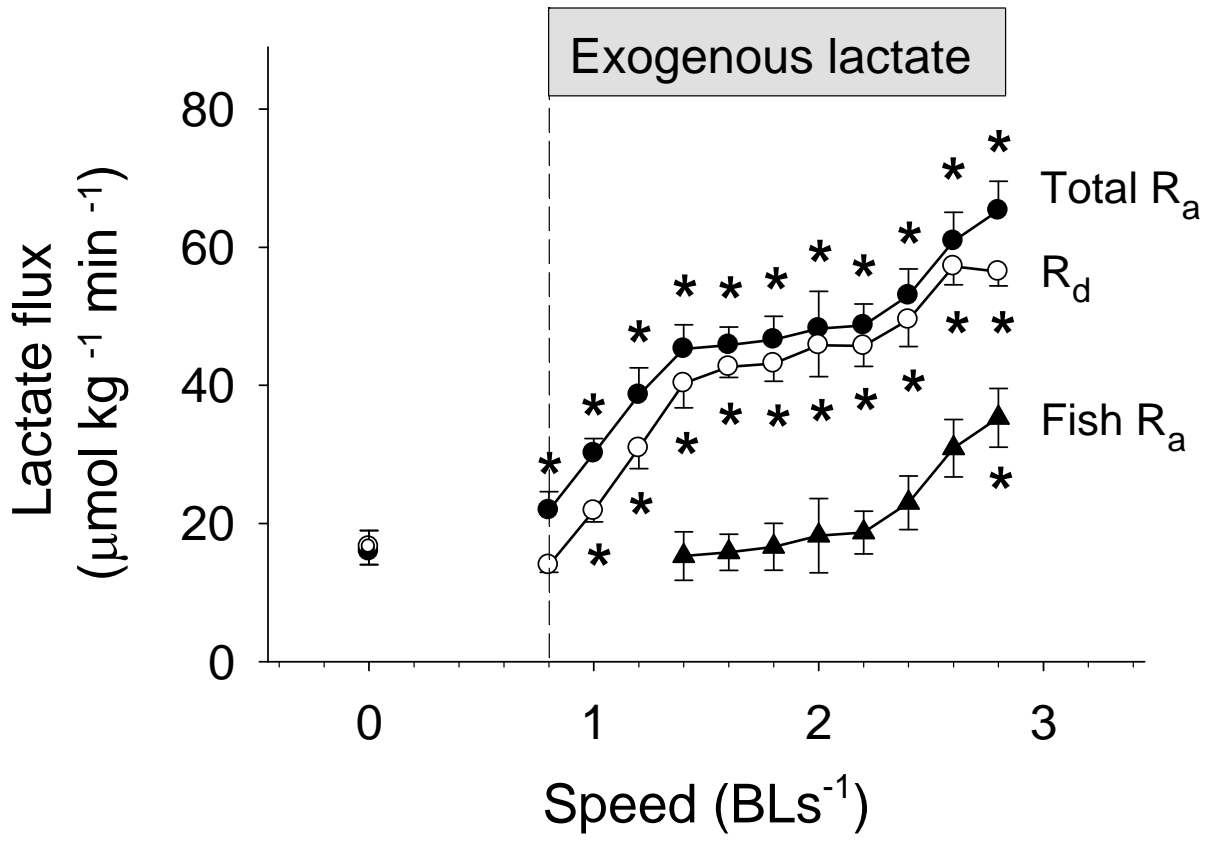


Figure 4.6: Blood glucose concentration and blood glucose specific activity in rainbow trout at rest (panel A) or during graded exercise (panel B). Filled circles represent glucose concentration and open circles represent glucose specific activities. For the resting experiments, tracer infusion was started 1 h before time 0, when infusion of the exogenous lactate was initiated. For the swimming fish, the tracer infusion was started 1 h before start of experiments (speed = 0 BLs⁻¹). Infusion of the exogenous lactate was initiated after the initial 1h, then the speed was increased by 0.2 BLs⁻¹ every 20 min. The tracer and the exogenous lactate infusions were maintained throughout experiments. Values are means ± s.e.m (N=7 for fish at rest and 5 for exercising fish). Differences from baseline (time 0) are indicated by * (P<0.05).

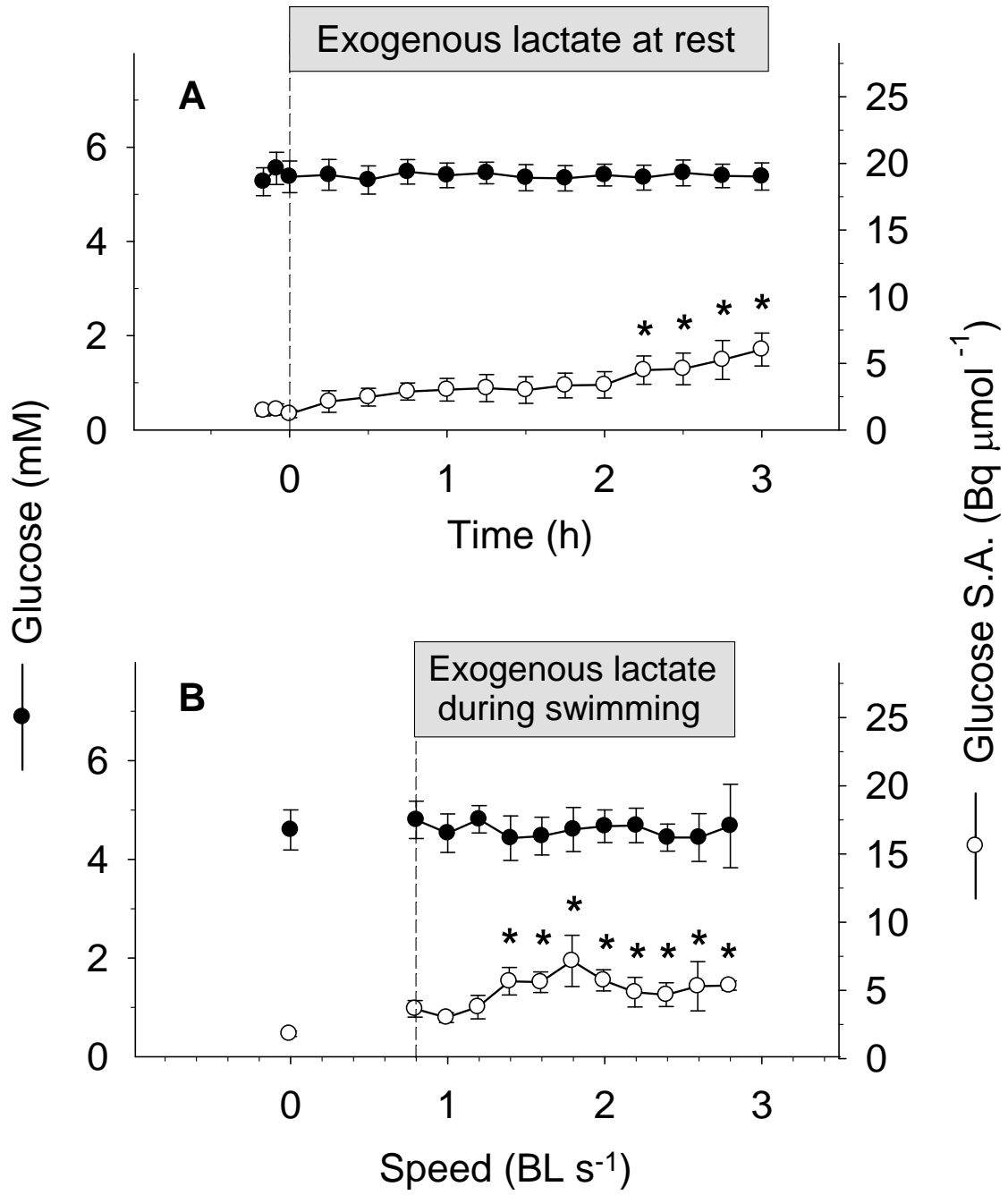


Figure 4.7: The rate of disappearance (R_d lactate) (panel A) and calculated percent MO_2 required to oxidize all the lactate metabolized (R_d lactate) (panel B) in exercising rainbow trout. Open circles represent fish receiving exogenous lactate and filled circles fish without exogenous lactate administration (Chapter 3). Values are means \pm s.e.m (N=5 for lactate and N=7 for controls). Differences from baseline (time 0) within each group are indicated by * (P<0.05).

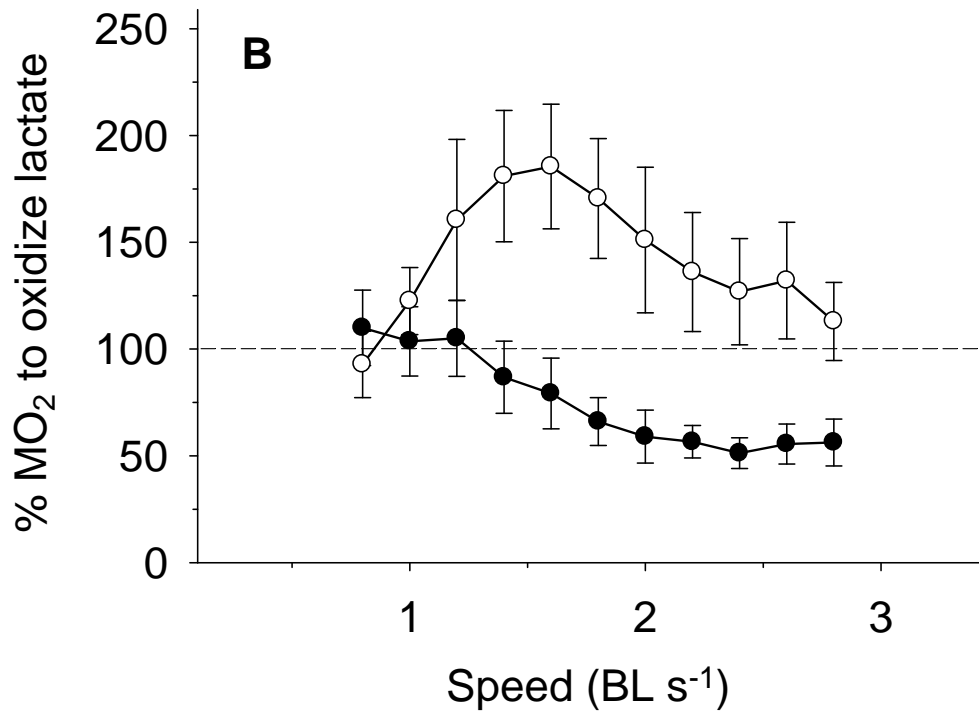
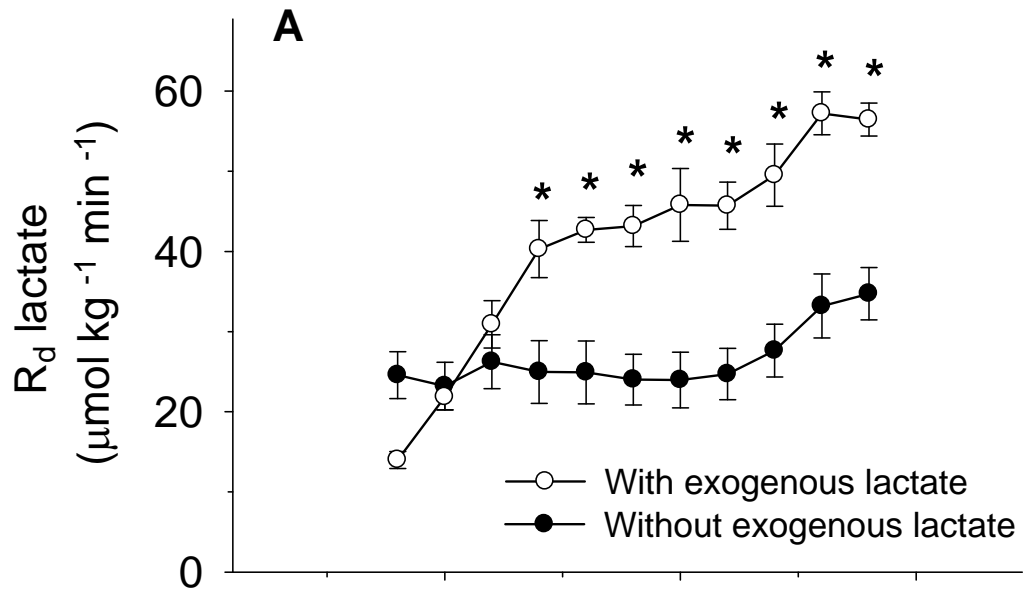


Table 4.1: Mean initial and final values for blood metabolite concentrations, specific activities, lactate fluxes (total R_a , R_d and endogenous R_a only) and metabolic rate, in resting or swimming rainbow trout infused with exogenous lactate.

	Resting fish		Swimming fish	
	Initial	Final	Initial	Final
Lactate (mM)	1.5 ± 0.1 (7)	13.4 ± 1.2 (7)*	1.2 ± 0.2 (5)	14.7 ± 1.4 (3)*
Glucose (mM)	5.2 ± 0.3 (7)	5.3 ± 0.3 (7)	4.5 ± 0.4 (5)	4.6 ± 0.8 (3)
Lactate S.A. (Bq μmol^{-1})	69.8 ± 4.9 (7)	42.2 ± 3.3 (7)*	117.8 ± 12.6 (5)	31.1 ± 5.4 (3)*
Glucose S.A. (Bq μmol^{-1})	1.4 ± 0.4 (7)	6.0 ± 1.2 (7)*	1.8 ± 0.1 (5)	5.3 ± 0.3 (3)*
Total R_a lactate ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	22.5 ± 1.1 (7)	41.9 ± 4.3 (7)*	21.9 ± 2.7 (5)	65.3 ± 4.2 (3)* ‡
Fish R_a lactate ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	14.5 ± 4.7 (7)	11.5 ± 4.3 (7)	15.2 ± 3.5 (5)	35.3 ± 4.2 (3)* ‡
R_d lactate ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	20.9 ± 1.0 (7)	39.9 ± 3.3 (7)*	14.0 ± 1.0 (5)	56.4 ± 2.0 (3)* ‡
MO_2 ($\mu\text{mol O}_2 \text{kg}^{-1} \text{min}^{-1}$)	46.2 ± 3.5 (7)	45.3 ± 2.5 (7)	52.3 ± 3.1 (5)	162.0 ± 7.4 (3)* ‡

Values are means ± s.e.m. with sample size in parentheses. S.A., specific activity; R_a , rate of appearance; R_d , rate of disposal. Statistical differences are indicated as * (Initial value vs. final value), and ‡ (resting final value vs. swimming final value) $P < 0.02$

**CHAPTER 5: Effects of hypoxia and exhausting
exercise on tissue-specific expression of
monocarboxylate transporters (MCTs) in rainbow
trout**

Partially based on:

Teye Omlin and Jean-Michel Weber

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

INTRODUCTION

Lactate plays essential roles as glycolytic end-product, energy source for oxidative tissues and gluconeogenic substrate (Brooks, 1991; Gladden, 2004). Mammals and fish maintain high basal rates of lactate production that are strongly stimulated during strenuous exercise and hypoxia, when aerobic pathways can no longer meet total ATP demand (Brooks, 1998; Omlin and Weber, 2010; Van Hall, 2010; Weber, 1991). Suitable mechanisms to regulate inter-tissue lactate trafficking are therefore required to cope with these common stresses and orchestrate recovery. Transmembrane lactate movements are mediated by monocarboxylate transport proteins (MCTs), a family of lactate/proton symporters particularly well characterized in mammals (Halestrap and Wilson, 2012; Meredith and Christian, 2008). Among the 14 known isoforms, MCT1, MCT2, and MCT4 are the most significant regulators of transmembrane lactate exchange (Halestrap and Meredith, 2004; Thomas et al., 2012). Each isoform has a dominant function and a different distribution: MCT1 is ubiquitous and specializes in lactate uptake by oxidative tissues; MCT2 predominantly controls lactate uptake by liver, brain and kidney, whereas MCT4 is mainly geared for lactate efflux from glycolytic muscle (Halestrap and Meredith, 2004; Halestrap and Price, 1999; Meredith and Christian, 2008). Partial or complete MCT sequences are now known for a few species of fish (Fig. 5.3). However, MCTs have not been characterized in rainbow trout (*Oncorhynchus mykiss*), even though several studies show that this common model species uses a proton-linked symporter to shuttle lactate between tissues (Laberee and Milligan, 1999; Wang et al., 1997)

Exposure to hypoxia for 1-3 hours increases blood lactate concentration from normoxic values of less than 1 mM to 6-15 mM (Dunn and Hochachka, 1986; Thomas et al.,

1992; van Raaij et al., 1996). Direct measurement of lactate fluxes revealed that the classic increase in blood lactate concentration observed during hypoxia results from a mismatch caused by the more rapid increase in R_a than in R_d (+98% vs. +52%) (Omlin and Weber, 2010). Moreover, the increase in R_d reduces the lactate load imposed on the circulation of hypoxic fish. The complex transport coordination that governs the dynamic lactate flux at the tissue level in rainbow trout is however unclear. Understanding the MCT expression pattern in trout exposed to hypoxia may determine the tissues responsible for stimulation lactate disposal during hypoxia.

