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Lipid mobilization and the role of lipoproteins during swimming in salmonids

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Lipid mobilization and the role of lipoproteins during swimming in salmonids

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Lipid mobilization and the role of lipoproteins during swimming in salmonids

SUMMARY

Lipids circulate as non-esterified fatty acids (NEFA) or as triacylglycerol (TAG) associated with phospholipids (PL), the two main components of lipoproteins. By analogy to mammals, previous studies have focused on NEFA, but lipoproteins have not been considered as an energy shuttle to working muscles. The main goal of this thesis was to study the effects of exercise on fish lipoprotein metabolism. Additional objectives of this thesis were to clarify the mechanisms of action of lipoprotein lipase (LPL) on circulating lipids and to study how lipolytic rate may be regulated *in vivo* by catecholamines in fish. Circulating NEFA, TAG and PL were measured in sockeye salmon (*Oncorhynchus nerka*) at different stages of their spawning migration (Chapter 2). NEFA represent less than 7% of total plasma fatty acids and only show a minor decrease during migration. In contrast, lipoproteins account for more than 93% of all the energy of circulating lipids, and their main constituents show a 27-fold decrease (TAG) and a 6-fold decrease (PL) in concentration. These results are consistant with the idea that lipoproteins may be used to cover the metabolic demands of exercise. In Chapter 3, I investigated the effects of endurance swimming on the lipoprotein metabolism of rainbow trout (*Oncorhynchus mykiss*) under controlled laboratory conditions. Red muscle LPL activity increased from 18 ± 5 (rest) to 49 ± 9 nmol FA min^{-1} g^{-1} (swimming for 4 days at 1.5 body lengths/s). In Chapter 4, I measured TAG turnover rate for the first time in a non-mammalian vertebrate. Results show that the baseline TAG turnover rate of trout ranges from 24 to 49 μmol TAG kg^{-1} min^{-1} and is not affected by prolonged exercise (6 h at 1.5 body length/s), exceeding all values

measured to date in endotherms. Finally, I measured lipolytic rate in intact rainbow trout at rest, and show contrasting effects of norepinephrine (NE) and epinephrine (Epi) administration on lipolysis (Chapter 5). Results show that trout maintain particularly high baseline lipolytic rate, because only 13% of their lipolytic rate can fuel resting energy metabolism entirely (87% reesterification). Baseline glycerol turnover rate (4.6 $\pm$ 0.4 μmol kg^{-1} min^{-1}) is inhibited by NE (-56%), instead of being stimulated as in mammals, whereas Epi has the same activating effect in both groups of vertebrates (+167%). I show that the TAG turnover rate in trout at rest can cover several times the energy requirements of locomotion. Additionally, I demonstrate that trout maintain high lipolytic rates, indicating that fatty acid mobilization and reesterification are particularly active in fish. Both characteristics of lipid metabolism can allow for the rapid cycling of fatty acids, and may be crucial for restructuring membrane phospholipids and, therefore, necessary in all ectotherms for adequate homeoviscous adaptation.

RÉSUMÉ

Les lipides circulent en tant qu'acides gras non-estérifiés (NEFA) ou comme du triacylglycérol (TAG) associé à des phospholipides (PL), les deux composantes principales des lipoprotéines. Par analogie aux mammifères, les études précédentes se sont concentrées sur les NEFA, mais les lipoprotéines n'ont pas été considérées comme une navette énergétique pour les muscles au travail. Le but principal de cette thèse était d'étudier les effets de l'exercice sur le métabolisme des lipoprotéines chez les poissons. Les objectifs secondaires de cette thèse étaient de clarifier les mécanismes d'action de la lipoprotéine lipase (LPL) sur les lipides circulant et d'étudier comment le taux de lipolyse peut être contrôlé *in vivo* par les catécholamines chez les poissons. Les NEFA, TAG et PL circulant furent mesurées chez le saumon Sockeye (*Oncorhynchus nerka*) à différents stades de leur migration de fraye (Chapitre 2). J'ai découvert que la concentration plasmatique des lipoprotéines du saumon Sockeye change dramatiquement durant la migration. Les NEFA représentent moins de 7% des acides gras plasmatiques totaux et leur importance baisse légèrement durant la migration. Au contraire, les lipoprotéines représentent plus de 93% de toute l'énergie contenue dans les lipides circulants et leurs constituants principaux présentent des concentrations plasmatiques 27 fois (TAG) et 6 fois (PL) moins importantes lors de la migration. De tels changements de concentrations des lipides plasmatiques des saumons en migration sont en accord avec l'idée que les lipoprotéines peuvent être utilisées pour combler les besoins métaboliques liés à l'exercice. Cependant, il n'est pas clair si cette réponse à la migration chez le saumon Sockeye est causée seulement par l'exercice ou par une

combinaison de facteurs de stress. Pour exclure les facteurs confondants, j'ai recherché les effets de la nage d'endurance sur le métabolisme des lipoprotéines chez la truite arc-en-ciel (*Oncorhynchus mykiss*) sous des conditions contrôlées en laboratoire (Chapitre 3). L'activité de la LPL des muscles rouges a augmentée de 8 ± 5 (repos) à 49 ± 9 nmol d'acides gras·min^{-1} g^{-1} (nage de 4 jours à 1.5 longueur corporelle·s^{-1}). Dans le Chapitre 4, j'ai mesuré le taux de renouvellement des lipoprotéines circulantes pour la première fois chez un vertébré autre qu'un mammifère. Les résultats démontrent que le taux basal de renouvellement des lipoprotéines chez la truite varient de 24 à 49 μmol de TAG·kg^{-1} min^{-1} et dépassent toutes les valeurs mesurées à ce jour chez les endothermes. Chez la truite arc-en-ciel, le flux de lipoprotéines n'est pas affecté par l'exercice prolongé (nage de 6 heures à 1.5 longueur corporelle·s^{-1}). Finallement, j'ai mesuré le taux de lipolyse chez des truites arc-en-ciel intactes au repos et je présente des effets contrastants de l'administration de norépinéphrine (NE) et d'épinéphrine (Epi) sur la lipolyse (Chapitre 5). Les résultats démontrent que la truite maintient un taux basal de lipolyse particulièrement élevé, car une faible fraction de 13% de ce taux de lipolyse permet de supporter le métabolisme au repos (87% de réestérification). Le taux basal de renouvellement du glycérol (4.6±0.4 μmol kg^{-1} min^{-1}) est inhibé par la NE (-56%), au lieu d'être stimulé comme chez les mammifères, alors que l'Epi a le même effet d'activation chez les deux groupes de vertébrés (+167%). Je démontre que le taux de renouvellement des lipoprotéines chez la truite au repos peut combler plusieurs fois les demandes énergétiques de la locomotion. De plus, je démontre que la truite maintient un taux de lipolyse élevé, ce qui indique que la mobilisation et la réestérification des

acides gras sont particulièrement actifs chez les poissons. Ces deux caractéristiques du métabolisme de lipides peuvent permettre un recyclage rapide des acides gras et peuvent jouer un rôle crucial dans la restructuration des phospholipides membranaires et sont ainsi nécessaires à tous les ectothermes pour permettre une adaptation homéovisceuse adéquate.

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CHAPTER 1.

GENERAL INTRODUCTION

I. Lipid mobilization in vertebrates

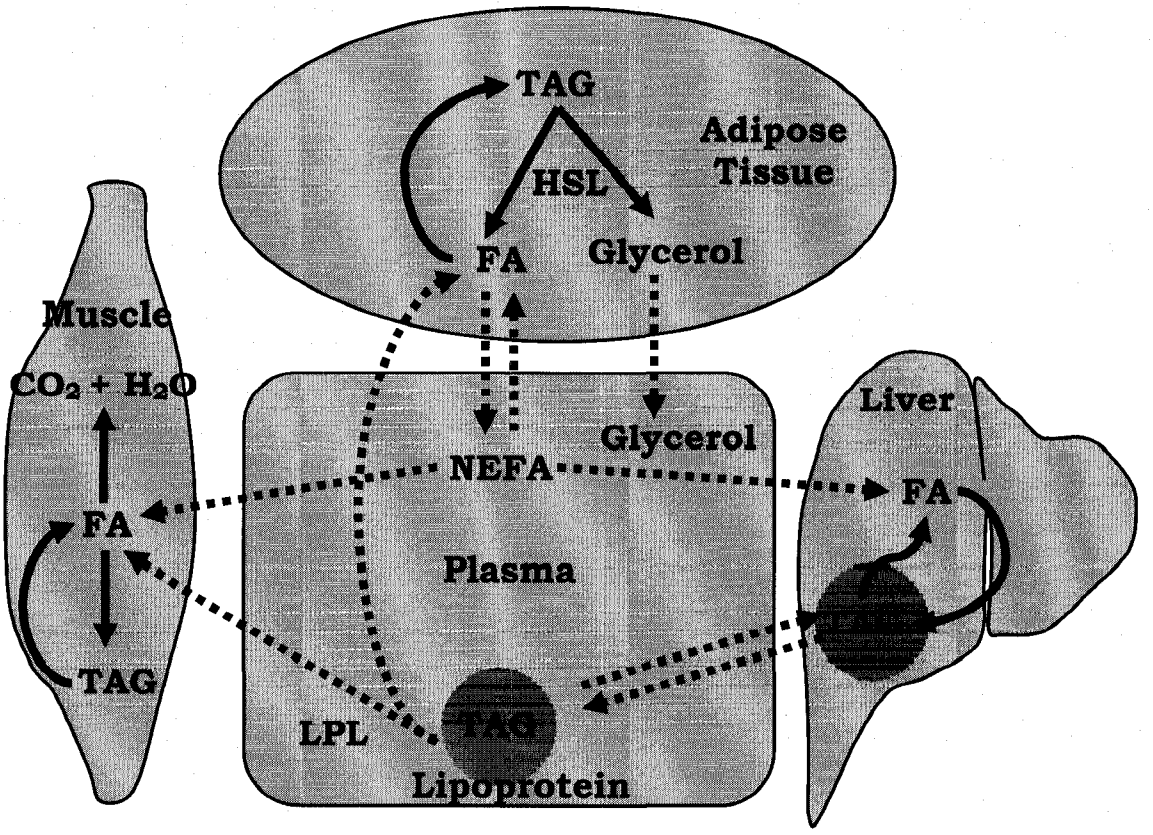
Lipids are structurally and functionally diverse in organisms. It is generally assumed that they have two key roles. First, triacylglycerols (TAG) are commonly used as metabolic substrates to obtain energy through oxidation. Second, lipids are a major structural component of biological membranes, mainly in the form of phospholipids (PL). Lipid metabolism can be regulated at different levels that include the synthesis, deposition, mobilization, and utilization of fatty acids (FA). Among these levels, FA mobilization is believed to play a fundamental role in energy metabolism. The complete hydrolysis of TAG and PL (lipolysis) generates FA and glycerol. Therefore lipid mobilization can generate FA, which can vary in carbon chain length and in degree of saturation. In recent years, the scientific knowledge of how lipids are mobilized in humans has advanced enormously, perhaps in relation to the study of how lipid metabolism can affect human health. In turn, this has driven basic research, looking to investigate the existence of fundamental mechanisms of lipid mobilization in other mammalian species. As a consequence, the process of lipid mobilization has been well described in mammals. It involves several regulated steps that include not only the release of lipids, but also the proper transport of lipolytic substrates and products between tissues.

Fish are located at the base of the vertebrate evolutionary phylogenetic tree. This group is represented by more than 20,000 species, and covers a heterogeneous collection of organisms (Romer, 1968). Fish are divided in two branches: Agnatha (hagfish and

lampreys) and Gnathostomes. The Gnathostomes (jawed fish) are in turn divided in two classes: Chondrichthyes (e.g. sharks, rays) and Osteichthyes (bony fish). The latter class is subdivided in two subclasses, the Crossopterygii that gave origin to land-based vertebrates, and the Actinopterygii (Nelson, 1994; Diogo, 2008). Salmoniformes (or Protacanthopterygii) is one of the various groups included in the subclass Actinopterygii that contain a broad number of commercially important species like sockeye salmon, Atlantic salmon, and rainbow trout. Therefore, the study of lipid transport systems in this group of fish is a very exciting field not only from an evolutionary perspective, but from an applied point of view.

It is believed that the development of a complex system for lipid transport in vertebrates occurred concomittantly with the appearance of a closed circulatory system (Fielding and Fielding, 1991). In this system, three main classes of lipids containing fatty acids (non-esterified fatty acids (NEFA), TAG, and PL) are used to shuttle fatty acids between tissues. Circulating lipids can be metabolized through exogenous or endogenous pathways. The exogenous pathway permits the bulk transport of dietary fatty acids to adipose tissue or other tissues for storage, and was not investigated in this thesis. The endogenous pathway allows the transport of fatty acids from tissue lipid stores in two different forms: 1) as NEFA, and 2) as TAG and PL (Figure 1.1).

Fig. 1.1 A simplified representation of lipid metabolism in vertebrates. Two major lipolytic enzymes, hormone-sensitive lipase (HSL) and lipoprotein lipase (LPL), act on lipids existing in adipocytes and on circulating lipoproteins, respectively. Fatty acids (FA) transported in a non-esterified form (NEFA) are taken up by muscle and other tissues for direct oxidation or reesterification to triacylglycerol (TAG). The action of HSL results in the release of glycerol and NEFA into the systemic circulation, but some of the FA may also be reesterified in adipose tissue. Lipoproteins, synthesized in the liver are hydrolyzed by LPL.


TAG
Adipose Tissue
HSL
FA
Glycerol
Muscle
$CO_2 + H_2O$
Glycerol
Liver
NEFA
FA
FA
Plasma
TAG
LPL
TAG
Lipoprotein

There exists a diversity of enzymes with lipase activity within organisms that may vary in their specificity for lipid substrates. In particular, mammals appear to be well characterized with respect to presence and regulation of lipases among tissues (Allen, 1976; Ryan and Van der Horst, 2000). In these organisms, hormone-sensitive lipase (HSL) and lipoprotein lipase (LPL) appear to be key enzymes regulating the overall availability of FA. The former enzyme controls the release of FA from lipids stored intracellulary as TAG in droplets, where the latter is in charge of hydrolyzing circulating lipoproteins rich in TAG.

Lipases have been measured in a number of fish tissues (Black and Skinner, 1987; Sheridan, 1988). In particular, fish red muscle possesses a lipase that shows many similarities with mammalian HSL (Bilinski and Lau, 1969). The other lipase, LPL, has been measured in a number of fish tissues including red muscle (Lindberg and Olivecrona, 2002; Albalat *et al.*, 2006), and presents similarities with its mammalian homologue (Lindberg and Olivecrona, 1995, 2002). Despite the measurement of lipase activity in different tissues of fish, limited information on lipid mobilization is available for this group of ectotherms even though it is frequently used as an experimental model.

II. Fuel supply for endurance exercise in vertebrates

Different vertebrate species store varying amounts of TAG, but, on average total energy stored in TAG is more than 60 times higher than in carbohydrates (Swinburn and Ravussin, 1993). Therefore, TAG stores represent the largest fuel reserve in the body.

The use of FA as a fuel requires hydrolysis of TAG from different lipid sources to supply FA to the mitochondria of different tissues for oxidation.

The majority of the energy needs of resting mammals are provided by the oxidation of NEFA derived from the hydrolysis of TAG reserves located in adipose tissue (Horowitz and Klein, 2000), whereas plasma TAG may only account for 5-10% of total fat oxidation (Wolfe and Durkot, 1985). Because the amount of FA released from adipose tissue typically exceeds the amount oxidized at rest, a large portion of FA is reesterified back into TAG before reaching the circulation (Klein and Wolfe, 1987). During exercise, locomotory muscle requires larger amounts of oxidative substrates than at rest and, depending on the duration and intensity of the activity, lipids can be the main source of energy. For example, moderate intensity exercise (25-65% of maximal oxygen consumption or VO_{2max}) is associated with a 5-10 fold increase in fat oxidation in mammals. Such an increase in fat oxidation is matched by a 2-3 fold increase in FA supply from TAG lipolysis in adipose tissue, whereas the percentage of FA that are reesterified is decreased by half (Wolfe *et al.*, 1990). Therefore, plasma NEFA accounts for nearly all the lipids oxidized during low intensity exercise (Romijn *et al.*, 1993). Other sources of lipids such as muscle TAG and plasma TAG only appear to make a minor contribution to working muscle (Olsson *et al.*, 1975; Mackie *et al.*, 1980; Hurley *et al.*, 1986; Havel, 1987; Kiens and Lithell, 1989; Nagel *et al.*, 1989; Turcotte *et al.*, 1992; Kiens *et al.*, 1993; Hardman, 1998). In contrast, the relative contribution of plasma TAG to energy production during exercise in fish remains unclear.

III. Sustained swimming in fish: Lipids and provision of energy

Carbohydrates, lipids, and proteins constitute alternative sources of metabolic energy that can be mobilized in animals. In fish, the relative importance of each source of metabolic fuel depends on: the type of food consumed (Walton and Cowey, 1982), the level of activity (Weber and Zwingelstein, 1995; Weber and Haman, 1996), and the life history pattern of the animal (Sheridan, 1994).

In particular, carbohydrate utilization has been comprehensively studied in fish, even though this fuel appears to be an important source of energy only during anaerobic exercise (Driedzic and Hochachka, 1978; Moyes and West, 1995). However, fuel utilization during aerobic exercise remains poorly investigated. It is widely accepted that lipids provide most of the energy for aerobic metabolism (Driedzic and Hochachka, 1978; Walton and Cowey, 1982; Henderson and Tocher, 1987; Moyes and West, 1995). Using indirect calorimetry, Lauff and Wood (1996; 1997) showed that 47-60% of the energy of resting rainbow trout comes from lipid oxidation and that the relative importance of this fuel increases further during aerobic exercise. In contrast, the use of proteins as a metabolic fuel in fish only appears to be important during the last stages of migrations, or during long term fasting, when the other energy reserves become depleted (Driedzic and Hochachka, 1978; Mommsen *et al.*, 1980).