Following exhausting exercise, lactate efflux is ~10 times slower in trout white muscle than in mammalian muscle (Wang et al., 1997; Watt et al., 1988). This well-known *lactate retention* capacity of fish white muscle has intrigued biologists for decades (Turner et al., 1983; Wardle, 1978), mainly because it prolongs recovery from maximal exercise (>12 h in trout vs. <1h in mammals (van Raaij et al., 1996)). Characterizing the expression of (putative) MCT isoforms in rainbow trout and their tissue distribution could prove important to understand the physiological basis for *lactate retention*. The few mammalian studies reporting the effects of exercise on MCT gene expression show variable responses depending on type of exercise, tissue, and isoform (Bickham et al., 2006; Bonen et al., 2000; Coles et al., 2004). Knowing how intense swimming alters expression in an athletic species like rainbow trout may explain inter-tissue lactate movements, especially during recovery.

Therefore, the goals of this study were: i) to clone rainbow trout MCTs and to identify which isoforms are expressed in this species, ii) to determine tissue-specific isoform distribution, and iii) to quantify the effects of acute and chronic hypoxia, and exhausting exercise on MCT gene expression in key tissues affecting whole-animal lactate kinetics:

white muscle (main lactate producer), red muscle, heart, brain, and gills (lactate oxidation), as well as liver (gluconeogenesis).

MATERIALS AND METHODS

Animals

Male and female rainbow trout, *Oncorhynchus mykiss* (Walbaum), ($302 \pm 14\text{g}$) were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and held in a 1,300-liter flow-through tank in dechlorinated, well-oxygenated water at 13°C under a 12:12 h light-dark photoperiod. The animals were acclimated to these conditions for at least 2 weeks before experiments. They were fed floating fish pellets (Martin Mills, Elmira, Ontario, Canada) 3 times a week to satiation. They were randomly assigned to a control group (rest; N=6) or treatment group (acute hypoxia N=6; chronic hypoxia N=6; exercise N=5). All procedures were approved by the Animal Care Committee of the University of Ottawa and adhered to the guidelines established by the Canadian Council on Animal Care.

Cloning, sequencing, and partial characterization of MCTs

MCT primers (*Zfish-MCT F* and *Zfish-MCT R*) were designed from Zebrafish MCT1a, MCT1b, MCT2, and MCT4 consensus sequence (regions 786 to 806 bp and 1332 to 1356 pb) to amplify trout MCTs by PCR. We reasoned that these conserved regions across zebrafish MCTs would likely be conserved in trout MCTs. It was reported previously for mammals that MCT1 is expressed in all tissues, MCT2 mainly in brain and MCT4 mainly in white muscle (Halestrap and Meredith, 2004). Therefore, total RNA (from two fish not used in the gene expression experiments) was isolated from white muscle and brain

using the TRIzol method (Invitrogen; Carlsbad, CA, USA). The RNA was quantified using NanoDrop 2000 (Thermo Scientific; Wilmington, DE, USA), and the purity and integrity verified on a 1.5% agarose gel prepared with 1X MOPS buffer. cDNA synthesis was performed using 5 µg of total RNA, 200 U SuperScript II reverse transcriptase, 250 ng random octamers (Invitrogen; Carlsbad, CA, USA), and DNase/RNase-free water for a total volume of 20 µl. The thermal profile was as per manufacturer's instructions. After cDNA synthesis, a PCR reaction was performed as follows: 1X PCR buffer, 1.5mM MgSO₄, 0.2 mM dNTP, 0.2mM of *Zfish-MCT F* and *Zfish-MCT R* each, 2 µl cDNA, 2U *Taq* polymerase (Bio Basic Inc; Amherst, NY, USA), and DNase/RNase-free water for a final volume of 50 µl. The thermal profile was started with 2 cycles (94°C/2 min; 63°C/1 min; 72°C/1 min), followed by 35 cycles (94°C/30 s; 63°C/30 s; 72°C/1 min), and a final extension step (72°C/5 min) using either Eppendorf Mastercycler (Hamburg, Germany) or BioRad S1000 thermal cycler (Hercules, CA, USA). The amplicons (565 base pair (bp)) obtained from both brain and white muscle were subcloned into TopoTA vector (Invitrogen; Carlsbad, CA, USA), and transformed in chemically competent *E.coli* cells. Clones (24 from brain and 28 from white muscle) were picked and screened by PCR in a reaction mix containing: 1X PCR buffer, 1.5mM MgSO₄, 0.2 mM dNTP, 0.2mM of M13 forward and reverse primers, 2U *Taq* polymerase (Bio Basic Inc; Amherst, NY, USA), and DNase/RNase-free water to a final volume of 50 µl. The thermal profile was as follows: an incubation step (94°C/5 min), followed by 40 cycles (94°C/30 s; 55°C/30 s; 72°C/1 min), and a final extension (72°C/5 min). Plasmids from clones with insert of the expected size (11/24 from brain and 12/28 from white muscle) were extracted using PureLink Miniprep kit (Invitrogen; Carlsbad, CA, USA) and sent for sequencing at Genome Quebec (McGill University, Montreal, QC,

Canada). The sequences were then identified by Basic Local Alignment Search Tool (BLAST). From the brain samples, 5/11 clones were identified as MCT1b, 4/11 as MCT2, and the remaining 2 were not positive for MCT. From the white muscle samples, 7/12 clones were identified as MCT1a, 4/12 as MCT1b, and 1/12 as MCT2. BLAST identified a sockeye salmon contiguous sequence (accession no. EZ815764) which had 90% nucleotide identity with trout MCT1b. Therefore, a new primer set *Sal-MCT* (from the salmon sequence) and *Trt-MCT* (from trout) was designed to amplify the upstream sequence of MCT1b using the white muscle sample. The PCR reaction and thermal profile were as described above for primer set *Zfish-MCT F* and *Zfish-MCT R*. A 460 bp amplicon was obtained and subcloned. Plasmids from clones with insert of the expected size (11/ 25) were extracted and sent for sequencing. Nine clones were identified as MCT1b, and the remaining 2 were not positive for MCT1b.

In an attempt to obtain full MCT sequences, rapid amplification of cDNA ends (RACE) was performed for MCT1a, MCT1b, and MCT2 using FirstChoice RLM-RACE Kit (Ambion; Austin, TX, USA) with total RNA extracted from white muscle. Both 5' and 3' ends could not be amplified for MCT1a, only the 3' RACE yielded a positive result for MCT1b and MCT2 (using primer sets *3'Trt-MCT1-outer* and *-inner*, and *3'Trt-MCT2-outer* and *inner*). Nested PCR was performed for RACE according to the manufacturer's instructions with the following thermal profiles: the first run was started with 2 cycles (94°C/2 min; 60°C/1 min; 72°C/1 min), followed by 35 cycles (94°C/30 s; 60°C/30 s; 72°C/1 min). The second run was the same as the first except for the annealing step (65°C/30 s). An array of amplicons (400-800 pb) were subcloned, and 26 clones each for MCT1b and MCT2 were screen by PCR (as described for *Zfish-MCT F* and *Zfish-MCT R*). Five clones were

identified as MCT1b, and 2 clones as MCT2. The 3' end sequences had 99% nucleotide identity in the overlapping region (82 bp for MCT1b, and 99 bp for MCT2). Given that amplification of complete sequences for MCT1b and MCT2 was unsuccessful, RACE was not repeated for MCT1a because the partial sequences obtained for all 3 MCTs were deemed adequate to design specific primers for MCT expression. We could not clone MCT4 in our experiments, however, we identified a contiguous sequence from rainbow trout (accession no. EZ780500.1; region 405 to 1071 pb) available in GenBank (NCBI) as MCT4 using BLAST. A schematic representation of the MCT cloning strategy is shown in Fig. 5.1, and the primer sequences are indicated in Table 5.1.

Sequence alignments and phylogenetic analysis.

The trout partial MCT nucleotide sequences were translated into putative protein sequences, and were aligned with known MCT proteins from various species using Clustal X version 2.0 (see Fig. 5.2). The transmembrane domains depicted for trout MCTs are as previously identified in Halestrap and Price (Halestrap and Price, 1999). A phylogenetic analysis of the partial trout MCT protein and known MCT proteins from various fish and mammals were generated as described in (Lemoine et al., 2010). Briefly, the phylogeny was generated by Bayesian analysis using a mixed-model approach [MrBayes version 3.2 (5)](Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The analysis was run for 10^6 generations on 3 heated chains and 1 cold chain for each simultaneous run, sampling at a frequency of 100 and a burn-in of 2500. MCT1 from *Saccharomyces cerevisiae* was included in the analysis as outgroup.

Catheterizations

All the animals were implanted with a single dorsal aorta catheter following the procedure of Haman and Weber (Haman and Weber, 1996) to monitor changes in plasma lactate concentration during experiments, and for rapid euthanasia (arterial injection of sodium pentobarbital) before tissue sampling for MCT expression. Briefly, fish were fasted for at least 24 h before surgery. They were anesthetized with ethyl-*N*-aminobenzoate sulfonic acid (MS-222; 60 mg L⁻¹) in well oxygenated water and their dorsal aorta was cannulated with PE-50 catheter (Intramedic, Clay-Adams, Sparks, MD, USA). Only animals with a hematocrit >20% after surgery were used in experiments. Catheters were kept patent by flushing with 0.2 ml Cortland saline (Wolf, 1963) containing 50 U/ml heparin (Sigma-Aldrich, St. Louis, MO, USA).

Acute and chronic hypoxia exposure

After cannulation, the animals were allowed to recover for 24 h in opaque Plexiglas chambers (50 x 12 x 12 cm) supplied with the same quality water as the acclimation tank. Oxygen saturation of the water was monitored using DAQ-PAC-G1 (Loligo Systems, Tjele, Denmark). The water was kept normoxic at 11.60 ± 0.01 mg O₂ l⁻¹ throughout the control experiments. For acute hypoxia exposure, the oxygen content was gradually reduced over 90 min from 11.60 ± 0.01 to 2.62 ± 0.04 mg O₂ l⁻¹ (25% oxygen saturation) and maintained for 90 min, as described in (Omlin and Weber, 2010). For chronic hypoxia exposure, the oxygen content was gradually reduced over 60 min from 11.30 ± 0.04 to 3.38 ± 0.04 mg O₂ l⁻¹ (35% oxygen saturation) and subsequently maintained for 8h. A blood sample (100 µl) was drawn before the start of each exposure and subsequent blood samples were taken every

45 min (acute hypoxia) or every 2h (chronic hypoxia) until the end of the experiments. The volume of blood sampled from each fish accounted for <5% of total blood volume. Blood samples were immediately deproteinized in 200 μ l perchloric acid (PCA; 6 % w/w) and centrifuged for 5 min at 16,000 G (Eppendorf 5424, Hamburg, Germany). Supernatants were stored at -20°C and lactate concentration was measured spectrophotometrically within 1 week. At the end of each exposure, the fish were euthanized by injection of an overdose of sodium pentobarbital through the catheter (Euthanyl, Abraxis Pharmaceutical/Products, Schaumburg, IL, USA). Liver, red muscle, and white muscle were harvested in random order within 5 min after death (~ 2 g per tissue). Tissues were immediately freeze-clamped in liquid N_2 .

Exhausting exercise

After surgery, the fish used for exercise experiments were individually transferred to a 110-liter flow-through circular tank supplied with the same quality water as the holding tank. They were first allowed to recover from surgery for at least 24 h, and manually chased around the tank until complete exhaustion (when they were no longer responding to touch after ~ 5 min of intense exercise). Resting controls and exercised fish were individually placed in a cylindrical respirometry chamber (47 cm long, 19 cm diameter) to monitor changes in blood lactate concentration. Recovering fish were at rest (no water current) as blood lactate levels were monitored. Blood samples were deproteinized with perchloric acid (6 % w/w) and centrifuged. Supernatants were stored at -20°C and lactate concentration was measured within 1 week as previously (Omlin and Weber, 2010).

The effects of swimming on MCT expression were quantified by comparing the tissues of resting controls with those of exercised fish, 23 h after exhaustion. This delay was selected because a previous study on mammals reported strong effects after this duration (Coles et al., 2004), and preliminary results on trout showed no effects after 6 h. After the experiments the fish were euthanized by overdose of sodium pentobarbital injected through the catheter (Euthanyl, Abraxis Pharmaceutical Products, Schaumburg, IL, USA). Gills, heart, liver, red muscle, white muscle and brain were harvested in random order within 5 min (~2 g of each tissue, except for heart and brain that were sampled entirely). Tissues were immediately freeze-clamped in liquid N₂.

Total RNA extraction and cDNA synthesis

Fifty to 100 mg subsamples were ground to a fine powder in liquid N₂ using mortar and pestle, homogenized in Qiazol (QIAGEN, Hilden, Germany) using a Polytron (Kinematica, Luzern, Switzerland), and stored at -80°C until RNA extraction. Total RNA extraction including a genomic DNA elimination step was performed using RNeasy Plus Universal kit as per manufacturer's instructions (QIAGEN, Hilden, Germany). The RNA was quantified, and the purity and integrity verified as described in the MCT cloning section. cDNA synthesis including a genomic DNA elimination step was performed with QuantiTect Reverse Transcription kit according to manufacturer's instructions using 1 µg of total RNA (QIAGEN, Hilden, Germany).

MCT expression by real-time PCR

Forward and reverse primer sets (*Trt-Exp-MCT1a*, *Trt-Exp-MCT1b*, *Trt-Exp-MCT2*, *Trt-Exp-MCT4*; Table 5.1) were designed from rainbow trout MCT sequences. The transcript levels of MCT1a, MCT1b, MCT2, and MCT4 were quantified by real-time PCR using the QuantiFast SYBR Green PCR kit (QIAGEN; Hilden, Germany) in a Bio-Rad CFX real-time PCR detection system (Hercules, CA, USA). The reaction mix contained 1X QuantiFast SYBR Green PCR master mix, 0.2 mM forward and reverse, 1 μ l DNase/RNase-free water, and 2 μ l of 2.5X diluted cDNA samples (10 μ l total reaction mix). All the samples were run in duplicate and fluorescence was measured at the end of every extension step. Each run included no-RT and no-template controls. Standard curves were constructed for MCT1a, MCT1b, MCT2, MCT4, elongation factor-1 α (EF-1 α), 18S and β -actin using serial dilutions of stock cDNA to ensure adequate amplification efficiency. Amplification efficiencies were 111% for MCT1a (linear Ct range: 23-29 cycles), 113% for MCT1b (21-27 cycles), 109% for MCT2 (24-30 cycles), 102% for MCT4 (19-31 cycles), 103% for EF-1 α (15-21 cycles), 111% for 18S (8-14 cycles), and 115% for β -actin (14-21 cycles). The thermal profile was 40 cycles (95°C/5min; 95°C/10 s; 60°C/30 s), followed by a final melt curve (65°C to 95°C with 5°C increment/5 s). Expression levels of MCTs were normalized to EF-1 α because comparisons of the 3 housekeeping genes (EF1- α , 18S, and β -actin) using excel-based software “BestKeeper” (Pfaffl et al., 2004) showed that EF1- α is the best housekeeping gene and that β -actin is too variable. The threshold cycles (Ct) for all the MCT amplifications were within the range of the standard curves. Changes in the expression of MCT1a, MCT1b, MCT2, and MCT4 transcript levels were determined using the “ $\Delta\Delta$ Ct method” (Pfaffl, 2001).

Calculations and statistics

Statistical comparisons were performed using one- or two-way repeated measure analysis of variance (RM-ANOVA) with the Bonferroni *post hoc* test to determine which means were different from controls. In cases where the assumptions of normality or homoscedasticity were not met, the data was normalized by \log_{10} transformation before parametric analysis. Friedman repeated measures ANOVA on ranks was used with Dunn's test when normality or homoscedasticity were not met after transformation. All values presented are means \pm SE and a level of significance of $P < 0.05$ was used in all tests.

RESULTS

Partial characterization of monocarboxylate transporters in rainbow trout

Partial sequences of the rainbow trout monocarboxylate transporters MCT1a, MCT1b, MCT2, and MCT4 were cloned and characterized. Figure 5.2 compares the amino acid sequences of MCT1a, MCT1b, MCT2, and MCT4 between rainbow trout and various other vertebrate species. It shows all the conserved regions (shaded in black) and the transmembrane domains (TMD) as previously identified (Halestrap and Price, 1999). For MCT1, two homologs were detected: MCT1a and MCT1b. The MCT1a sequence was 513 nucleotides long coding for part of the open reading frame (ORF)(171 amino acids). MCT1b was 1829 nucleotides long with 1155 coding for part of the ORF (385 amino acids) and 671 nucleotides in the 3' untranslated region (UTR). The amino acid sequences of trout MCT1a and MCT1b were 75% identical. MCT1a had 79 and 77% amino acids identities with zebrafish and cod respectively, as well as 64 and 66% identical to human and rat MCT1 amino acids (Fig. 5.2). The trout MCT1b was 85 and 81% identical to zebrafish and cod, as

well as 72 and 75% identical to human and rat MCT1 at the amino acid level (Fig. 5.2). Trout MCT2 was 1048 nucleotides long with 666 coding for part of the ORF (222 amino acids) and the remaining sequence in the 3' UTR. It had 78 and 81% identities to zebrafish and cod respectively, as well as 68 and 57% identities with its counterpart in human and rat at the amino acid level. The trout contiguous sequence (accession no. EZ780500.1; GenBank) identified as MCT4 was 1072 nucleotides long with a partial ORF of 666 (region 405 to 1071 pb) coding for 222 amino acids, and the remainder in the 5'UTR. It had 88 and 87% identities to zebrafish and cod respectively, as well as 79 and 81% identities with human and rat MCT4. Percent identities of trout MCTs with other vertebrate MCTs was obtained by comparing partial deduced amino acid sequences of trout MCTs. To confirm the identity of rainbow trout MCTs, we performed a Bayesian analysis (phylogenetic tree) of the MCT protein sequences for isoforms 1, 2 and 4 of various fish and mammalian species. This analysis shows that rainbow trout MCT1a, MCT1b, MCT2, and MCT4 (indicated by arrows) cluster with the corresponding isoforms from other fish species (Fig. 5.3).