In view of the importance of lipids as metabolic fuels in fish, it is not surprising that they are stored in large amounts, mainly as TAG (Moyes and West, 1995). Even though significant reserves can be stored intramuscularly in some fish species, the majority of

lipid stores are located outside muscle (Kiessling *et al.*, 2004). Therefore, most lipids must be transported through the circulation before oxidation.

The most remarkable case of lipid use for locomotion occurs during the natural migration of sockeye salmon. Most of their journey occurs at sustainable swimming speeds (Standen *et al.*, 2002), that mostly involves aerobic swimming. Sockeye salmon can swim for more than 1,000 km from the open ocean to natal freshwater sites, without feeding, in order to spawn (Forester, 1968; Burgner, 1991). These fish can utilize as much as 8,000 calories to complete their migration, >75% coming from lipid reserves and the remainder from proteins (< 25%). This represents the use of 75 -95% of total fat reserves (Gilhousen, 1980; Brett, 1995). In other words, a 2 kg fish can consume the impressive amount of 646 g of lipids to accomplish this metabolic challenge (Idler and Clemens, 1959).

Sustained or endurance swimming is mainly powered by the contraction of red muscle (Rome, 1998). *In vitro* measurements in fish show that this tissue has a high capacity for lipid oxidation (Moyes *et al.*, 1989; 1992). Although the use of lipids as a fuel to power this type of locomotion has been implied in a number of studies the mechanisms of FA delivery to working muscles remain unclear in fish (Van den Thillart, 1986; Moyes and West, 1995; Lauff and Wood, 1996; 1997; Richards *et al.*, 2002). In contrast, the effects of exercise on lipid metabolism of mammals is much better documented. In this group of vertebrates, prolonged exercise causes the stimulation of TAG hydrolysis that leads to a major increase in the rate of appearance of NEFA (R_a NEFA) and glycerol (R_a glycerol) in the circulation (Klein *et al.*, 1989; 1995; Wolfe *et*

al., 1990; Wolfe, 1992; Brooks, 1998; Patterson, 2002; Quisth *et al.*, 2005; Turner *et al.*, 2006).

A few studies have investigated the metabolism of plasma NEFA in fish (Ballantyne *et al.*, 1993; 1996; Bernard *et al.*, 1999; Booth *et al.*, 1999; Weber *et al.*, 2002), and it is generally accepted that NEFA derived from lipid stores in liver and mesenteric tissue represent an important metabolic fuel for endurance swimming in teleost (Driedzic and Hochachka, 1978; Walton and Cowey, 1982; Henderson and Tocher, 1987). However, NEFA only represent a small fraction of total plasma lipids (Plisetskaya, 1980; Babin and Vernier, 1989) and prolonged swimming does not increase the turnover rate of NEFA in trout (Bernard *et al.*, 1999). Other forms of fatty acids besides NEFA, such as TAG and PL, are available in the circulation of fish and can be used as a source of energy during swimming. However, this hypothesis has never been tested. Such a role may have been overlooked because most of the information available on fuel supply during exercise comes from mammalian studies, where NEFA-albumin complexes are used to bring energy to working muscles.

IV. Lipoprotein metabolism in fish

Using a "mammalian perspective", fish are usually categorized as hyperlipidemic because their plasma lipid concentrations (e.g. in species like rainbow trout) are more than three times higher than in rats (Serougne *et al.*, 1986; Babin and Vernier, 1989).

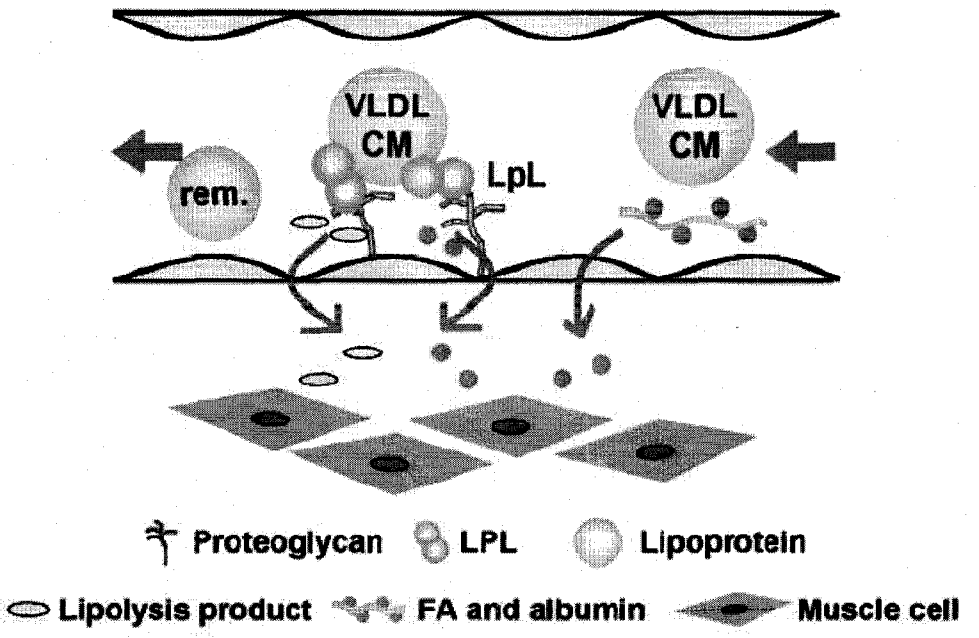
Most of these lipids (93-95% in adult rainbow trout) circulate as TAG and PL, two critical components of lipoproteins.

Lipoproteins are supra-molecular complexes ranging from 8 to 800 nm in diameter, used as lipid carriers in vertebrates. Most of our knowledge of lipoprotein metabolism comes from mammalian studies, where lipoprotein structure and metabolism have been extensively investigated (Cryer, 1981; Eckel, 1989; Goldberg and Merkel, 2001). These particles, formed by lipids and proteins combined in varying proportions, can be separated by ultracentrifugation and classified as chylomicrons, VLDL (very low density lipoproteins), LDL (low density lipoproteins) and HDL (high density lipoproteins), according to their decreasing size and increasing density. All lipoproteins share the same fundamental structural feature: they consist of a hydrophobic core formed by TAG and cholesterol ester surrounded by an amphipathic surface film of PL, cholesterol, and proteins (Jonas, 1991).

Lipoprotein particles are subjected to continuous modifications as they transfer their lipid and protein components to the tissues or exchange them between particles. Such processes can be catalyzed by specific enzymes or uncatalyzed (Fielding and Fielding, 1991). In mammals for example, TAG-rich particles (VLDL and chylomicra) are hydrolyzed by the action of lipoprotein lipase (LPL) to supply various tissues with fatty acids (Nilsson-Ehle, 1980; Zechner, 1997; Merkel *et al.*, 2002). LPL acts on circulating lipoproteins by controlling fatty acid release from the particles to neighbouring tissues, and, therefore, this enzyme plays a key role in the regulation of lipid deposition as well as lipid use (Figure 1.2). The lipolytic action of LPL on large lipoproteins yields smaller

particles with a lower TAG content. These smaller lipoproteins can be VLDL-remnants or LDL, and they are later removed from the circulation by peripheral tissues (Nilsson-Ehle, 1980). LPL is synthesized in parenchymal cells and transported to the capillary lumen. This enzyme is attached to the membrane of the capillary endothelium by interaction with heparan sulfate proteoglycan at the cell surface, and it can be released in the circulation by injecting heparin that has a higher affinity for the enzyme than the proteoglycan (Olivecrona and Bengston-Olivecrona, 1999). Although significant, the LPL activity of human and rat plasma is normally very low (Peterson *et al.*, 1990), because LPL released in the plasma is rapidly taken up by the liver and degraded (Wallinder *et al.*, 1984). LPL hydrolyzes most of the FA present in TAG and PL, but the enzyme appears to show selectivity for unsaturated over saturated FA, especially for oleate (Goldberg and Merkel, 2001). In addition, stereo-selectivity appears to occur since the FA in position 1 of TAG and PL are preferentially hydrolyzed over those in position 2 (Olivecrona *et al.*, 1995).

Fig. 1.2. Two possible pathways allowing the uptake of fatty acids (FA) by the tissues. Circulating FA can be delivered to the tissues as non-esterified fatty acids (NEFA) associated with albumin or after lipolysis of very low density lipoproteins (VLDL) and chylomicrons (CM) by lipoprotein lipase (LPL). Hydrolysis of those particles generates remnants (rem). LPL is attached to the endothelium by proteoglycans, in close proximity to the tissues. Based on Merkel *et al.* (2002).


VLDL
CM
rem.
LpL
VLDL
CM
Proteoglycan
LPL
Lipoprotein
Lipolysis product
FA and albumin
Muscle cell

In fish, fasting and spawning are known to cause changes in LPL activity in mesenteric fat, red muscle, and liver (Black and Skinner, 1986, 1987; Liang *et al.*, 2002; Albalat *et al.*, 2005b; 2006). Fasting and spawning also affect plasma lipoprotein concentration (Rogie and Skinner, 1981; Black and Skinner, 1986; Wallaert and Babin, 1992). Seasonal changes in lipoprotein concentration in relation to water temperature and reproduction have also been found in trout (Wallaert and Babin, 1994a, 1994b). It is thought that fish lipoprotein metabolism has several similarities with the well-described system of lipoprotein transport of mammals (Babin and Vernier, 1989; Gjoen and Berg, 1992). For example, the *in vitro* activation of LPL by Apo-CII (cofactor) or by heparin (Fremont *et al.*, 1987; Lindberg and Olivecrona, 1995, 2002) are thought to be mediated by the same mechanims in fish and mammals.

V. The regulation of lipid mobilization in fish

The regulation of lipolysis is crucial because this step influences the rate of FA utilization by the tissues. Lipid mobilization and FA transport have been well characterized in exercising mammals (Brooks, 1997; Ferguson *et al.*, 1998; Borsheim *et al.*, 1999). HSL and LPL are the main enzymes controlling FA availability in this group of vertebrates and they are modulated during exercise via several mechanisms including hormones (Chernick *et al.*, 1986; Ladu *et al.*, 1991a) and local signals associated with contractile activity (Hamilton *et al.*, 1998). In particular, the increased supply of FA from adipose tissue lipolysis is mediated by an increase in catecholamine release in

mammals (Arner *et al.*, 1990; Coppack *et al.*, 1994; Londos *et al.*, 1999). Specifically, lipolysis is stimulated by norepinephrine (NE) as well as epinephrine (Epi) (Fain and Garcia-Sainz, 1983; Nonogaki, 2000). The metabolic consequences of circulating catecholamines in the regulation of lipid metabolism in fish is not as well established as in mammals (Fabbri *et al.*, 1998). Much of the understanding of the effects of Epi and NE comes from *in vitro* studies in isolated cells from a number of fish species. It is known that Epi activates HSL in liver and possibly in mesenteric fat of salmonids through a cAMP-dependent protein kinase (Sheridan, 1987; Michelsen *et al.*, 1994). The effects of catecholamines on fish lipolytic rate (lipolysis) have not been measured *in vivo*. Overall, however, NE appears to inhibit lipolysis in fish adipose tissue (Farkas, 1967a, 1967b; Vianen *et al.*, 2002), although not in all species (Farkas, 1967b). Even for Epi, the response may be very different between fish and mammals.

VI. Goals of the investigation

Lipids are the main source of energy during aerobic exercise in fish, but mechanisms of lipid supply to working muscles are unclear. The main goal of this thesis was to study the effects of endurance exercise on fish lipoprotein metabolism, to clarify how an adequate FA supply to working muscle can be achieved. Additional objectives of this thesis were to clarify the mechanisms of action of lipoprotein lipase on circulating lipids and to study how lipolytic rate may be regulated *in vivo* by catecholamines in fish. To achieve these objectives, a series of studies were designed to investigate lipid

metabolism in salmonid fish (sockeye salmon, *Oncorhynchus nerka*, and rainbow trout, *Oncorhynchus mykiss*), a group of fish commonly using endurance swimming for locomotion.

The following questions were addressed:

1. How are circulating lipids (NEFA, TAG, and PL) affected by endurance swimming? (CHAPTERS 2 and 3)
2. Is red muscle LPL activity affected by endurance swimming? (CHAPTER 3)
3. How does heparin administration affect lipoprotein metabolism? (CHAPTER 3 and 4)
4. Is TAG turnover rate altered by exercise? (CHAPTER 4)
5. How do the lipolytic rate and TAG turnover rate compare with those measured in other vertebrates? (CHAPTER 4 and 5)
6. How is lipolysis regulated by catecholamines *in vivo*? (CHAPTER 5)

In more detail, CHAPTER 2 quantifies the effects that the long-distance migration of wild sockeye salmon has on plasma lipids containing fatty acids (NEFA, TAG and PL). Changes in the metabolism of lipoproteins were anticipated in view of their potential role in shuttling energy to muscle mitochondria.

To exclude confounding factors affecting lipid utilization during salmon migration, the goal of CHAPTER 3 was to investigate the effects of endurance swimming on the lipoprotein metabolism of rainbow trout under controlled laboratory conditions. Previous studies have shown that the catabolism of large lipoproteins rich in TAG yields smaller particles with a lower TAG content, such as VLDL-remnants or LDL. I hypothesize that both, LPL (enzyme) and plasma lipoproteins (substrate) are

modified by prolonged exercise. Therefore, it is predicted that endurance swimming will activate LPL in red muscle, and will alter circulating lipoprotein classes (HDL, LDL, and VLDL), and components (TAG, PL, and NEFA).

Because the activations of trout LPL elicited by prolonged swimming in red muscle or by heparin in plasma (CHAPTER 3) are not accompanied by significant changes in lipoprotein concentration, the objectives in CHAPTER 4 were to study the effects of endurance swimming and heparin administration on the TAG turnover rate of rainbow trout. I hypothesized that endurance swimming would stimulate TAG turnover rate to provide more fuel to working muscles, and that heparin would reduce it by releasing LPL in plasma, thereby preventing normal lipoprotein uptake.

The goal of CHAPTER 5 was to investigate the effects of catecholamines on R_a glycerol in intact fish to obtain an integrated hormonal response of total fatty acid supply. My aim was to quantify the effects of NE and Epi on the lipolytic rate of rainbow trout using in vivo tracer kinetics. I also examined the relationship between R_a glycerol and glycerol concentration to determine whether glycerol concentration could be used as a reliable index of fish lipolysis. It was hypothesized that trout lipolysis would be inhibited by NE and stimulated by Epi.

Finally, general conclusions are presented in CHAPTER 6 where the physiological significance of the high TAG turnover rate and lipolytic rate measured in trout, and the lack of change for both metabolic parameters are discussed.

CHAPTER 2.

EFFECTS OF LONG-DISTANCE MIGRATION ON CIRCULATING LIPIDS OF SOCKEYE SALMON (*ONCORHYNCHUS NERKA*)

Based on

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Introduction

The "once in a lifetime" spawning migration of sockeye salmon (*Oncorhynchus nerka*) involves swimming for hundreds of kilometers from the open ocean to natal freshwater sites (Forester, 1968). Migration can only be successful if enough energy is stored during the ocean phase because feeding stops upon river entry (Burgner, 1991). Several studies have investigated the energy cost of migration (Hinch and Rand, 1998; Rand and Hinch, 1998; Standen *et al.*, 2002) and characterized the relative roles of lipids, carbohydrates, and proteins as metabolic fuels for swimming (Idler and Clemens, 1959; Gilhousen, 1980; Mommsen *et al.*, 1980). In sockeye salmon, lipids provide most of the energy for migration, locomotory muscles being responsible for the oxidation of 75-95% of total fat reserves (Gilhousen, 1980; Brett, 1995). More importantly, most lipids must be transported through the circulation before oxidation because the majority of fat stores are located outside red muscle (Kiessling *et al.*, 2004).

Lipids circulate as non-esterified fatty acids (NEFA) or as triacylglycerol (TAG) associated with phospholipids (PL), the two main components of plasma lipoproteins. Few studies have investigated the metabolism of plasma NEFA in wild migrating salmon (Ballantyne *et al.*, 1996; Booth *et al.*, 1999) and captive rainbow trout (Bernard *et al.*, 1999; Weber *et al.*, 2002). However, NEFA only represent a small fraction of total plasma lipids (Plisetskaya, 1980; Babin and Vernier, 1989) and prolonged swimming does not increase the turnover rate of NEFA, at least in rainbow trout (Bernard *et al.*, 1999). Even though lipoproteins are clearly involved in gonad development (Greene and Selivonchick, 1987; Henderson, 1987; Wallaert and Babin, 1994a), they have never been considered as a potential energy shuttle between lipid stores and working muscles. Such

a role may have been overlooked because most of the information available on fuel supply to working muscles comes from mammalian studies, and exercising mammals do not follow this pattern. Instead, they use plasma NEFAs to bring energy to working muscles and maintain very low lipoprotein levels in plasma compared to fish (Nichols, 1967; Breslow, 1984; Babin and Vernier, 1989).

The consequences of changes in salinity on the plasma lipoproteins of salmon are not well known. The concentrations of TAG and PL in plasma of smolting salmon are both altered during the freshwater to seawater transition (Sheridan, 1988; 1989), but few data are available for adults returning to spawn (Patton *et al.*, 1970; French *et al.*, 1983). The effects of prolonged swimming on plasma lipoproteins have never been investigated in salmon. If lipoproteins are used as an oxidative fuel for muscle work, or if they play a role in coping with salinity transitions (e.g. gill membrane restructuring), significant changes in their concentration and fatty acid (FA) composition would be expected during migration. These changes would reflect overall mobilization and uptake, as well as the selective metabolism of particular FAs. For example, rainbow trout show selective mobilization of certain FAs during fasting, with chain length and degree of unsaturation varying between storage sites (Jezierska *et al.*, 1982). Also, isolated mitochondria from red muscle, the prime engine for sustained swimming (Walton and Cowey, 1982), show preferential oxidation of specific FAs (Bilinski and Lau, 1969; Kiessling and Kiessling, 1993). In this study, my goal was to characterize the plasma lipids containing fatty acids (NEFA, TAG and PL) in wild sockeye salmon and to monitor changes in their concentration and composition over a 500 km migration up the Fraser River (British Columbia, Canada). Important changes in lipoprotein metabolism are anticipated in view

of their potential roles in shuttling energy to muscle mitochondria and in the response to salinity changes.