Tissue distribution of monocarboxylate transporters

Figure 5.4 shows the presence of MCT1a, MCT1b, MCT2, and MCT4 in gill, heart, liver, red muscle, white muscle, and brain of rainbow trout. All the tissues investigated expressed the 4 isoforms, but mRNA levels varied greatly. Comparing the control levels of each isoform between tissues reveals the following: MCT1a expression was ~4-fold lower in white muscle than in gill, heart, and brain ($P < 0.05$; Fig. 5.4A). MCT1b was expressed at much higher levels in heart and red muscle than in other tissues (>10 times higher than in liver, 23 times higher than in white muscle, and >50 times higher than in gill and brain

($P < 0.001$; Fig. 5.4B)). The expression of MCT2 was ~40 times higher in gill and brain than in any other tissue ($P < 0.001$; Fig. 5.4C). MCT4 was > 40 times higher in the heart than in any other tissue ($P < 0.001$; Fig. 5.4D).

MCT expression during hypoxia

Changes in MCT1a, MCT1b, MCT2, and MCT4 mRNA levels in liver, red muscle and white muscle during acute and chronic hypoxia exposures are shown in Fig. 5.5 where values were normalized to EF1- α . The same effects were confirmed when using 18S as an alternative housekeeping gene (results not shown). Acute and chronic hypoxia exposure had no effects on MCT transcript levels.

Post-exercise MCT expression

The effects of exhausting exercise on the expression of MCT1a, MCT1b, MCT2, and MCT4 in rainbow trout tissues are presented in Fig. 5.6 where values were normalized to EF1- α . The same effects were confirmed when using 18S as an alternative housekeeping gene (results not shown). Transcript levels increased by 90% for MCT1a and by 50% for MCT1b in the heart of exercised fish ($P < 0.05$; Fig. 5.6A and B). However, swimming had no effect on MCT1a and MCT1b expression in other tissues (gill, liver, muscle, and brain; $P > 0.05$). The expression of MCT2 was strongly upregulated in the brain (+260 %; $P < 0.02$), but remained unchanged in all the other tissues ($P > 0.05$; Fig. 5.6C). Exhausting exercise had no effect on the expression of MCT4 ($P > 0.05$; Fig. 5.6D).

Hypoxia and post-exercise blood lactate concentration

Circulating lactate levels of resting controls remained low and steady at 1.3 ± 0.1 mM during the 24 h preceding tissue sampling ($P > 0.05$; result not shown). Changes in blood lactate concentration monitored through hypoxia exposure are shown in Fig. 5.7. The fish exposed to acute hypoxia showed progressive increase from 1.0 ± 0.1 to 4.6 ± 0.8 mM ($P < 0.001$, Fig. 5.7A). Fish exposed to chronic hypoxia had a transient increase of lactate from 1.0 ± 0.1 to 3.6 ± 0.7 mM after 4h, then slightly decreased to 3.0 ± 0.5 mM after 8h exposure ($P < 0.001$, Fig. 5.7B). For the exercise group, changes in lactate concentration during recovery from maximal swimming are presented in Fig. 5.8. Blood lactate was 10.3 ± 1.0 mM immediately after exercise (time 0), subsequently reached a maximum of 17.8 ± 2.1 mM after 3 h, and had returned to baseline after 22 h of recovery (0.6 ± 0.2 mM).

DISCUSSION

This study demonstrates the presence of four monocarboxylate transporter isoforms in rainbow trout. These proteins were present ubiquitously, but their level of expression varied greatly between tissues and isoforms. The main lactate exporter of glycolytic muscle in mammals, MCT4, was only expressed significantly in trout heart, but barely detectable in all other tissues, including white muscle. More importantly, hypoxia or exhausting exercise had no stimulating effect on the expression of any MCT isoform of white muscle. This study provides a possible functional explanation for the elusive lactate retention behaviour observed during recovery. The classic delay in exporting large lactate loads from white muscle to the circulation may be explained by the much lower expression of all MCTs in

white muscle than in other tissues and by the total lack of MCT upregulation by hypoxia or exercise in this tissue.

Cloning and identification of rainbow trout MCTs

This study shows the existence of MCT1a, MCT1b, MCT2, and MCT4 in *Oncorhynchus mykiss*. Using zebrafish sequences as a reference, we have characterized 37% of MCT1a (171 amino acids), 83% of MCT1b (385 aa), 56% of MCT2 (222 aa), and 44% of MCT4 (222 aa). To ensure that these peptides were properly identified, amino acid sequence alignments of the partial trout MCTs were performed with known MCTs from mammals and other fish species (Fig. 5.2). The partial rainbow trout isoforms showed higher identity with zebrafish and cod (72-85%) than with mammals (57-75%). The transmembrane domains identified in trout were extremely conserved across species: TMDs 6-10 for MCT1a, TMDs 2-12 for MCT1b, TMDs 6-11 for MCT2, and TMDs 1-6 for MCT4 (see Fig. 5.2). A Bayesian phylogenetic analysis of MCT1-4 was performed to compare known fish and mammalian sequences. The tree topology shows clades of fish and mammals in which rainbow trout MCTs (indicated by arrows in Fig. 5.3) cluster adequately with the corresponding fish homologs. MCT1a and b likely arose from the teleost genome duplication (Umezawa et al., 2012), and our phylogenetic analysis supports this idea by showing that the two MCT1 homologs of trout are also closely related (Fig. 5.3). Fish are known to express multiple copies of MCT2 (Umezawa et al., 2012) and the phylogenetic tree shows that the trout gene identified here is closely related to the MCT2a isoform previously characterized in other fish species (Fig. 5.3).

Tissue distribution of rainbow trout MCTs

All MCT isoforms were found in every tissue, but white muscle showed the lowest expression of these genes (Fig. 5.4). The fact that MCT1a, MCT1b, MCT2, and particularly MCT4 are barely expressed in white muscle may explain why lactate export from this tissue is so slow (Turner et al., 1983; Wang et al., 1997). Numerous mammalian studies have shown that MCT1 plays a key role in lactate uptake by oxidative tissues. With a K_m of 4-7 mM, the mammalian MCT1 is particularly important during recovery from intense exercise or hypoxia, when blood lactate levels are elevated (Bonen et al., 2006; Halestrap and Meredith, 2004). A similar lactate affinity was reported for the MCT1b of fugu (K_m of 4 mM) (Umezawa et al., 2012), suggesting that fish MCT1b has the same function as the unique MCT1 isoform expressed by mammals. High expression of MCT1b in heart and red muscle of trout (Fig. 5.4B) is consistent with the better lactate clearance seen during active recovery (low-intensity swimming), when both tissues increase their use of oxidative fuels over passive (resting) recovery (Milligan et al., 2000).

MCT2 was poorly expressed in rainbow trout. It was mostly found in brain and gill (Fig. 5.4C), but barely detectable in other tissues. Similarly, zebrafish MCT2 was dominant in the brain and expressed significantly in gills (Ngan and Wang, 2009). Mammalian studies show that MCT2 is specialized for lactate uptake, has high affinity for its substrate (K_m of 0.7 mM), and plays an important role in the brain to shuttle lactate from astrocytes to neurons for oxidation (Brooks, 2002; Gladden, 2004; Smith et al., 2003). The tissue distributions of MCT2 in trout and zebrafish suggest that this isoform has a similar function in fish. It could allow highly oxidative tissues such as brain and gill to use lactate as an oxidative fuel under baseline conditions, when blood lactate is ~1 mM (Fig. 5.7 and 5.8).

More work is needed to determine whether MCT2 has as low a K_m in fish as previously reported for mammals. Because MCT4 is recognized as the main lactate exporter from glycolytic muscles in mammals (Halestrap and Meredith, 2004; Halestrap and Price, 1999; Meredith and Christian, 2008), its extremely low expression in trout white muscle could well be partly responsible for lactate retention after maximal exercise (Turner et al., 1983; Wang et al., 1997; Wardle, 1978; Watt et al., 1988). A previous analysis of efflux kinetics using sarcolemmal vesicles supports this observation by showing that simple diffusion is the main mechanism for lactate export from white muscle (Sharpe and Milligan, 2003).

MCT expression during acute and chronic hypoxia

Liver, red muscle, and white muscle were strategically chosen to investigate MCT expression during hypoxia because they are highly metabolic under such condition, therefore they are more likely to alter their MCT expression. Unfortunately, the results show that none of the MCTs were affected by hypoxia these tissues ($P > 0.05$; Fig. 5.5). Similar results were observed in zebrafish muscle and gill where MCTs were not altered even after 96 h of severe hypoxia ($1.5 \text{ mg O}_2 \text{ l}^{-1}$) (Ngan and Wang, 2009). A trend towards increase in red muscle trout MCT1a transcripts during exposure to chronic hypoxia is however apparent. This small increase appears to be an attempt to stimulate lactate uptake for oxidative phosphorylation. This strongly suggest that the red muscle may be actively contributing to lactate disposal as observed in our previous study (Omlin and Weber, 2010). Future investigation of the MCT1a protein expression levels and transport kinetics in the red muscle will be necessary to confirm this idea.

Exhausting exercise and MCT expression

As circulating lactate becomes abundant after exercise (Fig. 5.8), some tissues increase their ability to import this valuable fuel (brain and heart), whereas others do not (muscle, liver and gills) (Fig. 5.6). Exhausting exercise stimulates the expression of MCT2 in brain (+260%), and of MCT1a and MCT1b in heart (+90% and +50%, respectively). The brain is well known to favor lactate as an oxidative fuel in birds and mammals (Izumi et al., 1997; Larrabee, 1995; Schurr et al., 1997; Schurr et al., 1988; Smith et al., 2003; Taberero et al., 1996), and the large MCT2 response observed here suggests that it is also true in fish. Perfused *in-situ* preparations show that the heart of rainbow trout oxidizes lactate preferentially (Lanctin et al., 1980; Milligan and Farrell, 1991). Our results are consistent with the higher capacity for lactate uptake potentially afforded by the increased expression of cardiac MCT1a and MCT1b (Fig. 5.6). Lactate clearance from the circulation would also be facilitated by improving the capacity for lactate entry in red muscle and gills for oxidation, as well as in liver for gluconeogenesis.

Immediately after maximal exercise, lactate concentration reaches 30-40 mM in white muscle before a progressive decline to baseline over the next 10 h (Turner et al., 1983). By contrast, the recovery pattern in blood is completely different. Blood lactate concentration continues to rise for 3-4 h into recovery to reach a maximum of only 15-20 mM, and subsequently returns to baseline over several hours (Turner et al., 1983)(Fig. 5.8). This study is the first to demonstrate the presence of monocarboxylate transporters in rainbow trout. Surprisingly, exercise only stimulates MCT expression in brain (MCT2) and heart (MCT1a, b), presumably to increase capacity for lactate uptake and to provide these highly oxidative tissues with better access to an abundant fuel source. Taken together, results provide a

possible functional explanation for the classic “lactate retention” phenomenon. Rainbow trout are unable to release large lactate loads rapidly from white muscle because: (i) MCT4, the main lactate exporter characterized in glycolytic muscles of mammals, is very poorly expressed in trout, (ii) the combined expression of all the trout MCT isoforms is much lower in white muscle than in other tissues, and (iii) exhausting exercise fails to upregulate MCT expression in white muscle.

Perspectives and significance

This study supports the idea that white muscle operates as a quasi-closed system with regard to carbohydrate metabolism. Hypoxia and intense swimming is fueled by local glycogen stores that are subsequently replenished *in situ* from lactate during recovery (Milligan and Girard, 1993; Turner et al., 1983). Resynthesis of glycogen from lactate is known to occur at significant rates in the glycolytic muscles of some ectotherms, possibly through a reversal of the pyruvate kinase reaction (Fournier and Guderley, 1992; Gleeson, 1996). Mammalian glycolytic muscles behave very differently because they favor the Cori cycle as a strategy for recovery, exporting lactate rapidly to the circulation and replenishing glycogen stores from hepatic glucose. In trout, white muscle glycogen acts as an “energy spring” that alternates between explosive power release during intense swimming or hypoxia and slow recoil during protracted recovery. Future studies on the time course of changes in gene expression and protein levels will determine whether the pattern of white muscle MCT expression reported here makes such a strategy possible.

Figure 5.1. (A) Agarose gel of rainbow trout and zebrafish MCT amplicon (565 bp). MCT from rainbow trout heart (H), red muscle (RM), and white muscle (WM) and from zebrafish brain (b), gill (g), and muscle (m) of both species was amplified using primer set *Zfish-MCT F* and *Zfish-MCT R*. (NT) represents a no-template control. (B) Diagram of the cloning strategy for MCT1a, MCT1b, and MCT2 of rainbow trout. Arrows indicate the primers used to amplify different regions of the gene (thick black bars). Asterisk (*) indicates primers from the Ambion FirstChoice RLM-RACE Kit.

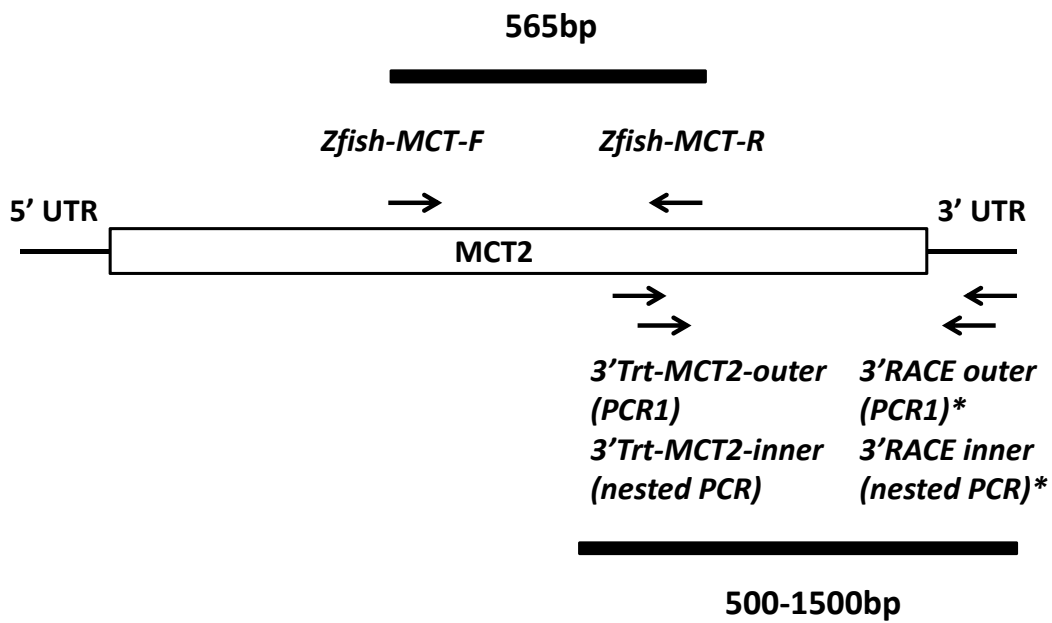
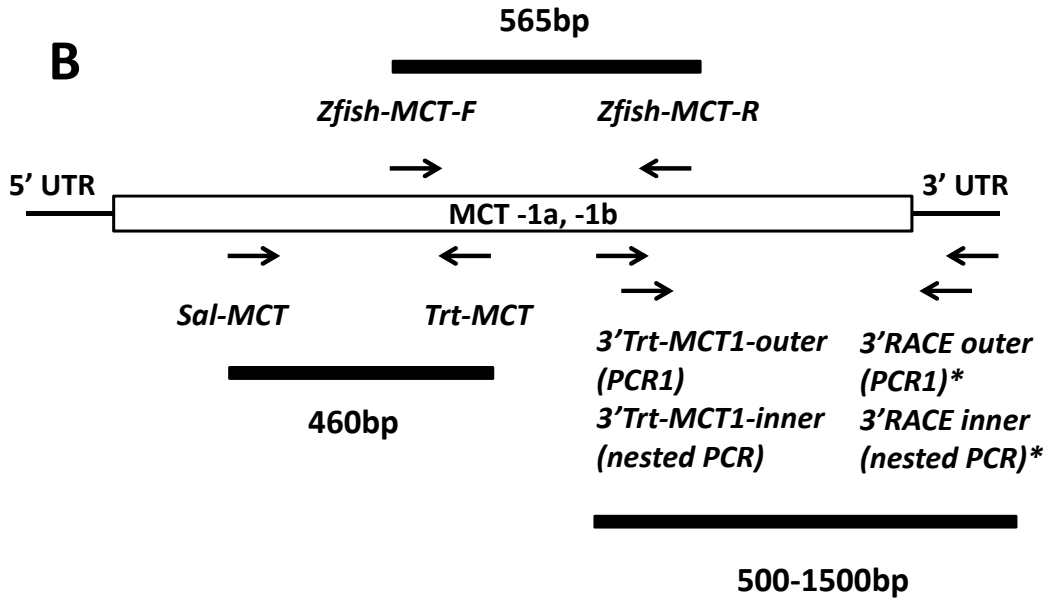
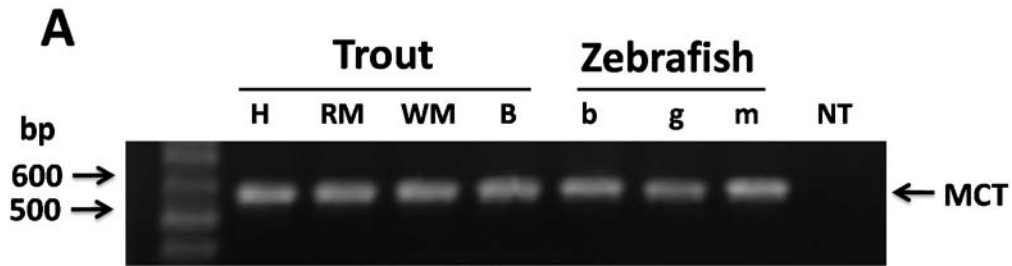


Figure 5.2. Comparisons of the amino acid sequences of MCT1a, MCT1b, MCT2, and MCT4 between rainbow trout and other species (zebrafish, cod, rat, mouse and human). Black areas show identical amino acids and grey areas similar amino acids. The transmembrane domains (TMDs) identified in Halestrap and Price (Halestrap and Price, 1999) are also indicated. Sequences were aligned using ClustalX 2.0 and Multiple Align Show (www.bioinformatics.org/SMS/multi_align.html).