Methods

Animal capture and blood sampling

Adult female sockeye salmon (*Oncorhynchus nerka*; 2,544 ± 497 g, n = 35) from the Lower Adams stock (Fraser River, British Columbia, Canada) were caught at four different stages of their spawning migration (Fig. 2.1). Positive stock identification was achieved by DNA analysis (Beacham *et al.*, 1995; 2004). Ocean-migrating animals were captured with a purse seine in the vicinity of Johnstone Strait (Aug 13-19, 2003). Freshwater-migrating salmon were collected with gill nets at the mouth of the Fraser River (Whonnock, British Columbia; Aug 15-Sep 12, 2003). Pre- (Oct 1, 2003) and post-spawning salmon (Oct 15, 2003) were caught with a beach seine in the Adams River. Immediately after capture, blood samples were collected by caudal puncture. Plasma was separated by centrifugation (800 g for 10 min) and stored at -80°C until analysis. The results of this study are based on plasma lipid analyses for 35 animals that were part of a more comprehensive research project that compared the reproductive success of different stocks of sockeye salmon in the Fraser River (see Patterson *et al.*, 2004).

Extraction of plasma lipids

Plasma lipids were extracted with a 2:1 (v/v) mixture of chloroform:methanol (Folch *et al.*, 1957) and centrifuged (2,000 g for 10 min). The pellet was discarded and

the supernatant was filtered before adding KCl (0.25%) to help eliminate water-soluble compounds. After shaking, the mixture was centrifuged (2,000 g for 10 min) to separate aqueous and organic phases. The aqueous phase was discarded and the organic phase was dried at 75°C under N_2. Total plasma lipids were immediately resuspended in chloroform before separating them into 3 classes.

Separation of neutral lipids (NL), non-esterified fatty acids (NEFA) and phospholipids (PL)

Total plasma lipids were loaded on Supelclean solid-phase extraction columns (100 mg LC NH_2, Sigma, St. Louis, MO, USA) that were used to separate NL, NEFA, and PL by sequential elution as described previously by Bernard *et al.* (1999). In preliminary experiments, TAGs were separated from NL by thin-layer chromatography (TLC). Results showed that the fatty acid compositions of the complete NL fraction and of the TLC-purified TAG fraction were not significantly different because >80% of NL are TAG.

Gas chromatography analysis

The concentrations of plasma NEFA, TAG and PL as well as their FA composition were measured by gas chromatography after methylation (NEFA), or acid transesterification (TAG and PL) (Chapelle and Zwingelstein, 1984; Abdul-Malak *et al.*, 1989). Heptadecanoic acid (17:0) was used as an internal standard because preliminary experiments showed that this acid is absent from the NEFA, NL and PL of sockeye salmon plasma. The fatty acid methyl esters obtained from each fraction were analyzed

on a Hewlett Packard 5890 series II gas chromatograph equipped with a Supelco 2330 capillary column (30 m x 0.32 mm ID, 0.2 μm film thickness), using helium as carrier gas, and the temperature conditions described in McClelland *et al.* (1999). Individual fatty acids were identified by determining exact retention times with authentic standards (Sigma-Aldrich, St. Louis). Only FA representing more than 1% of total fatty acids within each plasma lipid fraction are reported.

Calculations and statistical analyses

The double bond index (DBI) was used to express the level of fatty acid unsaturation in each class of plasma lipids. It was calculated as follows (expressing percentages as ratios):

$$DBI = \frac{\text{average number of double bonds}}{\text{\% saturated FA}}$$

The average number of double bonds (also called degree of unsaturation) was calculated as:

Average number of double bonds = (1 x % monoenes) + (2 x % dienes) + (3 x % trienes) + ... + (n x % FA with n double bonds)

Results were evaluated using one-way analysis of variance (ANOVA). In cases where the assumptions of normality or homoscedasticity were not met, a non-parametric ANOVA based on ranks (Dunn's test) was carried out. Percentages were transformed to the arcsine of their square root before analysis, and all values given are means ± standard deviation (SD).

Results

Effects of migration on the levels of plasma lipid classes

Changes in the abundance of plasma TAG, PL and NEFA throughout the spawning migration of sockeye salmon are summarized in Fig. 2.2. Absolute concentrations of the three lipid fractions are shown in Fig. 2.2a, where they are all expressed as fatty acid concentrations to allow meaningful comparisons. Plasma TAG and plasma PL showed a strong decrease throughout the spawning migration ($P<0.001$). TAG decreased abruptly by over six-fold from 27.2 to 4.3 μmol FA ml^{-1} between the ocean stage and river entry, before declining more progressively to 1 μmol ml^{-1} until the end of migration. Plasma PL decreased more gradually from 30.3 to 5.2 μmol FA ml^{-1} throughout the complete migration. Plasma NEFA stayed constant during most of the migration, but showed a small decrease during spawning (from 0.76 to 0.50 μmol ml^{-1}; $P<0.05$).

Changes in the percent distribution of total plasma fatty acids between TAG, PL and NEFA are presented in Fig. 2.2b. Together, TAG and PL accounted for 93-98% of total plasma FA at all times, whereas the contribution of NEFA always remained minor (2-7% of total plasma FA). The relative importance of TAG decreased from 46 to 15% of total plasma FA during migration, with the most rapid decline occurring between the ocean phase and river entry (46 to 26%). Percent total plasma fatty acid in PL increased from 52 to 76% during the seawater to freshwater transition and remained at this high

level until the end of migration. The relative abundance of plasma NEFA increased from 2 to 7% throughout migration.

Effects of migration on the fatty acid composition of plasma NEFA, TAG and PL

Figure 2.3 summarizes changes in the relative abundance of fatty acids with different degrees of saturation in the 3 classes of plasma lipids (NEFA, TAG, and PL). Overall, the percentages of saturated (SFA), monounsaturated (MUFA) and polyunsaturated FA (PUFA) within each lipid class were relatively constant as migration only had minor effects. Saturation levels of plasma NEFA are presented in Fig. 2.3a. In this class, the only changes observed were a decrease in PUFA at the end of migration (40 to 19% of FA in plasma NEFA) and an increase in SFA before spawning (31 to 44%). Saturation levels of plasma TAG are presented in Fig. 2.3b. The only observable changes in TAG occurred at river entry: a decrease in SFA (15 to 9% of FA in plasma TAG) and an increase in PUFA (51-55%). Saturation levels of plasma PL are presented in Fig. 2.3c. In this class, SFA ranged from 20 to 25% (of FA in PL), MUFA from 19 to 24%, and PUFA from 55-59%.

Overall saturation levels are summarized in Fig. 2.4 that shows changes in the double bond index (DBI) of the 3 lipid classes throughout migration. The most notable changes are a large increase in the DBI of plasma TAG between the ocean phase and river entry (from 20 to 36), and a progressive decrease in the DBI of plasma NEFA throughout migration (from 10 to 3).

Saturation levels were very different between NEFA, TAG and PL (Figs. 2.3 and 2.4). The fatty acid composition of the 3 lipid classes varied as follows: TAG < PL <

NEFA (for % SFA), PL < NEFA < TAG (for % MUFA), and NEFA < TAG < PL (for % PUFA). On average, plasma NEFA had 33% SFA, 35% MUFA and 31% PUFA, whereas plasma TAG had 13% SFA, 37% MUFA, 51% PUFA, and plasma PL had 23% SFA, 21% MUFA and 56% PUFA. Therefore, the average DBI were drastically different between NEFA (6), PL (14), and TAG (25).

The effects of migration on the FA composition of plasma TAG are presented in Table 2.1. Eight main FAs accounted for >85% of total TAG fatty acids, the remaining being represented by 10-12 minor FAs (1-2% each). Oleic acid (18:1), eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) were the most abundant fatty acids in TAG (on average accounting for 25%, 20%, and 17% of TAG fatty acids, respectively). Overall, the FA composition of plasma TAG remained relatively constant, but some significant changes were noticed. The decrease in palmitic acid (16:0) and increase in EPA (20:5), taking place between the ocean phase and river entry, were largely responsible for the observed increase in DBI (see Fig. 2.4). The only PUFA showing a consistent change was docosapentaenoic acid (22:5) whose relative contribution kept decreasing throughout the migration.