MCT2

	TMD6		TMD7	TMD8	TMD9	
Trout	ILGGILLNCVAGALMRPLGPPLG-----KIKKDE-----ELVVAKTTTKKETTTCCLGTVNRFIDLSLEKHRGFLIYLS	70	GNVIMFLGFFSPIVFLLTAYAKDIGVDEYSAAFLLSILAFVDMFARFMSGLLANSKWIRPRIQVFFSEAVLNGVCHLLCPVLESYACL	V	160	
Zebrafish	ILGGILLNCVAGSLMRPLVAPAGSIAPPVVEVKHAEKE-----KLTAWQT-----VNRKLDLSLEKHRGFLIYLS	67	GNVIMFLGFFAPIVFLSPYAKDNGVDEYQAAFLLSILAFVDMFARFMSGLLANSRWVPRIQVFFSEAVLNGVCHLLCPVLESYTCM	VV	157	
Cod	ILGGILLNCVAGALMRPLVPPRA-----DGPKPE-----KLSWET-----VNRKLDLSLEKHRGFLIYLS	57	GNVIMFLGFFSPIVFLAAYAKDMGVDEYSAAFLLSILAFVDMFARFMTGLLANSRWIRPRIQVFFCFVAVLNGVCHLLCPVSDY	TGLVV	147	
Mouse	ILGGIFLHS CVAGCLMRPLGPSPTKKS-----KSKVGSRHDSTLKKASKVSTAQK-----VNRFLDESLEKHRGFLIYLS	71	GNVIMFLGFFAPIIFLAQYAKHIGVDDYNSAFLLSVMAFIDMFARFVGLLANTSLIRPRIQVLFSSAIFFTGICHLCPVLA	TSALVV	161	
Human	ILGSLLLNACVAGSLMRPLGPNQTTSKS----KNKTGKTEDDSSPKKIKTKKSTWEK-----VNRKLDLSLEKHRGFLIYLS	73	GNVIMFLGFFAPIIFLAQYAKDQGVDEYSAAFLLSVMAFVDMFARFVGLLANSKYIRPRIQVFFSEAVLNGVCHLLCPVLAQDY	TSLVL	163	
	TMD10	TMD11				
Trout	YVVFQFAFGVSSVLFETLMDLVGAORFSSAVGLTTIVECCPVLLGPPLAG	212				
Zebrafish	YVVFQFAFGVSAVLFETLMDLVGAORFSSAVGLTTIVECCPVLLGPPLAG	209				
Cod	YVVFQFAFGVSSVLFETLMDLVGAORFSSAVGLTTIVECCPVLLGPPLAG	199				
Mouse	YVVFQFGFGSISVLFETLMDLVGAORFSSAVGLTTIVECCPVLLGPPLAG	213				
Human	YVVFQFGFGSISVLFETLMDLVGAORFSSAVGLTTIVECCPVLLGPPLAG	215				

MCT4

	TMD1	TMD2	
TROUT	MGCVAVDDEPSDKAPDGGWGWAVLXGCVITGFSYAFPKAVSVFFKELIREFGVGYSDTAWISSILLAMLYGTGPLCSVIVNRFGCRPV	90	
ZEBRAFISH	MGCVAVDDEPSDKAPDGGWGWAVLXGCVITGFSYAFPKAVSVFFKELIREFGVGYSDTAWISSILLAMLYGTGPLCSVIVNRFGCRPV	90	
COD	MGCVAVDDEPSDKAPDGGWGWAVLXGCVITGFSYAFPKAVSVFFKELIREFGVGYSDTAWISSILLAMLYGTGPLCSVIVNRFGCRPV	90	
RAT	MGCVAVDDEPSDKAPDGGWGWAVLXGCVITGFSYAFPKAVSVFFKELIREFGVGYSDTAWISSILLAMLYGTGPLCSVIVNRFGCRPV	90	
HUMAN	MGCVAVDDEPSDKAPDGGWGWAVLXGCVITGFSYAFPKAVSVFFKELIREFGVGYSDTAWISSILLAMLYGTGPLCSVIVNRFGCRPV	90	
	TMD3	TMD4	TMD5
TROUT	MIVGGLEFASLGMVLAASFATSIIHLYICTGVITGLGIALNFQPSLIMLNRYSEKREHANGLSAAGSPVALCCLSPLGQVLCYBYGWRGGE	180	
ZEBRAFISH	MIVGGLEFASLGMVLAASFATSIIHLYICTGVITGLGIALNFQPSLIMLNRYSEKREHANGLSAAGSPVALCCLSPLGQVLCYBYGWRGGE	180	
COD	MIVGGLEFASLGMVLAASFATSIIHLYICTGVITGLGIALNFQPSLIMLNRYSEKREHANGLSAAGSPVALCCLSPLGQVLCYBYGWRGGE	180	
RAT	MIVGGLEFASLGMVLAASFCR-IIIQYIITGVITGLGIALNFQPSLIMLNRYSEKREHANGLSAAGSPVALCCLSPLGQVLCYBYGWRGGE	179	
HUMAN	MIVGGLEFASLGMVLAASFCRSIIIQYIITGVITGLGIALNFQPSLIMLNRYSEKREHANGLSAAGSPVALCCLSPLGQVLCYBYGWRGGE	180	
	TMD6		
TROUT	LILGGILLNCCVAGALMRPLVGPVK 205		
ZEBRAFISH	LILGGILLNCCVAGALMRPLVPPKS 205		
COD	LILGGILLNCCVAGALMRPLVPPKA 205		
RAT	LILGGILLNCCVAGALMRPLVAPQA 204		
HUMAN	LILGGILLNCCVAGALMRPLVWTAQ 205		

Figure 5.3. Phylogeny of monocarboxylate transporter proteins (MCT1a and b, MCT2a, b and c, and MCT4a and b) in selected vertebrate species. The tree was obtained through Bayesian inference of phylogeny (Mr. Bayes v.3.2.(5)). Nodes are supported by >80% posterior probabilities, except where indicated by numerical values or by (*) when <50%. Arrows indicate the retrieved rainbow trout MCTs in the reconstructed phylogeny. The scale bar (0.1) represents the estimated mean number of amino acid substitutions per site for all species. Protein accession numbers (ENSEMBL) are also indicated.

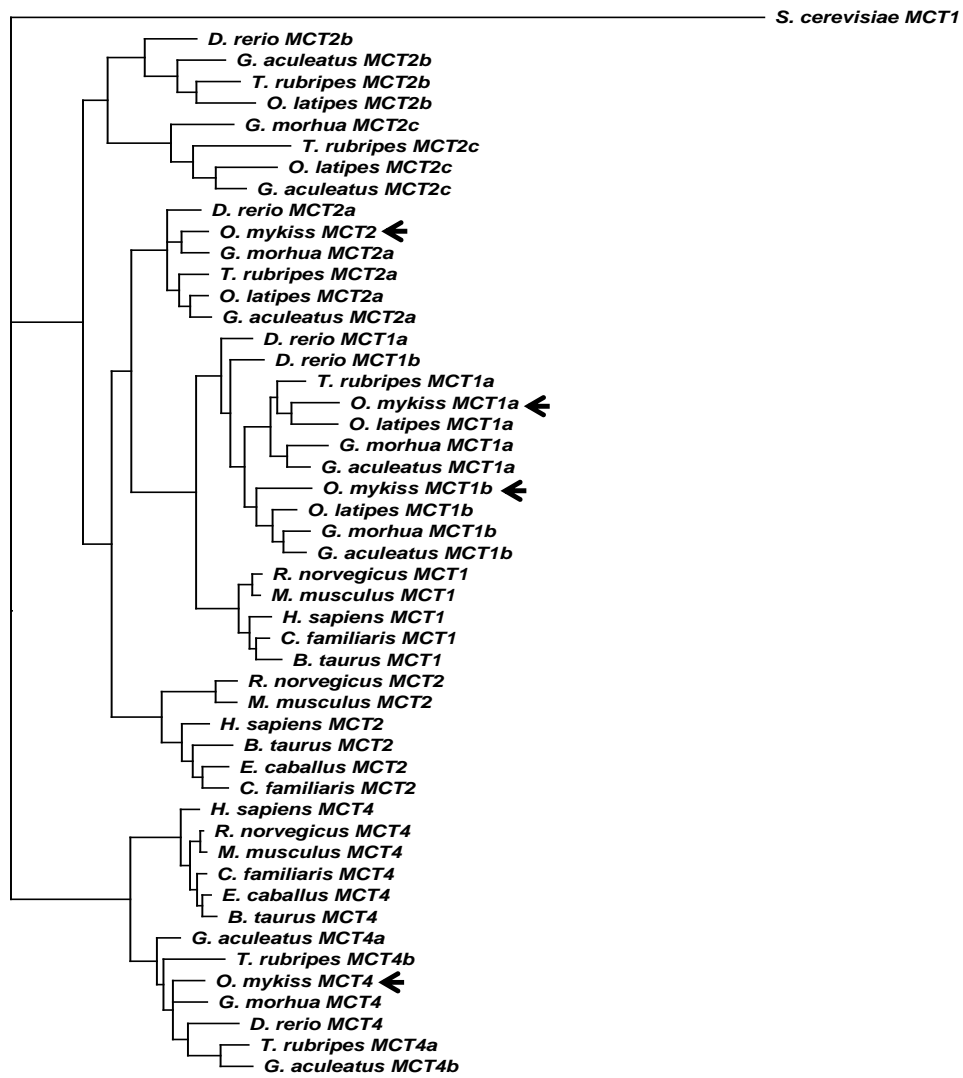


Figure 5.4. Distribution of MCT1a (panel A), MCT1b (panel B), MCT2 (panel C), and MCT4 (panel D) in rainbow trout gill (G), heart (H), liver (L), red muscle (RM), white muscle (WM), and brain (B). Expressions are standardized to elongation factor-1 α (EF-1 α). Values are means \pm SE (n=6). Symbols (*a,b,c*) indicates tissue significantly differences (P<0.05).

Expression relative to EF-1 α

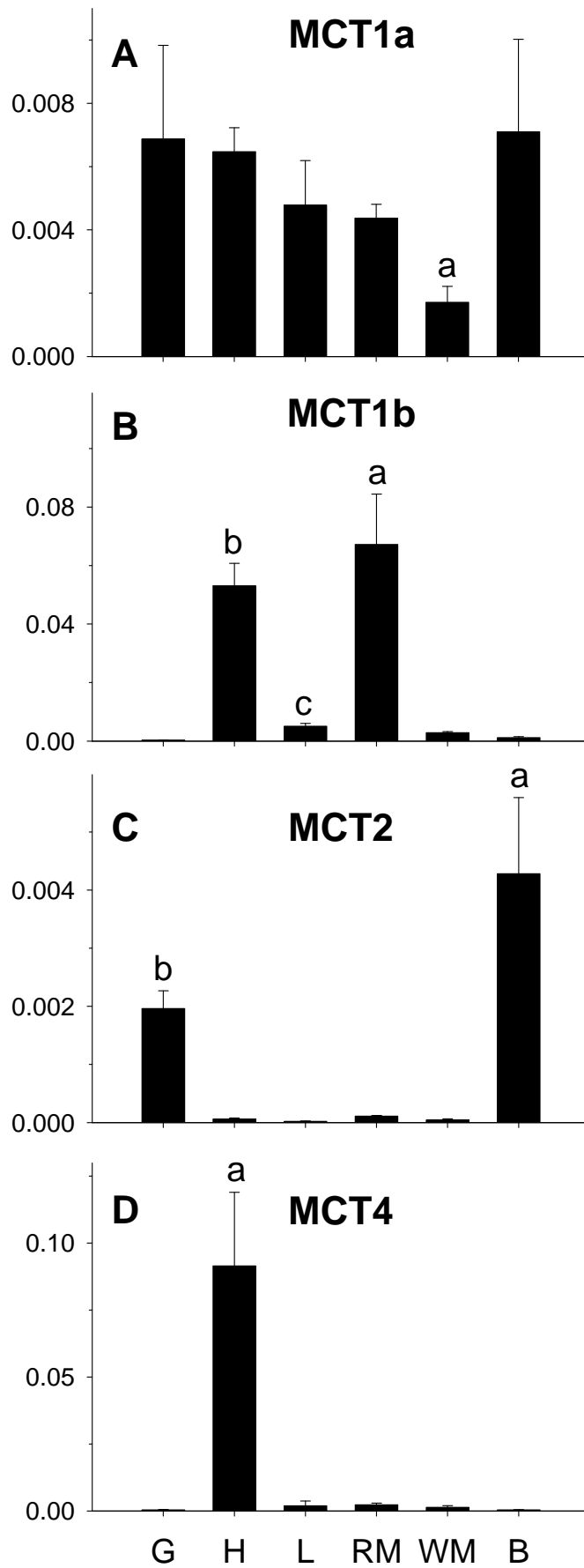


Figure 5.5. Effects of acute hypoxia (25% O₂; 90 min; grey bars) and chronic hypoxia (33% O₂; 8h; black bars) on expression of MCT1a (panel A), MCT1b (panel B), MCT2 (panel C), and MCT4 (panel D) in rainbow trout tissues: Liver (L), red muscle (RM), white muscle (WM). Control group (normoxia) are represented by white bars. Values are means \pm s.e.m. (n=5).

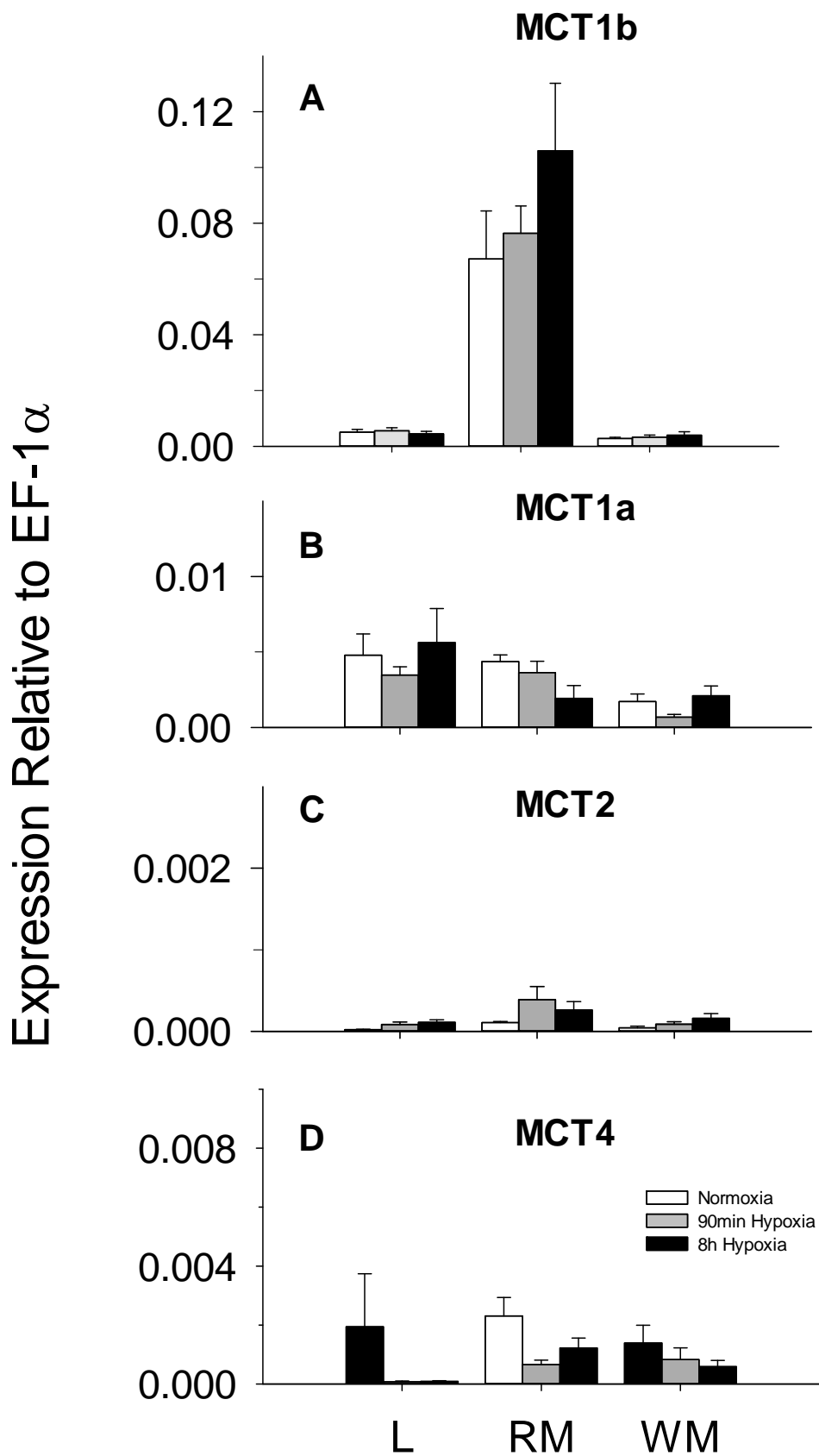


Figure 5.6. Effects of intense exercise on the expression of MCT1a (panel A), MCT1b (panel B), MCT2 (panel C), and MCT4 (panel D) in rainbow trout tissues: gills (G), heart (H), liver (L), red muscle (RM), white muscle (WM), and brain (B). Histograms show pre-exercise, control levels (white bars) and post-exercise levels (black bars; 23 h after intense swimming). Values are means \pm s.e.m. (n=6 for controls and 5 for post-exercise). Differences between controls and exercised fish are indicated by * (P<0.02).