The FA composition of plasma PL during migration is summarized in Table 2.2. Eight main fatty acids accounted for >90% of total PL FA, the remaining being represented by 8-10 minor FAs (1-2% each). DHA (22:6), palmitic acid (16:0), oleic acid (18:1), and EPA (20:5) were the most abundant fatty acids in PL, on average accounting for 33%, 16%, 13%, and 12 % of PL fatty acids, respectively. Overall, the FA composition of plasma PL remained relatively constant, but a significant decrease in the most abundant acid (DHA, 22:6) was observed between the beginning of migration and

the spawning site (Table 2.2). At the beginning of migration, the level of DHA in plasma PL was 1015 μmol · mL^{-1} (ocean stage): the highest concentration observed in this study for all FA in all lipid fractions. DHA concentration in plasma PL decreased nearly seven-fold during migration to reach 154 μmol · mL^{-1} after spawning.

Discussion

Salmon migration mainly occurs at sustainable swimming speeds (Hinch and Rand, 1998; Hinch *et al.*, 2002), when muscles favor the use of lipids as an energy source (Moyes and West, 1995; Lauff and Wood, 1996). Total energy costs and depletion of body lipids are relatively well understood, but little information is available on the mechanisms of lipid mobilization and oxidation. This study shows that long-distance migration has dramatic effects on the plasma lipoprotein constituents of wild sockeye salmon, implying that these circulating lipids are involved in key physiological processes necessary for successful spawning. In sockeye salmon, plasma lipoproteins account for over 93% of the energy transported in circulating lipids and the concentration of their main constituents shows a 27-fold decrease (TAG) and 6-fold decrease (PL) throughout the complete migration (Fig. 2.2).

Limitations of study

My goal was to measure the metabolic response of wild fish in their real environment, along their natural migration route, to eliminate potential artifacts caused by holding fish under laboratory conditions. As always, such a choice imposes a

compromise because the obvious advantages gained through this approach must be shared with the inability to control conditions, one variable at a time. Salmon migration requires a complex, integrated response involving simultaneous changes linked to exercise, temperature, salinity, fasting and reproduction, making it difficult to associate the observed response to a single cause. Therefore, this first attempt to examine the metabolism of lipoprotein fatty acids during migration was to identify promising avenues for future experiments where individual causes could be monitored separately.

Changes in plasma TAG and PL

Sockeye salmon show an abrupt decline in plasma TAG when they move from the ocean to freshwater (Fig. 2.2). Similar observations have been documented for various salmonids, not only during the spawning migration (Patton *et al.*, 1970; French *et al.*, 1983), but also for smolting animals undergoing the opposite salinity transition (Sheridan, 1988; 1989). The timing of the sockeye response suggests that it is related to changes in feeding and/or environmental salinity. By itself, the cessation of feeding could have this effect because it stops the synthesis of chylomicrons by intestinal cells when the necessary lipid substrates start lacking (Rogie and Skinner, 1985; Sheridan *et al.*, 1985; Burgner, 1991). However, the effects of salinity alone are difficult to separate form the effects of fasting, even in "controlled" laboratory experiments, because the stress elicited by changes in salinity can strongly affect feeding.

The osmoregulatory response of fish is partly mediated by changes in cortisol, growth hormone and prolactin levels, and these hormones also play important roles in lipid metabolism (McCormick, 2001). They are known to contribute to the regulation of

lipid mobilization in smolting salmon (Sheridan, 1986). Recent experiments also show that the seawater to freshwater transition causes a strong decrease in the activity of phosphatidylethanolamine N-methyltransferase in euryhaline fish (*Anguilla anguilla*) and crustaceans (*Carcinus maenas*) (Zwingelstein *et al.*, unpublished). This enzyme catalyses the synthesis of phosphatidylcholine and its inhibition is known to impair the production of very low density lipoproteins (VLDL) by the liver (Vance and Vance, 1986; Noga *et al.*, 2002). Such a mechanism could explain the rapid reduction in circulating TAG levels observed here when sockeye salmon enter freshwater.

Migration also caused a progressive decline in plasma PL concentration (Fig. 2.2), and this change is consistent with the idea that energy from lipid reserves is transported to working muscles as lipoproteins. Because the declines in PL and in TAG levels do not proceed at the same pace, the PL/TAG ratio increases along the migration path, suggesting that the average size of circulating lipoproteins decreases from (large) VLDL to (smaller and denser) high density lipoproteins (HDL). It is interesting to note that the oxidation of VLDL to support muscle work would progressively convert them to HDL. In pink salmon, it has actually been reported that VLDL levels decrease sharply during migration, whereas HDL levels remain unchanged (Nelson and Shore, 1974).

Alternately, it could be argued that the observed declines in TAG and PL are related to egg production. Vitellogenin is considered the main lipoprotein used to shuttle lipids from storage sites to the gonads (Babin and Vernier, 1989), and its plasma levels decrease during migration, at least in pink salmon (Dye *et al.*, 1986). More recently, evidence supporting a similar role for HDL has also been proposed in Atlantic salmon (Vegusdal *et al.*, 2004). However, it is important to keep in mind that gonad development

only represents a minor sink for lipids compared with locomotion. In sockeye salmon, it has been estimated that only 6% of total lipids used during migration is directed to reproduction, whereas the remaining 94% is allocated to locomotion (Idler and Clemens, 1959; Brett, 1973). Therefore, the large overall response to migration monitored in my study is more likely to primarily reflect the lipid requirements of swimming rather than gonad development.

In fish, changes in plasma NEFA have been used frequently to evaluate the effects of swimming and reproduction on lipid metabolism (Plisetskaya, 1980; Ballantyne *et al.*, 1993; 1996; Booth *et al.*, 1999). However, NEFA represent a minor fraction of total FAs in plasma lipids (< 7%, see Fig. 2.2b) and migration only causes a very small decrease in the plasma NEFA concentration of sockeye salmon (Fig. 2.2a). These results suggest that using changes in NEFA concentration to draw conclusions about total plasma FA utilization for metabolic requirements are probably misleading. They also support the idea that circulating lipids other than NEFAs are involved in supplying energy for swimming and egg production. A previous study on rainbow trout demonstrated that endurance swimming does not stimulate NEFA fluxes above resting levels (Bernard *et al.*, 1999). This intriguing observation could be explained if the TAG and PL components of lipoproteins, rather than NEFA, play a prominent role in shuttling energy from storage sites to the muscles of swimming salmonids.

FA composition of plasma lipids

After characterizing changes in total plasma TAG, PL and NEFA, the FA composition of these three lipid fractions was examined. Over the migration, I was

expecting changes in composition as a result of selective metabolism in target tissues such as muscles, gills and gonads. Lipoprotein lipase (LPL) has been thoroughly characterized in mammals, where it plays a key role in orchestrating FA mobilization from lipoproteins. LPL shows selectivity for various types of fatty acids, and, in particular, is known to release MUFAs preferentially (Goldberg and Merkel, 2001). Fish LPL has also been identified (e.g. see Black and Skinner, 1987) and it generally shares most characteristics with its mammalian counterpart (Lindberg and Olivecrona, 2002). It would be surprising if fish LPL did not show selectivity, but this particular property still needs to be investigated. *In vitro* evidence indicates that fish tissues show selective oxidation of different FAs (Henderson and Sargent, 1985; Sidell and Driedzic, 1985; Kiessling and Kiessling, 1993; Eggington, 1996). For example, MUFAs are the preferred fuel for red muscle mitochondria of Antarctic fish (Sidell *et al.*, 1995) and the amount of oleic acid (18:1) in lipid reserves of Atlantic salmon is correlated with swimming performance (McKenzie *et al.*, 1998). Saturated (16:0) and monounsaturated FAs (18:1) are not incorporated similarly into tissue TAG and PL because these two fatty acids play different physiological roles (Weber *et al.*, 2002). Taken together, these previous observations led me to predict that sockeye salmon would show changes in the FA composition of plasma lipids throughout their long-distance migration. Contrary to expectation, very few changes in FA composition were observed in TAG and PL, and, in particular, both lipid fractions kept the same MUFA levels throughout migration (Fig. 2.3, Tables 2.1 and 2.2). The exact reasons why FA composition remains relatively constant during long-distance migration are presently unknown. One possibility is that

selectivity affects the flux of MUFAs rather than their concentration, but additional experiments are needed to address this issue (e.g. see Haman *et al.*, 1997).

The most noticeable change in FA composition was observed at the beginning of migration when the percentage of unsaturated FAs in plasma TAG showed an increase between the ocean and river entry, as indicated by a sharp rise in double bond index from 20 to 36 (Fig. 2.4). This change was mainly due to a decrease in palmitic acid (16:0) and to an increase in EPA (20:5) (Table 2.1). In view of the well known health benefits from dietary PUFAs of marine origin, such an increase in (omega 3) EPA may have positive nutritional consequences for native populations that traditionally fish in the lower Fraser river. The timing of this response also suggests that TAG from circulating lipoproteins are used to supply FAs for modifying membrane fluidity as the animals experience a rapid change in salinity. Homeoviscous adjustments could therefore be achieved by altering the PUFA / SFA ratio in plasma without changing membrane cholesterol content or the composition of PL head groups (Bell *et al.*, 1986; Hazel and Williams, 1990). Finally, migration also caused a decline in the DHA content of plasma PL (Table 2.2). It has been reported that embryonic membranes are particularly rich in DHA-containing PL (March, 1993) and this decline may therefore be related to egg production.