Expression relative to EF-1 α

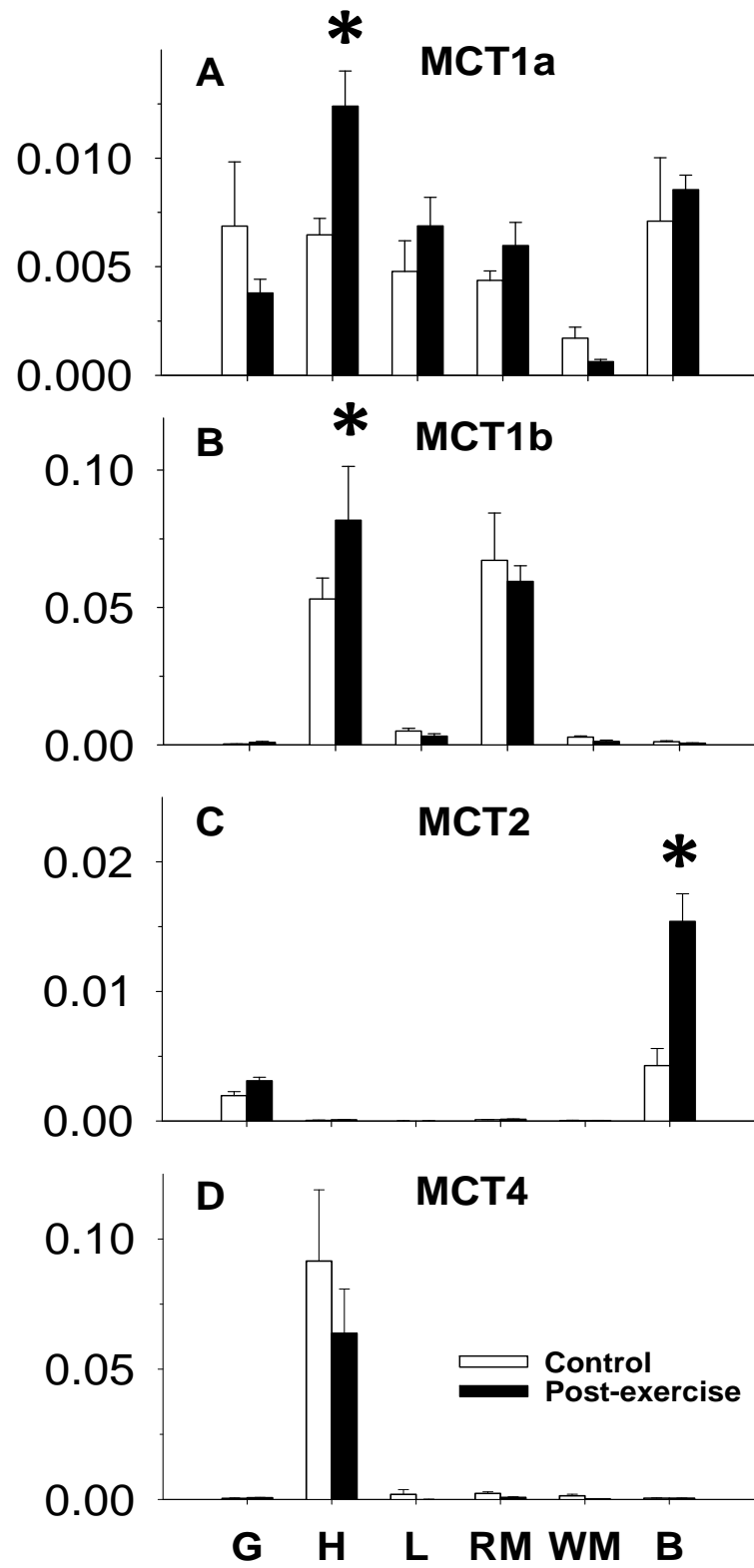


Figure 5.7. Changes in blood lactate concentration of rainbow trout during exposure to short hypoxia (25% O₂; 90 min; panel A) and long hypoxia (33% O₂; 8h; panel B). Values are means ± SE (n=5). Error bars are not visible when smaller than the symbols. Differences from control (normoxia) are indicated by * (P<0.05), ** (P<0.01) or *** (P<0.001).

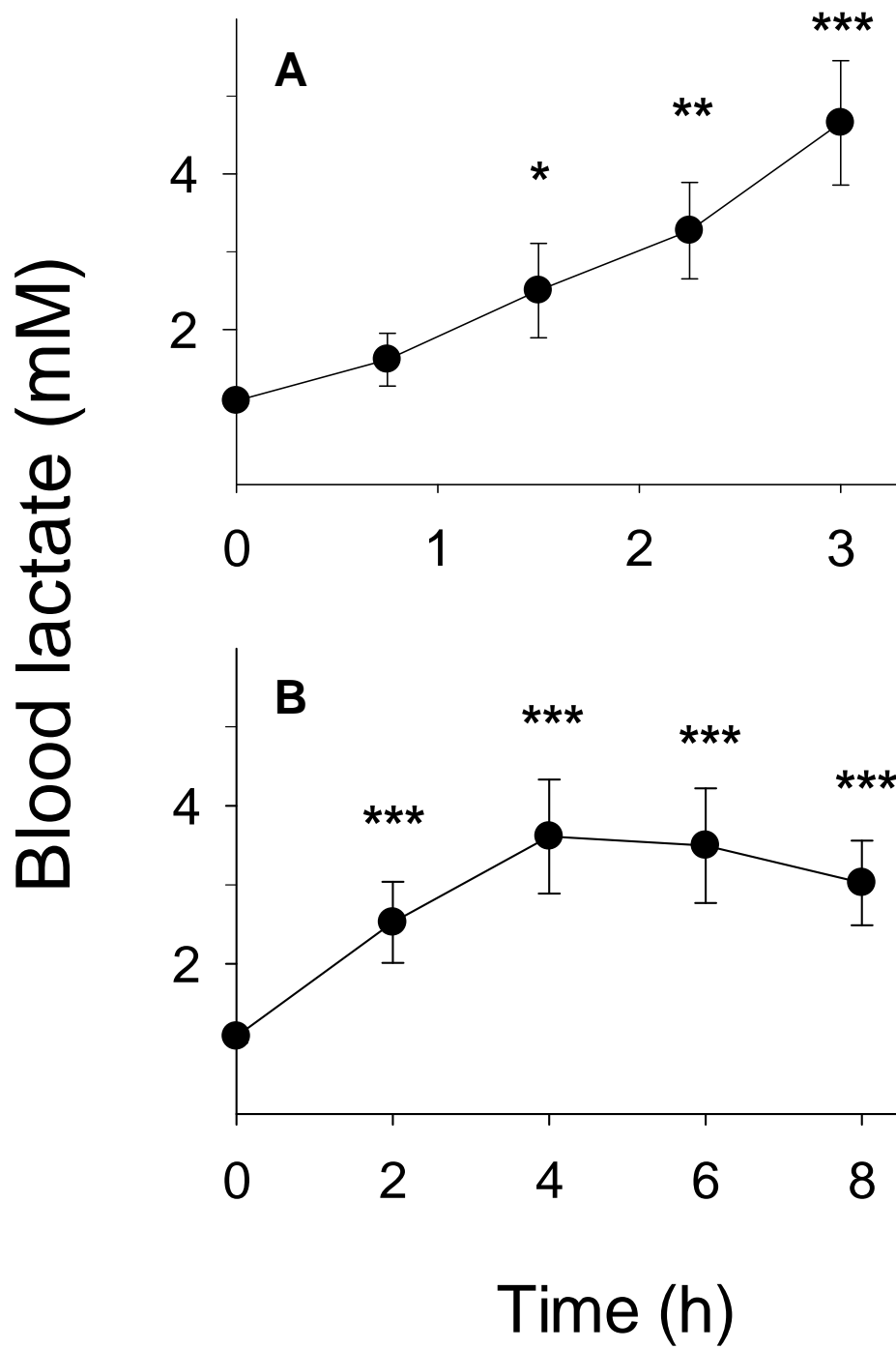


Figure 5.8. Changes in blood lactate concentration of rainbow trout after maximal exercise.

Values are means \pm SE (n=5). Error bars are not visible when smaller than the symbols. *

Indicates differences from baseline values reached 23 h after exercise (P<0.01).

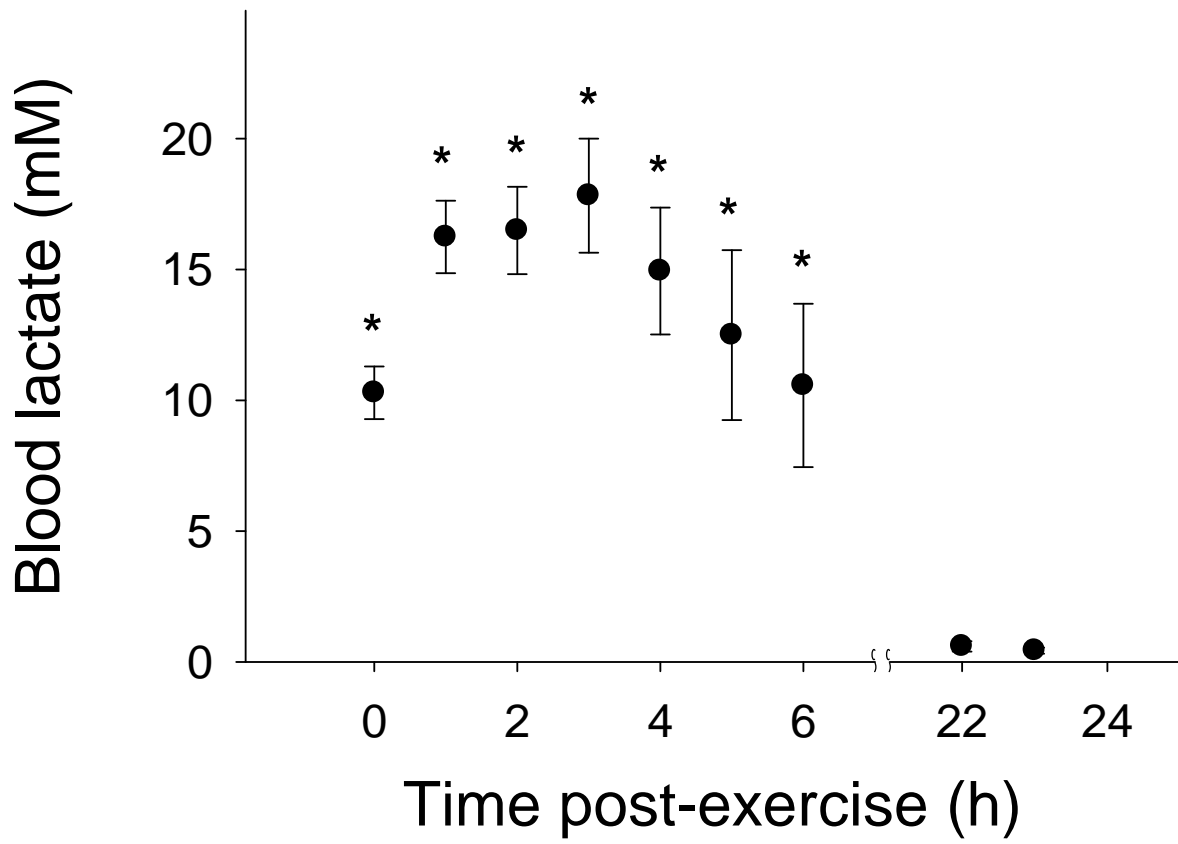


Table 5.1. Primers for cloning rainbow trout monocarboxylate transporter isoforms 1a, -b, and -2 (MCT1a, -1b, and -2) were based on zebrafish MCTs sequences. Primers denoted by an asterisk were taken from the Ambion FirstChoice RLM-RACE Kit. Trt-MCT, 3'Trt-MCT1-outer and -inner, 3'Trt-MCT2-outer and -inner, Trt-Exp-MCT1a, -MCT1b, MCT2, and MCT4, were designed based on rainbow trout MCTs sequences. EF-1 α was previously published (Morash et al).

Primer	Forward (5' to 3')	Reverse (5' to 3')	Amplicon size (bp)
Zfish-MCT (F+R)	cggctggaggggcagcttcn	caccaggtccatcagtgctcgaan	565
Sal-MCT;Trt-MCT	ccttcactccatcggtctctcn	aggaagctgcccctccagn	460
3'Trt-MCT1-outer	cccaggrtrcagtactctctygc		-
3'RACE outer*		gcgagcacagaattaatacgact	-
3'Trt-MCT1-inner	cggtgtgtgycacgctcytg		-
3'RACE inner*		cgcggatccgaattaatacgactcactatagg	-
3'Trt-MCT2-outer	cggcccaggatccagtactcttc		-
3'Trt-MCT2-inner	cggggtgtgccacatcctctg		-
Trt-Exp-MCT1a	tgcagcgtgtcaatgccgtg	ccttactcttagcgtagttggagagg	138
Trt-Exp-MCT1b	acgacgtgcgggcagaagtg	tccctgctcttgcaaagttac	149
Trt-Exp-MCT2	cgaccactgtttggggactgtc	tatgtccttggcataggccgtc	149
Trt-Exp-MCT4	tactgggtctctctacgc	cactggccgacacccaaac	179
EF-1α	cattgacaagagaaccattga	ccttcagcttgccagcac	98

CHAPTER 6: General conclusions

THESIS OVERVIEW

The main purpose of this thesis was to study the effects of **hypoxia** and **exercise** on lactate metabolism in rainbow trout (*Oncorhynchus mykiss*), a commonly used ectotherm model in energy metabolism. I was particularly interested in determining how the rates of appearance (R_a) and disposal (R_d) of lactate account for the observed changes in lactate concentration when these animals are exposed to hypoxia or exercise. My interest stems from the fact that current understanding of lactate metabolism in fish is almost entirely based on concentration measurements. I was also interested in studying the role of monocarboxylate transporters (**MCTs**) in transmembrane lactate movements because previous evidence demonstrated that lactate transport in rainbow trout is carrier mediated (Laberee and Milligan, 1999; Wang et al., 1997). However, the actual presence of MCTs had never been characterized in this species. Consequently, this thesis includes two different approaches: (i) *in vivo* experiments to investigate the effects of hypoxia and swimming on lactate kinetics (CHAPTERS 2-4), and (ii) *in vitro* experiments to clone and characterize rainbow trout MCTs, as well as to investigate the effects of hypoxia and exercise on the expression of these MCTs (CHAPTER 5).

Lactate kinetics experiments

In chapter 2, my aim was to determine the baseline lactate turnover rate of rainbow trout using the most reliable tracer method presently available: *continuous tracer infusion*. Then, I wanted to establish how changes in rates of lactate appearance (R_a) and disposal (R_d) account for the increase in blood lactate concentration elicited by hypoxia, and finally, to

identify the main tissues responsible for lactate production. I anticipated that hypoxia would stimulate R_a lactate by activating anaerobic glycolysis in muscle and possibly liver, and that R_d lactate would decrease as the result of the reduced capacity of tissues to oxidize lactate.

In chapter 3, my aim was to use the continuous tracer infusion method to measure the effects of steady-state and graded swimming on lactate fluxes, and to determine how R_a and R_d lactate vary with exercise intensity. **In chapter 4**, I have determined the effects of exogenous lactate infusion on lactate kinetics in rainbow trout, at rest and during graded exercise, and investigated whether supplemental lactate could improve critical swimming speed (U_{crit}). I anticipated that exogenous lactate supply would depress endogenous lactate production of resting fish. During exercise, supplying extra lactate was used as a tool to examine what might limit maximal rates of lactate disposal. I also hypothesized that exogenous lactate would improve U_{crit} by increasing the supply of a valuable oxidative substrate.

Cloning and characterization of MCTs

In chapter 5, the goal was to clone and characterize rainbow trout MCTs to identify which isoforms are expressed in this species. I also wanted to determine the tissue-specific isoform distribution, and finally, quantify the effects of acute and chronic hypoxia, as well as exhausting exercise on MCT gene expression in key tissues affecting whole-animal lactate kinetics: white muscle (main lactate producer), red muscle, heart, brain, and gills (lactate oxidation), as well as liver (gluconeogenesis).

SUMMARY OF PRINCIPAL FINDINGS

CHAPTER 2: Hypoxia stimulates lactate disposal in rainbow trout

1. I report the first measurements of non-steady state lactate kinetics in an ectotherm. In the control (resting, normoxic) fish, R_a and R_d lactate were 18.2 and $19.0 \mu\text{mol kg}^{-1} \text{min}^{-1}$ at time 0. After 3 h, these fluxes decreased slightly to 13.1 and $12.8 \mu\text{mol kg}^{-1} \text{min}^{-1}$, respectively.
2. The acute hypoxia group did not only increase R_a lactate (from 18.4 to $36.5 \mu\text{mol kg}^{-1} \text{min}^{-1}$), but also strongly stimulated R_d lactate (from 19.9 to $30.3 \mu\text{mol kg}^{-1} \text{min}^{-1}$). The increase in blood lactate concentration observed during hypoxia (from 1 to 9 mM) resulted from the mismatch caused by the more rapid increase in R_a than in R_d (+98% vs. +52%). The stimulation of R_d lactate was a surprising because the animals were in a low oxygen environment. I concluded that this metabolic response is essential because it greatly reduces the lactate load imposed on the circulation of hypoxic fish.
3. Differential accumulation of lactate in various tissues revealed that white muscle was the main lactate producer with a major increase in lactate concentration from 3.6 to $19.2 \mu\text{mol g}^{-1}$.

CHAPTER 3: Lactate kinetics in swimming rainbow trout: graded and steady state exercise

1. In the graded swimming group, R_a lactate increased from a baseline value of $24.2 \pm 2.9 \mu\text{mol kg}^{-1} \text{min}^{-1}$ to $40.4 \pm 0.7 \mu\text{mol kg}^{-1} \text{min}^{-1}$ and R_d lactate increased from $24.6 \pm 2.9 \mu\text{mol kg}^{-1} \text{min}^{-1}$ to $34.7 \pm 3.3 \mu\text{mol kg}^{-1} \text{min}^{-1}$, causing an increase in blood

lactate from 1.3 to 5.1 mM. By contrast, steady-state submaximal exercise caused R_a and R_d lactate to increase similarly from ~ 20 to $\sim 30 \mu\text{mol kg}^{-1} \text{min}^{-1}$, with a trivial mismatch between production and disposal that only affected blood concentration minimally (from 0.7 to 1.4 mM).

2. This thesis reports the first measurements characterizing the relationship between exercise intensity and lactate kinetics in an ectotherm. In rainbow trout, the increase in blood lactate concentration only starts at speeds greater than 2.4 BL s^{-1} when R_a lactate starts diverging from R_d lactate.

CHAPTER 4: Supplemental lactate does not affect swimming performance and endogenous lactate production in rainbow trout.

1. This thesis is the first to demonstrate the high capacity of rainbow trout for R_d lactate when supplied with exogenous lactate. During graded exercise combined with exogenous lactate supply, R_d lactate was stimulated by 300% (from 14.0 to 56.4 $\mu\text{mol kg}^{-1} \text{min}^{-1}$: a value 63% higher than the maximal flux measured without providing exogenous lactate). By contrast, resting fish only increased R_d lactate by 90% (from 20.9 to 39.9 $\mu\text{mol kg}^{-1} \text{min}^{-1}$).
2. I also show that the infusion of exogenous lactate has no impact on endogenous lactate production at rest or during exercise. The endogenous lactate production (fish R_a) remained at baseline levels (17 $\mu\text{mol kg}^{-1} \text{min}^{-1}$), whereas it was significantly stimulated in swimming fish (from 22.5 to 35 $\mu\text{mol kg}^{-1} \text{min}^{-1}$)

3. Infusion of exogenous lactate did not improve or impair swimming performance.

The U_{crit} of fish receiving exogenous lactate or saline (control) were almost identical (2.8 vs. 2.9 BL s^{-1} , respectively)

CHAPTER 5: Effects of hypoxia and exhausting exercise on tissue specific expression of monocarboxylate transporters (MCTs) in rainbow trout.

1. This thesis reports the presence of four monocarboxylate transporter isoforms (MCT1a, MCT1b, MCT2, and MCT4) in rainbow trout for the first time. Using zebrafish sequences as a reference, I characterized 37% of MCT1a (171 amino acids), 83% of MCT1b (385 aa), 56% of MCT2 (222 aa), and 44% of MCT4 (222 aa).
2. MCT1a, MCT1b, MCT2, and MCT4 were ubiquitously present but expressed at different levels in the tissues investigated. In the white muscle, all the MCTs were expressed at low levels, particularly MCT4 (the isoform specialized for lactate export)
3. Acute or chronic hypoxia did not affect the expression levels of MCTs, whereas, exhausting exercise stimulated the expression of MCT2 in brain (+260%), and of MCT1a and MCT1b in heart (+90% and +50%, respectively).
4. Hypoxia or exhausting exercise had no stimulating effect on the expression of any MCT isoform in white muscle. This thesis provides a functional explanation for the elusive lactate retention behaviour observed for decades in recovering salmonids. The classic delay in exporting large lactate loads from white muscle to the circulation may be explained by the much lower expression of all MCTs in white

muscle than in other tissues and by the total lack of MCT upregulation (MCT4) by hypoxia or exercise in this tissue.

GENERAL DISCUSSION

I. Lactate kinetics

Using the *continuous tracer infusion* technique, R_a and R_d lactate of rainbow trout was quantified at rest (baseline levels), during acute exposure to hypoxia, during steady-state and graded exercise, and during infusion of exogenous lactate. I show that the lactate fluxes of resting and swimming trout are much higher than estimated in previous experiments relying on the bolus injection method: an obsolete approach with significant limitations (Dunn and Hochachka, 1987; Milligan and McDonald, 1988; Weber, 1991). The information obtained from the flux measurements in this thesis provides a new understanding of fish lactate metabolism that previous approaches did not make possible. Knowledge about rates of production and utilization was crucial to characterize how fish regulate glycolysis when coping with stresses such as environmental hypoxia and exercise. A simple interpretation of changes in lactate concentration (the most common approach used in the past), would not have allowed me to discover the following new features of lactate metabolism in trout: Both, R_a and R_d lactate are stimulated during hypoxia (+98% and +52%, respectively) and during graded exercise (+67% and +41 %), accounting for increases in blood lactate concentration to 8.9 mM (hypoxia) and 5.3 mM (exercise). Clearly, the stimulation of R_d lactate plays a crucial role in minimizing the lactate load imposed on the circulation, a phenomenon that simple measurements of changes in concentration could not have shown. By contrast, the increases in R_a and R_d lactate

measured during steady-state submaximal exercise were almost exactly matched (+50%), accounting for a trivial change in blood lactate concentration (from 0.7 to 1.4 mM).

Administering exogenous lactate allowed me to demonstrate that the maximal R_d lactate observed during graded exercise was not limited by the capacity of oxidative tissues to use lactate.

II. Correlation between lactate concentration and lactate flux

It had been suggested that increasing lactate flux at the whole organism level can be done via 2 distinct mechanisms: 1) by increasing blood lactate concentration (blood-to-tissue gradient), and 2) by increasing cardiac output (convective transport) (Weber et al., 1987a; Weber et al., 1987b). The data collected in this thesis provided a good opportunity to test whether this idea was supported in rainbow trout, because values obtained during hypoxia (low cardiac output) and swimming (high cardiac output) could be compared. Therefore, I have plotted the relationships between blood lactate concentrations and lactate fluxes for the 2 physiological conditions (Fig. 6.1). The linear regression lines plotted for R_a and R_d lactate show an excellent correlation between circulating lactate concentrations and fluxes. Overall, the graph shows that the slopes of the linear regression lines of R_a and R_d lactate vs. concentration during exercise are higher than those during hypoxia. This is because, as the exercise intensity of the fish increases, muscle and systemic blood flow (cardiac output) of the animal are stimulated, when compared to animals at rest (Randall and Daxboeck, 1982). As a consequence, the fluxes of metabolites such as lactate are higher for a given concentration during exercise than hypoxia.

The accurate measurement of R_a and R_d lactate by *continuous infusion* in fish is technically challenging (surgical placement of two catheters: one to infuse, the other for blood sampling) (Haman and Weber, 1996), as well as use of metabolic tracers. Therefore, using blood lactate concentration to predict lactate flux may be useful if the relationship between the 2 parameters is known. The results from this study have allowed me to develop four equations (from Fig. 6.1) to estimate lactate flux from concentration for rainbow trout exposed to hypoxia (25% air saturation) or graded exercise (from 0.5-3 BL s^{-1}). Using these equations after simple measurements of blood lactate concentration, would allow to estimate R_a and R_d lactate without a need for tracer infusion. Four separate equation were obtained from the regressions of Fig. 6.1 as follows:

- Flux (R_a or R_d) = $\mathbf{mx} + \mathbf{b}$

Where flux is obtained in $\mu\text{mol kg}^{-1} \text{min}^{-1}$ \mathbf{m} = slope, \mathbf{x} = blood lactate concentration in mmol l^{-1} , and \mathbf{b} = y-intercept

For hypoxia at rest:

- $\mathbf{R_a lactate} = 2.52 [\text{lactate}] + 10.18$ (*Equation 1*)
- $\mathbf{R_d lactate} = 1.84 [\text{lactate}] + 13.14$ (*Equation 2*)

For exercise:

- $\mathbf{R_a lactate} = 9.56 [\text{lactate}] + 11.07$ (*Equation 3*)
- $\mathbf{R_d lactate} = 6.49 [\text{lactate}] + 15.25$ (*Equation 4*)

III. Monocarboxylate transporters (MCTs) and lactate fluxes

For the first time, I report the presence of monocarboxylate transporters (MCTs) in rainbow trout tissues. The MCT1 with low lactate affinity is mainly expressed in heart and red muscle, whereas the high affinity MCT2 is expressed mainly in brain and gill. The pattern of expression of these various isoforms provides differential tissue specificity for lactate transport. MCTs facilitate the clearance of blood lactate during recovery from hypoxia or exercise as illustrated in the theoretical model of Fig. 6.2. However, the overall low expression of MCTs in trout tissues (Chapter 5) may be the cause for low lactate uptake. Consequently, the increased “mass-action” effect resulting from infusion of exogenous lactate stimulated lactate uptake. I identified white muscle as the main lactate producer in rainbow trout during acute hypoxia or exercise, but this tissue also poorly expresses all MCTs, and fails to upregulate them (particularly MCT4, the main lactate exporter previously characterized in the glycolytic muscles of other animals). Consequently, the high level of lactate produced in the white muscle remain trapped (*lactate retention*) as there little MCTs present for export. So what causes such prolonged high blood lactate concentration in rainbow trout after hypoxia and exhausting exercise? Due to low expression and lack of upregulation of MCTs, export of lactate from this tissue may rely primarily on free diffusion (Sharpe and Milligan, 2003; Wang et al., 1997). Diffusion is very slow compared to carrier-mediated transport, and this is probably why large lactate loads produced within white muscle take such a long time to be cleared therefore prolonging blood lactate concentration for 8-12h. I investigated MCT expressions at the gene level, and the results suggest that MCTs may not play an important role in blood lactate appearance. The expression of MCT proteins may be increased in white muscle (without any changes in RNA expression) to

facilitate lactate export to the circulation, but since no specific antibodies are currently available for fish MCT detection, and this possibility remains to be investigated

GENERAL CONCLUSION

This thesis addresses important questions about rainbow trout lactate fluxes and transport mechanisms that were puzzling for decades for an animal model commonly used to study the effects of hypoxia and exhausting exercise. For the first time, this thesis clarifies the effects of hypoxia and exercise on in vivo lactate kinetics of rainbow trout using the continuous tracer infusion method. I demonstrate that these fish have higher baseline lactate fluxes than previously estimated by bolus injection. Under baseline conditions, I show that R_a and R_d lactate are matched to maintain low and stable blood lactate concentrations (<1mM). Results also reveal that R_a lactate increases by 98% and 67% during hypoxia and graded exercise, respectively, while R_d lactate changes by +52% and +41%. This strategy allowed the fish to reduce the lactate load imposed on the circulation. This thesis illustrates the idea that simple changes in metabolite concentrations can generally not be used to make guesses about changes in lactate flux, unless the actual relationship between these 2 parameters has been determined. For example, the same observed change in blood lactate concentration during hypoxia or during exercise reflects widely different changes in lactate fluxes (see Fig. 6.1), because cardiac output is different. Here, I developed a set of regression equations that may provide a tool to estimate fluxes from blood lactate concentration for hypoxia and swimming in rainbow trout. I showed that white muscle is the major lactate producer during hypoxia and that increased lactate oxidation by aerobic tissues is probably responsible for the increased R_d lactate observed during exercise, and

presumably, during hypoxia. For the first time we demonstrated a higher capacity for lactate disposal than previously measured (+300% R_d lactate), probably as a consequence of “mass-action” effect, when the fish are supplied with exogenous lactate. Surprisingly, these higher rates of lactate uptake do not improve the swimming performance of the fish. Results also contradict the erroneous notion that lactate is a noxious waste product that causes muscle fatigue, because supplying extra end-product did not impair swimming performance. The presence of MCTs in rainbow trout was suggested for decades, however, in this thesis, I confirm for the first time their presence. Overall their expression is low and unaffected by hypoxia, however, exhausting exercise stimulated the expression of MCT2 in brain, and of MCT1 in heart. In white muscle, surprisingly, hypoxia and intense exercise had no effects on the expression of any MCT in particular. My study provides a molecular evidence that has been lacking for decades to explain the elusive lactate retention behavior in fish: low MCT expression particularly MCT4 and the lack of upregulation by exhausting exercise. Finally, I show that white muscle operates as a quasi-closed system where glycogen stores acts as an “energy spring” that alternates between explosive power release during intense swimming and slowly recoil in situ during protracted recovery.

The broad implications of the study: (i) Lactate metabolism has always been important in sports. My study provides a different animal model, particularly in a very athletic animal, to further understand how lactate is produced and utilized during exercise. (ii) Metabolite and drug kinetics are important in drug development in the pharmaceutical industry. My study provides detailed metabolite kinetics in non-steady state conditions using an ectotherm as animal model. (ii) Recent advances in cancer research have revealed that cancer cells uptake lactate through the high affinity lactate transporter MCT2. Given that I have now cloned

MCTs in rainbow trout, my study provides an ectotherm system as possible model to study drug delivery through MCTs.

Future experiments: (i) Clone the full sequences of MCT1, MCT2, and MCT4 in rainbow trout. (ii) Develop antibodies for identifying MCT proteins in rainbow trout. (iii) Determine expression of MCT proteins during hypoxia and exercise and correlate lactate kinetics to the change in protein expression.

Figure 6.1 Rates of appearance (R_a) and disappearance (R_d) of lactate in rainbow trout during hypoxia and exercise as function of concentration. Values of R_a during hypoxia (open circles) and exercise (open triangles), and R_d during hypoxia (filled circles) and exercise (filled triangles) are presented as s.e.m (N=8 for hypoxia; for exercise N=7 up to 2 mM lactate, and N=4 and 2 for the last 2 points). The regression lines for R_a / R_d exercise, and R_a / R_d hypoxia, respectively are: R_a (exercise) = $9.56 x + 11.07$ ($r^2 = 0.96$, $P = 0.0005$); R_d (exercise) = $6.49 x + 15.25$ ($r^2 = 0.94$, $P = 0.002$); R_a (hypoxia) = $2.52 x + 10.18$ ($r^2 = 0.98$, $P < 0.0001$); and R_d (hypoxia) = $1.84 x + 13.14$ ($r^2 = 0.83$, $P = 0.0006$).