Conclusions

This study shows that sockeye salmon undergo major changes in plasma lipoprotein metabolism during long-distance migration. The timing and direction of these changes suggest for the first time that lipoproteins are used as an important oxidative fuel in fish muscles and possibly play a role in the response to abrupt salinity transitions.

Previous investigations of lipid use by migrating salmon have focused on plasma NEFAs as the only shuttle system between fat stores and working muscles. Results from this study shift attention to lipoproteins because: 1) migration affects them most, and 2) they account for over 93% of total plasma lipids. The exact contributions of this pathway to locomotion, osmoregulation and reproduction remain to be established. Quantifying the effects of prolonged swimming on lipoprotein turnover and LPL activity are promising areas for further research.

TABLE 2.1. Fatty acid composition (%) and total fatty acid concentration ($\mu mol \cdot mL^{-1}$) in plasma triacylglycerol (TAG) of migrating sockeye salmon.

Fatty acids	Ocean	River entry	Pre-spawning	Post-spawning
16:0	9.7^{a} (1.3)	5.3 (2.2)	8.8^{a} (0.6)	8.3^{a} (1.7)
18:0	$3.6^{a\,b}$ (0.7)	$2.6^{b\,c}$ (0.6)	2.5^{c} (0.6)	4.1^{a} (0.9)
18:1	$24.1^{a\,b}$ (3.1)	27.1^{a} (2.7)	$25.3^{a\,b}$ (2.5)	22.4^{b} (2.7)
20:1	4.1^{a} (0.7)	$2.9^{a\,b}$ (1.9)	$2.0^{a\,b}$ (1.1)	1.5^{b} (1.8)
22:1	1.4^{a} (0.6)	2.4^{a} (1.2)	$3.2^{a\,b}$ (1.4)	12.5^{b} (5.7)
20:5	16.0 (1.2)	21.5^{a} (1.6)	22.8^{a} (3.3)	20.2^{a} (3.2)
22:5	7.0^{a} (0.8)	$6.6^{a\,b}$ (0.9)	$4.9^{b\,c}$ (0.8)	3.6^{c} (1.4)
22:6	21.7^{a} (3.0)	17.2^{b} (2.5)	15.6^{b} (2.6)	$17.8^{a\,b}$ (2.6)
Others*	12.4^{a} (1.5)	14.3^{a} (3.4)	14.8^{a} (8.0)	9.6^{a} (6.0)
[Total FA]†	27.2 (5.5)	4.3 (0.7)	2.7 (0.5)	1.0 (0.2)

* Others consist of 10 to12 fatty acids that represent 1-2% of the total fatty acid content in plasma TAG. † Plasma concentration of each fatty acid in TAG can be calculated by multiplying its percentage by total fatty acid concentration (bottom row). Values are means of 10 fish (except for the ocean stage where n= 5) with SD in parenthesis. Letters indicate statistical comparisons between the 4 stages within TAG. Values lacking letters or with different letters are different from each other ($P < 0.05$). Values sharing the same letter are not statistically different.

TABLE 2.2. Fatty acid composition (%) and total fatty acid concentration ($\mu mol \cdot mL^{-1}$) in plasma phospholipids (PL) of migrating sockeye salmon.

Fatty acids	Ocean	River entry	Pre-spawning	Post-spawning
16:0	$17.0^{a\,b}$ (0.6)	14.0^{c} (1.7)	17.5^{a} (1.0)	$14.2^{b\,c}$ (1.9)
18:0	$6.6^{a\,b}$ (0.6)	6.9^{a} (1.1)	5.7^{b} (0.4)	4.7 (0.6)
18:1	$12.7^{a\,c}$ (0.9)	12.4^{c} (0.5)	$15.5^{a\,b}$ (1.4)	16.5^{b} (1.6)
20:1	$1.8^{a\,b}$ (0.2)	1.3^{b} (1.0)	2.2^{a} (0.5)	2.7^{a} (1.1)
22:1	0.4^{a} (0.1)	1.1^{b} (0.7)	$0.49^{a\,b}$ (0.1)	$0.7^{a\,b}$ (0.3)
20:5	$12.4^{a\,b}$ (0.9)	8.6^{a} (5.6)	$15.0^{b\,c}$ (1.2)	15.6^{c} (1.7)
22:5	5.5^{a} (0.7)	5.7^{a} (1.1)	6.3^{a} (0.7)	6.0^{a} (0.9)
22:6	33.5 (2.5)	37.3 (2.0)	29.4^{a} (1.6)	29.7^{a} (1.9)
Others*	10.1^{a} (1.0)	$12.8^{a\,b}$ (6.8)	7.9^{b} (1.6)	9.8^{a} (1.3)
[Total FA]†	30.3 (0.3)	16.2 (2.0)	10.8 (2.6)	5.2 (1.4)

* Others consist of 8 to10 fatty acids that represent 1-2% of the total fatty acid content in plasma PL. † Plasma concentration of each fatty acid in PL can be calculated by multiplying its percentage by total fatty acid concentration (bottom row). Values are means of 10 fish (except for the ocean stage where n= 5) with SD in parenthesis. Letters indicate statistical comparisons between the 4 stages within PL. Values lacking letters or with different letters are different from each other ($P < 0.05$). Values sharing the same letter are not statistically different.

Fig. 2.1. Map showing the sampling sites for Adams run sockeye salmon (*Oncorhynchus nerka*) on the Fraser River basin, British Columbia. Black dots indicate the sampling locations: **1,** Johnstone Strait (Ocean); **2,** Whonnock (River entry); and **3,** Adams River (Pre and Post-spawning).

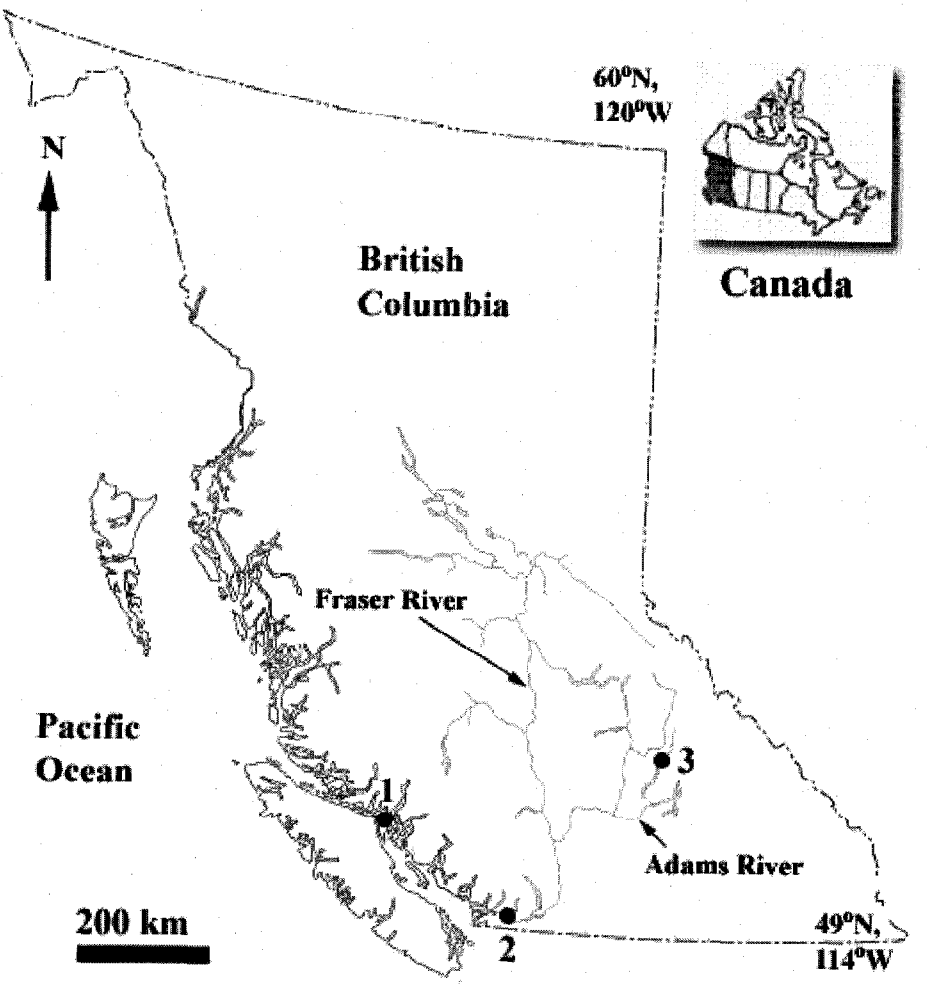

60°N,
120°W
N
British
Columbia
Canada
Fraser River
Pacific
Ocean
1
2
3
Adams River
200 km
49°N,
114°W

Fig. 2.2. Changes in concentration **(a)**, and percent contribution to total plasma fatty acids **(b)** for non-esterified fatty acids (gray bars), triacylglycerol (black bars), and phospholipids (white bars) during sockeye salmon migration. To allow meaningful comparisons between the 3 lipid classes, concentrations are all given in μmol of fatty acids per ml plasma. Absolute phospholipid and TAG concentrations can be obtained by dividing the values presented in **(a)** by 2 (2 fatty acids per PL) and 3 (3 fatty acids per TAG), respectively. River = River entry, Pre-S. = Pre-spawning, and Post-S. = Post-spawning stages. Values are means + SD for 10 fish, except for the ocean stage (n= 5). Letters indicate statistical comparisons between the 4 stages within each lipid class. Bars lacking letters or with different letters are different from each other ($P < 0.05$). Bars sharing the same letter are not statistically different.