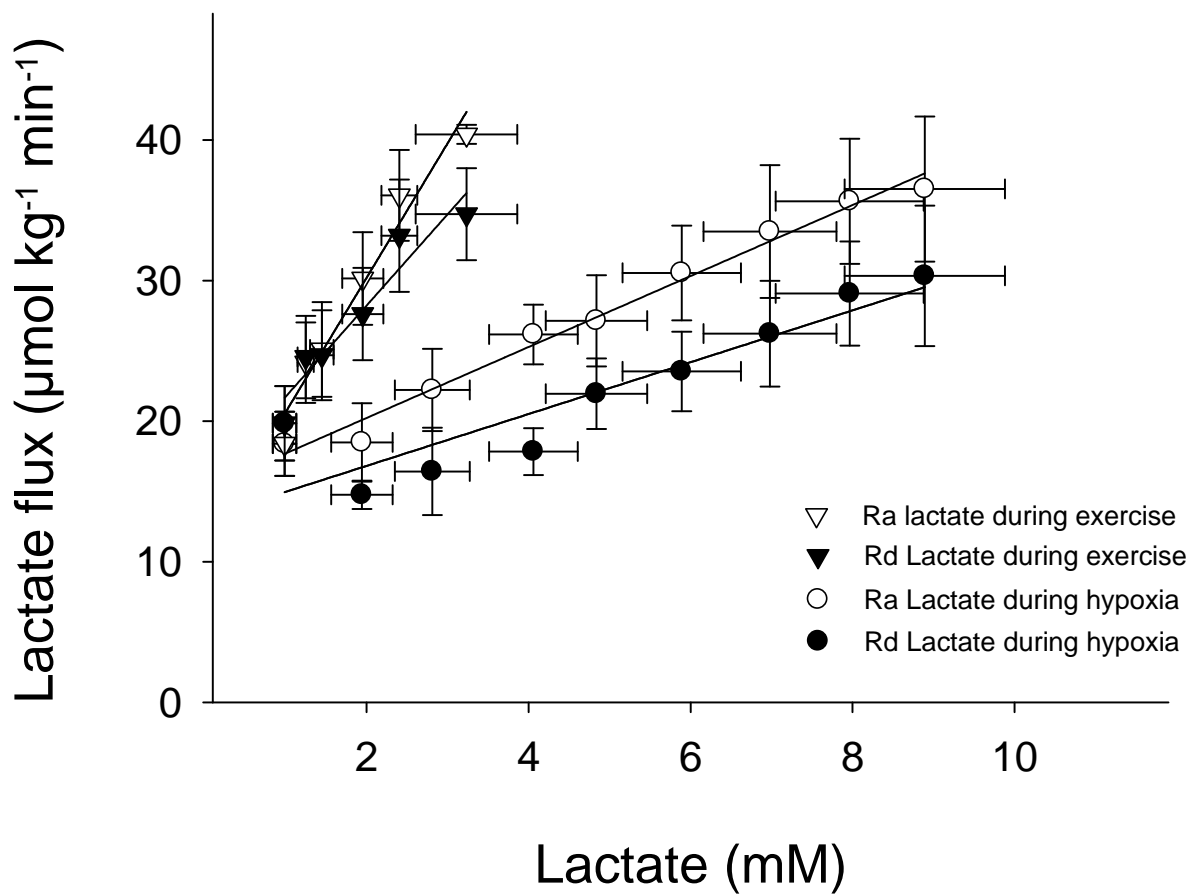
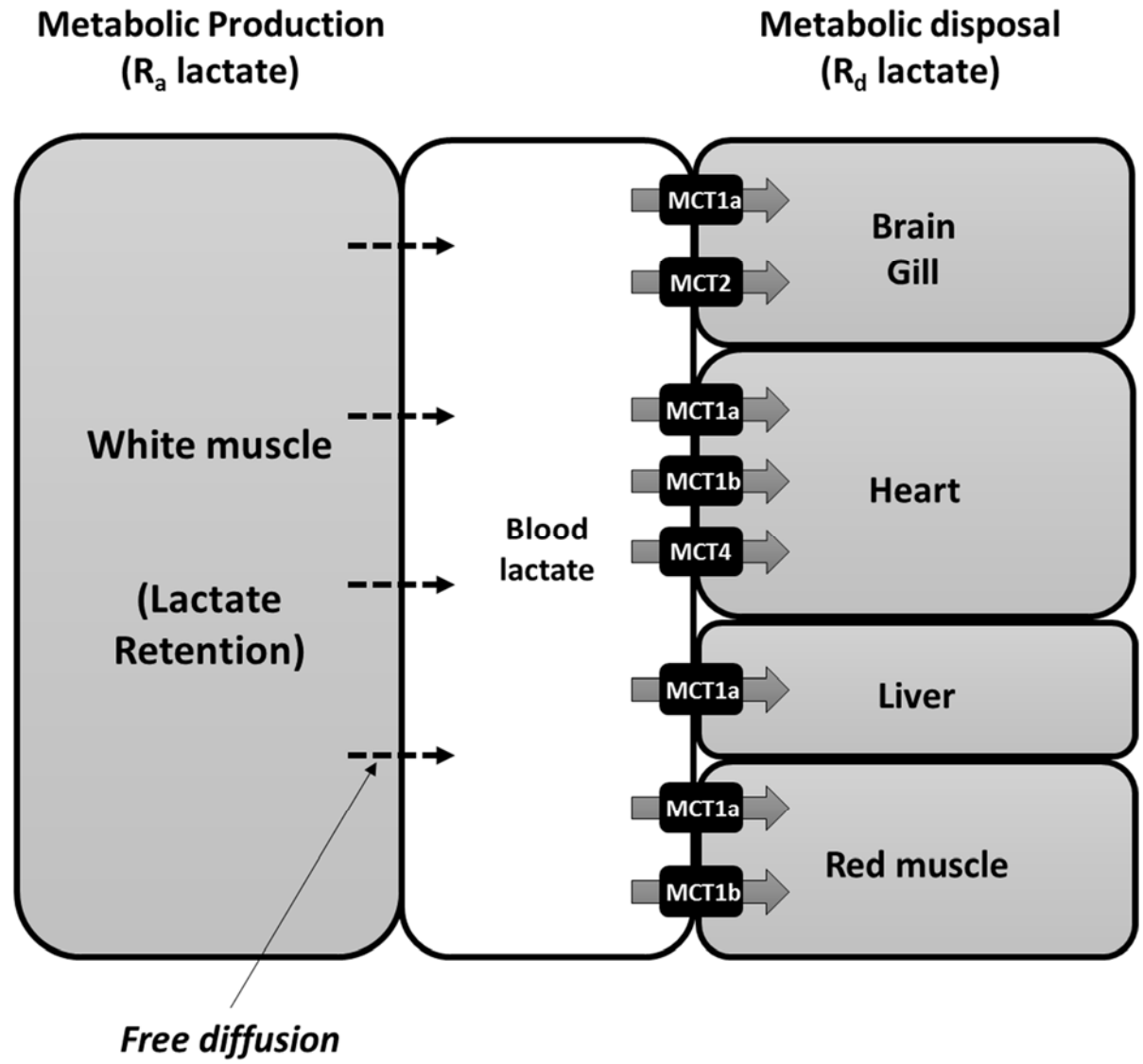


Figure 6.2 Proposed major transmembrane lactate movement resulting in the net blood lactate production (R_a) and disposal (R_d) in rainbow trout during acute hypoxia or intense exercise. Dashed arrows represent free diffusion of lactate from white muscle (main producer of lactate) and black boxes with arrow MCT transport into oxidative tissues.



REFERENCES

- Allsop, J. R., Wolfe, R. R. and Burke, J. F.** (1978). The reliability of rates of glucose appearance in vivo calculated from constant tracer infusions. *Biochemical Journal* **172**, 407-416.
- Allsop, J. R., Wolfe, R. R., Distephano, J. J. and Burke, J. F.** (1979). Reliability of rates of glucose appearance in vivo calculated from single tracer injections. *Canadian Journal of Physiology and Pharmacology* **57**, 1267-1274.
- Baba, M., Inoue, M., Itoh, K. and Nishizawa, Y.** (2008). Blocking CD147 induces cell death in cancer cells through impairment of glycolytic energy metabolism. *Biochemical and Biophysical Research Communications* **374**, 111-116.
- Bergman, B. C., Wolfel, E. E., Butterfield, G. E., Lopaschuk, G. D., Casazza, G. A., Horning, M. A. and Brooks, G. A.** (1999). Active muscle and whole body lactate kinetics after endurance training in men. *J. Appl. Physiol.* **87**, 1684-1696.
- Bergmeyer, H. U.** (1985). *Methods of Enzymatic Analysis*: Weinheim: VCH.
- Bernard, S. F., Reidy, S. P., Zwingelstein, G. and Weber, J.-M.** (1999). Glycerol and fatty acid kinetics in rainbow trout: effects of endurance swimming. *Journal of Experimental Biology* **202**, 279-288.
- Bernier, N., Harris, J., Lessard, J. and Randall, D.** (1996). Adenosine receptor blockade and hypoxia-tolerance in rainbow trout and Pacific hagfish. I. Effects on anaerobic metabolism. *J Exp Biol* **199**, 485-495.
- Bickham, D. C., Bentley, D. J., Le Rossignol, P. F. and Cameron-Smith, D.** (2006). The effects of short-term sprint training on MCT expression in moderately endurance-trained runners. *European Journal of Applied Physiology* **96**, 636-643.
- Bilinski, E. and Jonas, R. E. E.** (1972). Oxidation of Lactate to Carbon Dioxide by Rainbow Trout (*Salmo gairdneri*) Tissues. *J. Fish. Res. Bd. Can.* **29**, 1467-1471.
- Bonen, A., Heynen, M. and Hatta, H.** (2006). Distribution of monocarboxylate transporters MCT1-MCT8 in rat tissues and human skeletal muscle. *Applied Physiology Nutrition and Metabolism-Physiologie Appliquee Nutrition Et Metabolisme* **31**, 31-39.
- Bonen, A., Tonouchi, M., Miskovic, D., Heddle, C., Heikkila, J. J. and Halestrap, A. P.** (2000). Isoform-specific regulation of the lactate transporters MCT1 and MCT4 by contractile activity. *American Journal of Physiology-Endocrinology and Metabolism* **279**, E1131-E1138.

Boutilier, R. G., Dobson, G., Hoeger, U. and Randall, D. J. (1987). Acute exposure to graded levels of hypoxia in rainbow trout (*Salmo gairdneri*): Metabolic and respiratory adaptations. *Respiration Physiology* **71**, 69-82.

Brett, J. R. (1964). The respiratory metabolism and swimming performance of young sockeye salmon. *Journal of the Fisheries Board of Canada* **21**, 1183-1226.

Brett, J. R. (1972). The metabolic demand for oxygen in fish, particularly salmonids, and a comparison with other vertebrates. *Resp. Physiol.* **14**, 151-170.

Brooks, G. A. (1991). Current concepts in lactate exchange. *Medicine and Science in Sports and Exercise* **23**, 895-906.

Brooks, G. A. (1998). Mammalian fuel utilization during sustained exercise. *Comp. Biochem. Physiol.* **120B**, 89-107.

Brooks, G. A. (2002). Lactate shuttles in nature. *Biochemical Society Transactions* **30**, 258-264.

Brooks, G. A., Brown, M. A., Butz, C. E., Sicurello, J. P. and Dubouchaud, H. (1999). Cardiac and skeletal muscle mitochondria have a monocarboxylate transporter MCT1. *Journal of Applied Physiology* **87**, 1713-1718.

Brooks, G. A., Butterfield, G. E., Wolfe, R. R., Groves, B. M., Mazzeo, R. S., Sutton, J. R., Wolfel, E. E. and Reeves, J. T. (1991). Decreased reliance on lactate during exercise after acclimatization to 4,300 m. *Journal of Applied Physiology* **71**, 333-341.

Cameron, J. N. and Cech Jr., J. J. (1990). Lactate kinetics in exercised channel catfish, *Ictalurus punctatus*. *Physiol. Zool.* **63**, 909-920.

Choi, J. H., Park, M. J., Kim, K. W., Choi, Y. H., Park, S. H., An, W. G., Yang, U. S. and Cheong, J. (2005). Molecular mechanism of hypoxia-mediated hepatic gluconeogenesis by transcriptional regulation. *FEBS Letters* **579**, 2795-2801.

Claireaux, G., Couturier, C. and Groison, A. L. (2006). Effect of temperature on maximum swimming speed and cost of transport in juvenile European sea bass (*Dicentrarchus labrax*). *Journal of Experimental Biology* **209**, 3420-3428.

Clark, M. G., Bloxham, D. P., Holland, P. C. and Lardy, H. A. (1974). Estimation of the fructose 1,6-diphosphatase-phosphofructokinase substrate cycle and its relationship to gluconeogenesis in rat liver in vivo. *J. Biol. Chem.* **249**, 279-90.

Coles, L., Litt, J., Hatta, H. and Bonen, A. (2004). Exercise rapidly increases expression of the monocarboxylate transporters MCT1 and MCT4 in rat muscle. *Journal of Physiology-London* **561**, 253-261.

Donohoe, P. H. and Boutilier, R. G. (1999). The use of extracellular lactate as an oxidative substrate in the oxygen-limited frog. *Respir Physiol* **116**, 171-179.

- Donovan, C. M. and Brooks, G. A.** (1983). Endurance training affects lactate clearance, not lactate production. *Am J Physiol* **244**.
- Drake, A. J., Haines, J. R. and Noble, M. I. M.** (1980). Preferential uptake of lactate by the normal myocardium in dogs. *Cardiovascular Research* **14**, 65-72.
- Drury, D. R. and Wick, A. N.** (1956). Metabolism of lactic acid in the intact rabbit *American Journal of Physiology* **184**, 304-308.
- Dubouchaud, H., Butterfield, G. E., Wolfel, E. E., Bergman, B. C. and Brooks, G. A.** (2000). Endurance training, expression, and physiology of LDH, MCT1, and MCT4 in human skeletal muscle. *American Journal of Physiology - Endocrinology and Metabolism* **278**, E571-E579.
- Dunn, J. and Hochachka, P.** (1987). Turnover rates of glucose and lactate in rainbow trout during acute hypoxia. *Can J Zool* **65**, 1144-1148.
- Dunn, J. F. and Hochachka, P. W.** (1986). Metabolic Responses of Trout (*Salmo Gairdneri*) to Acute Environmental Hypoxia. *J Exp Biol* **123**, 229-242.
- Emhoff, C. A. W., Messonnier, L. A., Horning, M. A., Fattor, J. A., Carlson, T. J. and Brooks, G. A.** (2013). Gluconeogenesis and hepatic glycogenolysis during exercise at the lactate threshold. *Journal of Applied Physiology* **114**, 297-306.
- Ferrannini, E., Natali, A., Brandi, L. S., Bonadonna, R., De Kreutzemberg, S. V., DelPrato, S. and Santoro, D.** (1993). Metabolic and thermogenic effects of lactate infusion in humans. *American Journal of Physiology - Endocrinology and Metabolism* **265**, E504-E512.
- Fournier, P. A. and Guderley, H.** (1992). Metabolic fate of lactate after vigorous activity in the leopard frog, *Rana pipiens*. *American Journal of Physiology* **262**, R245-R254.
- Fournier, R. A. and Weber, J. M.** (1994). Locomotory energetics and metabolic fuel reserves of the Virginia opossum. *J Exp Biol* **197**, 1-16.
- Garcia, C. K., Goldstein, J. L., Pathak, R. K., Anderson, R. G. W. and Brown, M. S.** (1994). Molecular characterization of a membrane transporter for lactate, pyruvate, and other monocarboxylates: Implications for the Cori cycle. *Cell* **76**, 865-873.
- Gladden, L. B.** (2004). Lactate metabolism: a new paradigm for the third millenium. *J. Physiol.* **558.1**, 5-30.
- Gleeson, T.** (1996). Post-exercise lactate metabolism: A Comparative review of sites, pathways, and regulation. *Annu. Rev. Physiol.* **58**, 565-581.
- Haesler, E., Schneiter, P., Temler, E., Jequier, E. and Tappy, L.** (1995). Effects of lactate infusion on hepatic gluconeogenesis and glycogenolysis. *Clinical Physiology* **15**, 581-595.

Halestrap, A. P. and Meredith, D. (2004). The SLC16 gene family - From monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflugers Archiv European Journal of Physiology* **447**, 619-628.

Halestrap, A. P. and Price, N. T. (1999). The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochemical Journal* **343**, 281-299.

Halestrap, A. P. and Wilson, M. C. (2012). The monocarboxylate transporter family-Role and regulation. *IUBMB Life* **64**, 109-119.

Haman, F., Powell, M. and Weber, J.-M. (1997a). Reliability of continuous tracer infusion for measuring glucose turnover rate in rainbow trout. *J Exp Biol* **200**, 2557-2563.

Haman, F. and Weber, J. M. (1996). Continuous tracer infusion to measure in vivo metabolite turnover rates in trout. *Journal of Experimental Biology* **199**, 1157-1162.

Haman, F., Zwingelstein, G. and Weber, J. M. (1997b). Effects of hypoxia and low temperature on substrate fluxes in fish: plasma metabolite concentrations are misleading. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **42**, R2046-R2054.

Hashimoto, T., Masuda, S., Taguchi, S. and Brooks, G. A. (2005). Immunohistochemical analysis of MCT1, MCT2 and MCT4 expression in rat plantaris muscle. *Journal of Physiology* **567**, 121-129.

Heath, A. G. (1995). Water Pollution and Fish Physiology. *Published by CRC Press*, 359 pages.

Huelsenbeck, J. P. and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754-755.

Issekutz, B., Jr., Shaw, W. A. and Issekutz, A. C. (1976). Lactate metabolism in resting and exercising dogs. *J Appl Physiol* **40**, 312-319.

Izumi, Y., Benz, A. M., Katsuki, H. and Zorumski, C. F. (1997). Endogenous monocarboxylates sustain hippocampal synaptic function and morphological integrity during energy deprivation. *Journal of Neuroscience* **17**, 9448-9457.

Jain, K., Hamilton, J. and Farrell, A. (1997). Use of a ramp velocity test to measure critical swimming speed in rainbow trout (*Onchorhynchus mykiss*). *Comp. Biochem. Physiol. A* **117**, 441-444.

Jenssen, T., Nurjhan, N., Consoli, A. and Gerich, J. E. (1993). Dose-response effects of lactate infusions on gluconeogenesis from lactate in normal man. *European Journal of Clinical Investigation* **23**, 448-454.

Katz, J., Okajima, F., Chenoweth, M. and Dunn, A. (1981). The determination of lactate turnover in vivo with 3H- and 14C-labelled lactate. The significance of sites of tracer administration and sampling. *Biochemical Journal* **194**, 513-524.

Laberee, K. and Milligan, C. L. (1999). Lactate transport across sarcolemmal vesicles isolated from rainbow trout white muscle. *Journal of Experimental Biology* **202**, 2167-2175.

Lai, J. C. C., Kakuta, I., Mok, H. O. L., Rummer, J. L. and Randall, D. (2006). Effects of moderate and substantial hypoxia on erythropoietin levels in rainbow trout kidney and spleen. *Journal of Experimental Biology* **209**, 2734-2738.

Lanctin, H. P., McMorran, L. E. and Driedzic, W. R. (1980). Rates of glucose and lactate oxidation by the perfused isolated trout (*Salvelinus fontinalis*) heart. *Canadian Journal of Zoology* **58**, 1708-1711.

Larrabee, M. G. (1995). Lactate metabolism and its effects on glucose metabolism in an excised neural tissue. *Journal of Neurochemistry* **64**, 1734-1741.

Lemoine, C. M. R., Lougheed, S. C. and Moyes, C. D. (2010). Modular evolution of PGC-1 α in vertebrates. *Journal of Molecular Evolution* **70**, 492-505.

Lenz, T., Nadansky, M., Gossmann, J., Oremek, G. and Geiger, H. (1998). Exhaustive exercise-induced tissue hypoxia does not change endothelin and big endothelin plasma levels in normal volunteers[ast]. *Am J Hypertens* **11**, 1028-1031.

Magnoni, L., Vaillancourt, E. and Weber, J.-M. (2008a). High resting triacylglycerol turnover of rainbow trout exceeds the energy requirements of endurance swimming. *Am J Physiol Regul Integr Comp Physiol* **295**, R309-R315.

Magnoni, L., Vaillancourt, E. and Weber, J.-M. (2008b). In vivo regulation of rainbow trout lipolysis by catecholamines. *Journal of Experimental Biology* **211**, 2460-2466.

Marcinek, D. J., Kushmerick, M. J. and Conley, K. E. (2010). Lactic acidosis in vivo: Testing the link between lactate generation and H⁺ accumulation in ischemic mouse muscle. *J App Physiol* **108**, 1479-1486.

Martinez, M. L., Landry, C., Boehm, R., Manning, S., Cheek, A. O. and Rees, B. B. (2006). Effects of long-term hypoxia on enzymes of carbohydrate metabolism in the Gulf killifish, *Fundulus grandis*. *J Exp Biol* **209**, 3851-3861.

Mazer, C. D., Stanley, W. C., Hickey, R. F., Neese, R. A., Cason, B. A., Demas, K. A., Wisneski, J. A. and Gertz, E. W. (1990). Myocardial metabolism during hypoxia: maintained lactate oxidation during increased glycolysis. *Metabolism* **39**, 913-918.

McClelland, G. B. and Brooks, G. A. (2002). Changes in MCT 1, MCT 4, and LDH expression are tissue specific in rats after long-term hypobaric hypoxia. *Journal of Applied Physiology* **92**, 1573-1584.

McClelland, G. B., Khanna, S., Gonza?lez, G. F., Butz, C. E. and Brooks, G. A. (2003). Peroxisomal membrane monocarboxylate transporters: Evidence for a redox shuttle system? *Biochemical and Biophysical Research Communications* **304**, 130-135.

Meredith, D. and Christian, H. C. (2008). The SLC16 monocarboxylate transporter family. *Xenobiotica* **38**, 1072-1106.

Miller, B. F., Fattor, J. A., Jacobs, K. A., Horning, M. A., Navazio, F., Lindinger, M. I. and Brooks, G. A. (2002a). Lactate and glucose interactions during rest and exercise in men: effect of exogenous lactate infusion. *Journal of Physiology-London* **544**, 963-975.

Miller, B. F., Fattor, J. A., Jacobs, K. A., Horning, M. A., Suh, S. H., Navazio, F. and Brooks, G. A. (2002b). Metabolic and cardiorespiratory responses to "the lactate clamp". *American Journal of Physiology-Endocrinology and Metabolism* **283**, E889-E898.

Milligan, C. L. (1996). Metabolic recovery from exhaustive exercise in rainbow trout. *Comparative Biochemistry and Physiology - A Physiology* **113**, 51-60.

Milligan, C. L. and Farrell, A. P. (1991). Lactate utilization by an *in Situ* perfused trout heart: effects of workload and blockers of lactate transport. *J Exp Biol* **155**, 357-373.

Milligan, C. L. and Girard, S. S. (1993). Lactate metabolism in rainbow-trout. *Journal of Experimental Biology* **180**, 175-193.

Milligan, C. L., Hooke, G. B. and Johnson, C. (2000). Sustained swimming at low velocity following a bout of exhaustive exercise enhances metabolic recovery in rainbow trout. *J Exp Biol* **203**, 921-926.

Milligan, C. L. and McDonald, D. G. (1988). In vivo lactate kinetics at rest and during recovery from exhaustive exercise in coho salmon (*Oncorhynchus kisutch*) and starry flounder (*Platichthys stellatus*). *Journal of Experimental Biology* **135**, 119-131.

Moyes, C. D. and Schulte, P. M. (2008). Principles of animal physiology. **Second edition.**

Moyes, C. D. and West, T. G. (1995). Exercise metabolism of fish. *Biochemistry and molecular biology of fishes* **4**, 367-392.

Ngan, A. K. and Wang, Y. S. (2009). Tissue-specific transcriptional regulation of monocarboxylate transporters (MCTs) during short-term hypoxia in zebrafish (*Danio rerio*). *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* **154**, 396-405.

Nilsson, G. E. and Ostlund-Nilsson, S. (2008). Does size matter for hypoxia tolerance in fish? *Biological Reviews* **83**, 173-189.

Omlin, T. and Weber, J.-M. (2010). Hypoxia stimulates lactate disposal in rainbow trout. *J Exp Biol* **213**, 3802-3809.

Omlin, T. and Weber, J. M. (2013). Exhausting exercise and tissue-specific expression of monocarboxylate transporters in rainbow trout. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **304**, R1036-R1043.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**.

Pfaffl, M. W., Tichopad, A., Prgomet, C. and Neuvians, T. P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - Excel-based tool using pair-wise correlations. *Biotechnology Letters* **26**, 509-515.

Philp, A., Macdonald, A. L. and Watt, P. W. (2005). Lactate - A signal coordinating cell and systemic function. *Journal of Experimental Biology* **208**, 4561-4575.

Polakof, S. and Soengas, J. L. (2008a). Differential effects of in vivo and in vitro lactate treatments on liver carbohydrate metabolism of rainbow trout. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* **151**, 205-210.

Polakof, S. and Soengas, J. L. (2008b). Involvement of lactate in glucose metabolism and glucosensing function in selected tissues of rainbow trout. *Journal of Experimental Biology* **211**, 1075-1086.

Randall, D. and Shelton, G. (1963). The effects of changes in environmental gas concentrations on the breathing and heart rate of a teleost fish. *comparative Biochemistry and Physiology* **9**, 229-239.

Randall, D. J. and Daxboeck, C. (1982). Cardiovascular changes in the rainbow trout (*Salmo gairdneri*, Richardson) during exercise. *Canadian Journal of Zoology-Revue Canadienne De Zoologie* **60**, 1135-1140.

Richards, J. G., Farrell, A. P. and Brauner, C. J. (2009). Hypoxia. In *Fish Physiology*, vol. 27 eds. A. P. Farrell and C. J. Brauner), pp. 528. London: Elsevier, Academic Press.

Richards, J. G., Heigenhauser, G. J. F. and Wood, C. M. (2002). Glycogen phosphorylase and pyruvate dehydrogenase transformation in white muscle of trout during high-intensity exercise. *Am J Physiol Regul Integr Comp Physiol* **282**, R828-R836.

Richards, J. G., Wang, Y. S., Brauner, C. J., Gonzalez, R. J., Patrick, M. L., Schulte, P. M., Choppari-Gomes, A. R., Almeida-Val, V. M. and Val, A. L. (2007). Metabolic and ionoregulatory responses of the Amazonian cichlid, *Astronotus ocellatus*, to severe hypoxia. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **177**, 361-374.

Roef, M. J., De Meer, K., Kalhan, S. C., Straver, H., Berger, R. and Reijngoud, D. J. (2003). Gluconeogenesis in humans with induced hyperlactatemia during low-intensity exercise. *American Journal of Physiology - Endocrinology and Metabolism* **284**.

Ronquist, F. and Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572-1574.

Roth, D. A. and Brooks, G. A. (1990). Lactate transport is mediated by a membrane-bound carrier in rat skeletal muscle sarcolemmal vesicles. *Archives of Biochemistry and Biophysics* **279**, 377-385.

Schurr, A., Payne, R. S., Miller, J. J. and Rigor, B. M. (1997). Brain lactate is an obligatory aerobic energy substrate for functional recovery after hypoxia: Further in vitro validation. *Journal of Neurochemistry* **69**, 423-426.

Schurr, A., West, C. A. and Rigor, B. M. (1988). Lactate-supported synaptic function in the rat hippocampal slice preparation. *Science* **240**, 1326-1328.

Searle, G. L., Feingold, K. R., Hsu, F. S. F., Clark, O. H., Gertz, E. W. and Stanley, W. C. (1989). Inhibition of endogenous lactate turnover with lactate infusion in humans. *Metabolism: Clinical and Experimental* **38**, 1120-1123.

Shanghavi, D. S. and Weber, J.-M. (1999). Effects of sustained swimming on hepatic glucose production of rainbow trout. *Journal of Experimental Biology* **202**, 2161-2166.

Sharpe, R. L. and Milligan, C. L. (2003). Lactate efflux from sarcolemmal vesicles isolated from rainbow trout *Oncorhynchus mykiss* white muscle is via simple diffusion. *Journal of Experimental Biology* **206**, 543-549.

Shoubridge, E. A. and Hochachka, P. W. (1980). Ethanol: Novel end product of vertebrate anaerobic metabolism. *Science* **209**, 308-309.

Smith, D., Pernet, A., Hallett, W. A., Bingham, E., Marsden, P. K. and Amiel, S. A. (2003). Lactate: A preferred fuel for human brain metabolism in vivo. *Journal of Cerebral Blood Flow and Metabolism* **23**, 658-664.

Soengas, J. L. and Aldegunde, M. (2002). Energy metabolism of fish brain. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **131**, 271-296.

Stanley, W. C., Gertz, E. W., Wisneski, J. A., Morris, D. L., Neese, R. A. and Brooks, G. A. (1985). Systemic lactate kinetics during graded exercise in man. *Am J Physiol* **249**.

Steele, R. (1959). Influence of glucose loading and of injected insulin on hepatic glucose output. *Ann, NY Acad Sci* **82**, 420-430.

Tabernerero, A., Vicario, C. and Medina, J. M. (1996). Lactate spares glucose as a metabolic fuel in neurons and astrocytes from primary culture. *Neuroscience Research* **26**, 369-376.

Thomas, C., Bishop, D. J., Lambert, K., Mercier, J. and Brooks, G. A. (2012). Effects of acute and chronic exercise on sarcolemmal MCT1 and MCT4 contents in human skeletal muscles: current status. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **302**, R1-R14.

Thomas, S. and Hughes, G. M. (1982a). Effects of hypoxia on blood gas and acid-base parameters of sea bass. *Journal of Applied Physiology Respiratory Environmental and Exercise Physiology* **53**, 1336-1341.

Thomas, S. and Hughes, G. M. (1982b). A study of the effects of hypoxia on acid-base status of rainbow trout blood using an extracorporeal blood circulation. *Respiration Physiology* **49**, 371-382.

Thomas, S., Perry, S. F., Pennec, Y. and Maxime, V. (1992). Metabolic alkalosis and the response of the trout, *Salmo fario*, to acute severe hypoxia. *Respiration Physiology* **87**, 91-104.

Turner, J. D. and Wood, C. M. (1983). Factors affecting lactate and proton efflux from pre-exercised, isolated-perfused rainbow trout trunks. *J Exp Biol* **105**, 395-401.

Turner, J. D., Wood, C. M. and Clark, D. (1983). Lactate and Proton Dynamics in the Rainbow Trout (*Salmo Gairdneri*). *J Exp Biol* **104**, 247-268.

Umezawa, T., Kato, A., Ogoshi, M., Ookata, K., Munakata, K., Yamamoto, Y., Islam, Z., Doi, H., Romero, M. F. and Hirose, S. (2012). O₂-filled swimbladder employs monocarboxylate transporters for the generation of O₂ by lactate-induced root effect hemoglobin. *PloS one* **7**, e34579.

Van Hall, G. (2000). Lactate as a fuel for mitochondrial respiration. *Acta Physiol Scand* **168**, 643-656.

Van Hall, G. (2010). Lactate kinetics in human tissues at rest and during exercise. *Acta Physiologica* **199**, 499-508.

van Hall, G., Jensen-Urstad, M., Rosdahl, H., Holmberg, H.-C., Saltin, B. and Calbet, J. A. L. (2003). Leg and arm lactate and substrate kinetics during exercise. *American Journal of Physiology - Endocrinology and Metabolism* **284**, E193-E205.

van Raaij, M. T. M., van den Thillart, G. E. E. J. M., Vianen, G. J., Pit, D. S. S., Balm, P. H. M. and Steffens, A. B. (1996). Substrate mobilization and hormonal changes in rainbow trout (*Oncorhynchus mykiss*, L.) and common carp (*Cyprinus carpio*, L.) during deep hypoxia and subsequent recovery. *Journal of Comparative Physiology - B Biochemical, Systemic, and Environmental Physiology* **166**, 443-452.

Voet, D., Voet, J. and Pratt, C. (1999). Fundamentals of Biochemistry. *John Wiley and Sons, Inc.*

Wang, Y., Wright, P. M., Heigenhauser, G. J. F. and Wood, C. M. (1997). Lactate transport by rainbow trout white muscle: Kinetic characteristics and sensitivity to inhibitors. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **272**.

Wardle, C. S. (1978). Non-release of lactic acid from anaerobic swimming muscle of plaice *Pleuronectes platessa* L.: a stress reaction. *Journal of Experimental Biology* **77**, 141-155.

Watt, P. W., MacLennan, P. A., Hundal, H. S., Kuret, C. M. and Rennie, M. J. (1988). I(+)-Lactate transport perfused rat skeletal muscle: kinetic characteristics and sensitivity to pH and transport inhibitors. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **944**, 213-222.

Weber, J.-M., Brichon, G., Bodennec, J. and Zwingelstein, G. (2002). Palmitate and oleate metabolism of rainbow trout *in vivo*. *Comp. Biochem. Physiol. A* **131**, 409-416.

Weber, J.-M., Klein, S. and Wolfe, R. R. (1990). Role of the glucose cycle in control of net glucose flux in exercising humans. *J App Physiol* **68**, 1815-1819.

Weber, J.-M., Parkhouse, W. S., Dobson, G. P., Harman, J. C., Snow, D. H. and Hochachka, P. W. (1987a). Lactate kinetics in exercising Thoroughbred horses: regulation of turnover rate in plasma. *Am J Physiol* **253**, R896-903.

Weber, J.-M. and Shanghavi, D. S. (2000). Regulation of glucose production in rainbow trout: Role of epinephrine *in vivo* and in isolated hepatocytes. *Am. J. Physiol. Regulatory Integrative Comp. Physiol.* **278**, R956-R963.

Weber, J. M. (1991). Effect of endurance swimming on the lactate kinetics of rainbow trout. *Journal of Experimental Biology* **158**, 463-476.

Weber, J. M. (2001). Energy Cycle in Vertebrates. *Encyclopedia of Life Sciences*/www.els.net.

Weber, J. M. (2011). Metabolic fuels: regulating fluxes to select mix. *Journal of Experimental Biology* **214**, 286-294.

Weber, J. M., Brill, R. W. and Hochachka, P. W. (1986). Mammalian metabolite flux rates in a teleost: Lactate and glucose turnover in tuna. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **250**.

Weber, J. M., Dobson, G. P., Parkhouse, W. S., Wheeldon, D., Harman, J. C., Snow, D. H. and Hochachka, P. W. (1987b). Cardiac-output and oxygen consumption in exercising thoroughbred horses. *American Journal of Physiology* **253**, R890-R895.

Wolf, K. (1963). Physiological saline for fresh water teleosts. *Prog Fish-Cult* **25**, 135-140.

Wolfe, R. R. (1992). Radioactive and stable isotope tracers in biomedicine. Principles and practice of kinetic analysis. New York: Wiley-Liss.

Wright, P. A., Perry, S. F. and Moon, T. W. (1989). Regulation of hepatic gluconeogenesis and glycogenolysis by catecholamines in rainbow trout during environmental hypoxia. *J Exp Biol* **147**, 169-188.

Yoon, H. and Philp, N. J. (1998). Genomic structure and developmental expression of the chicken monocarboxylate transporter MCT3 gene. *Experimental Eye Research* **67**, 417-424.