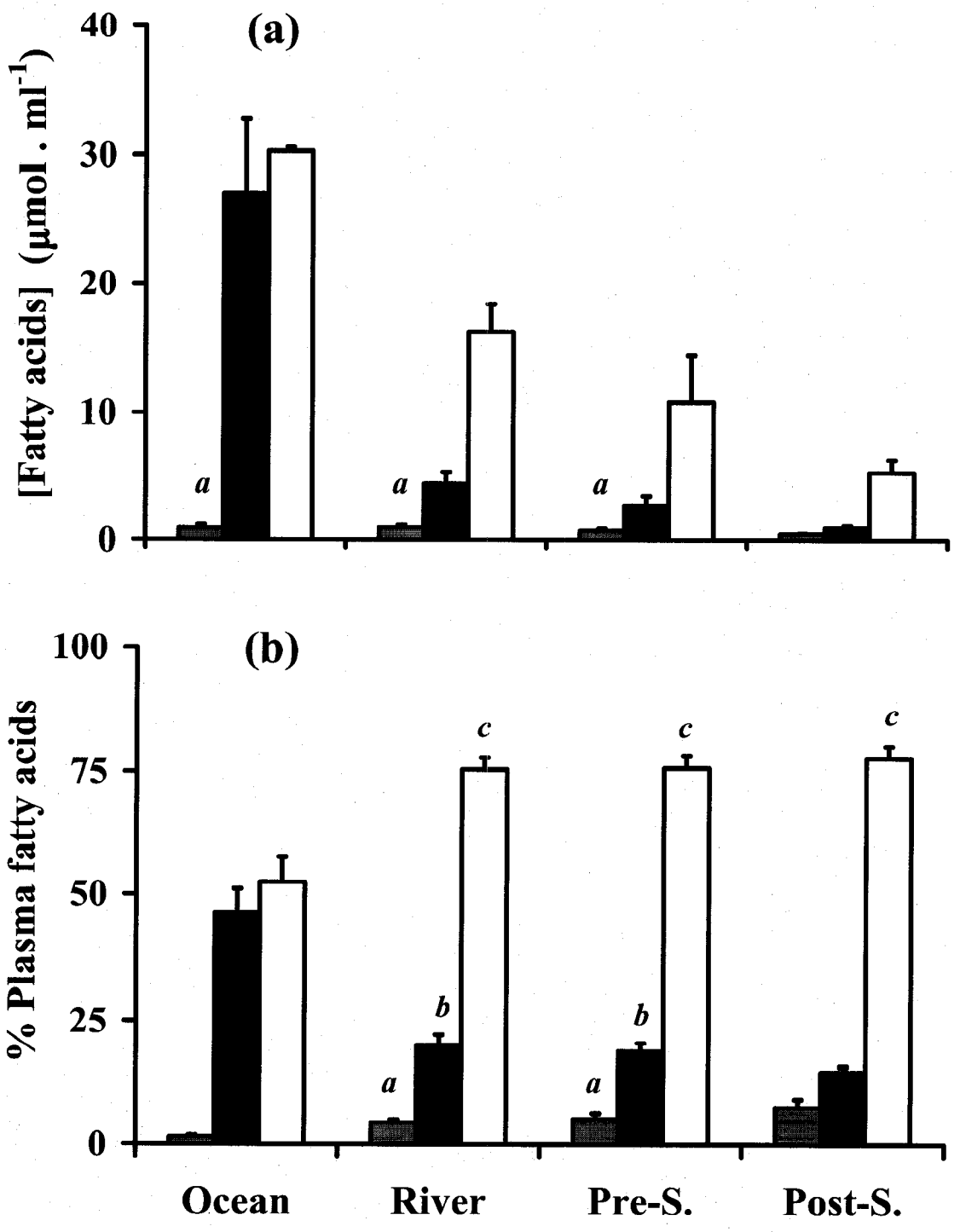

(a)
[Fatty acids] (µmol . ml-1)
40
30
20
10
0
a
a
a
(b)
% Plasma fatty acids
100
75
50
25
0
a
b
c
a
b
c
c
Ocean
River
Pre-S.
Post-S.

Fig. 2.3. Changes in the percentages of saturated fatty acids (white bars), monounsaturated fatty acids (black bars), and polyunsaturated fatty acids (hatched bars) transported in plasma as non-esterified fatty acids **(a)**, triacylglycerol **(b)**, and phospholipids **(c)** in migrating sockeye salmon. River = River entry, Pre-S. = Pre-spawning, and Post-S. = Post-spawning stages. Values are means + SD for 10 fish, except for the ocean stage (n = 5). Letters indicate statistical comparisons between the 4 stages within each lipid class. Bars lacking letters or with different letters are different from each other ($P < 0.05$). Bars sharing the same letter are not statistically different.

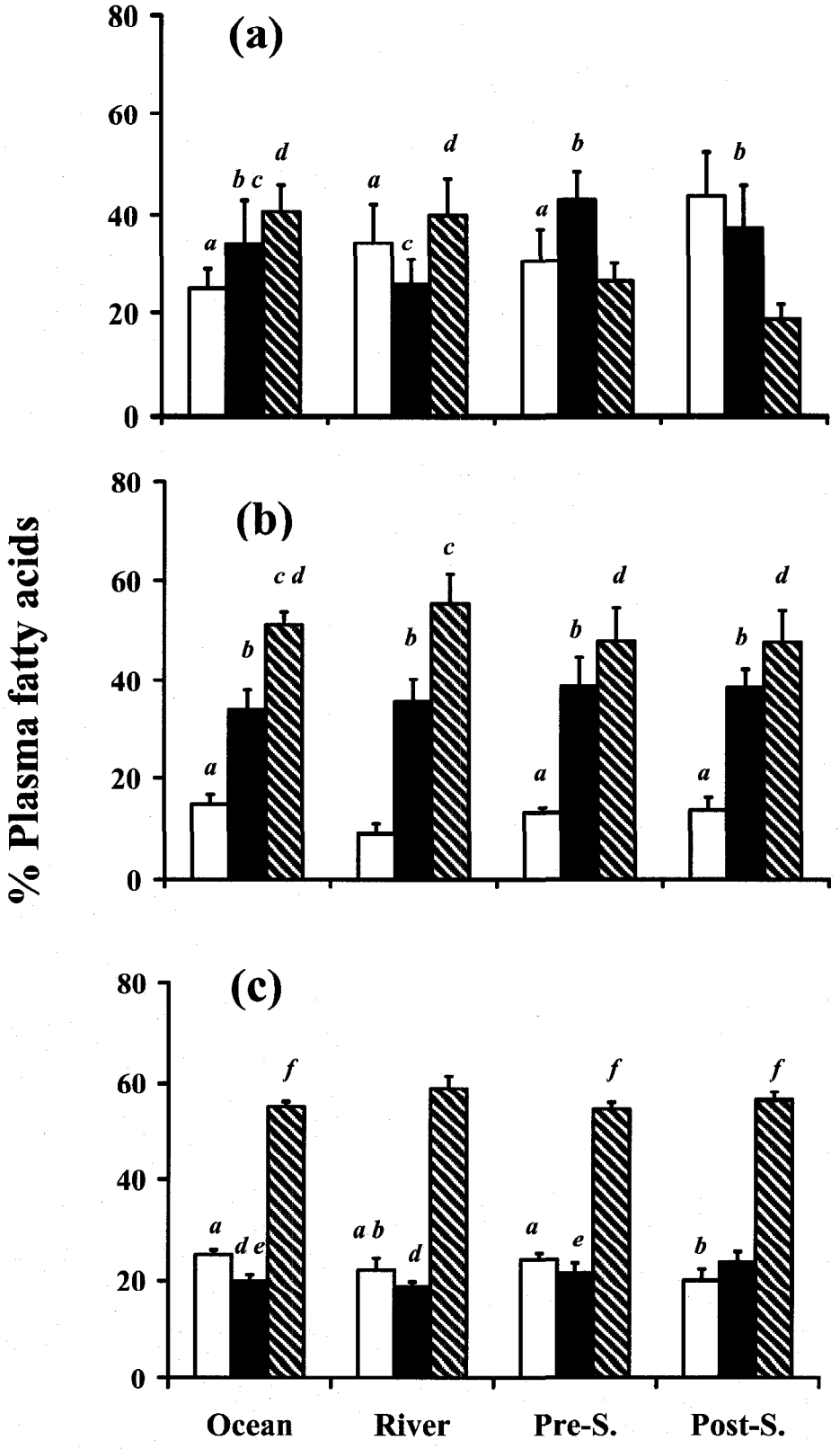
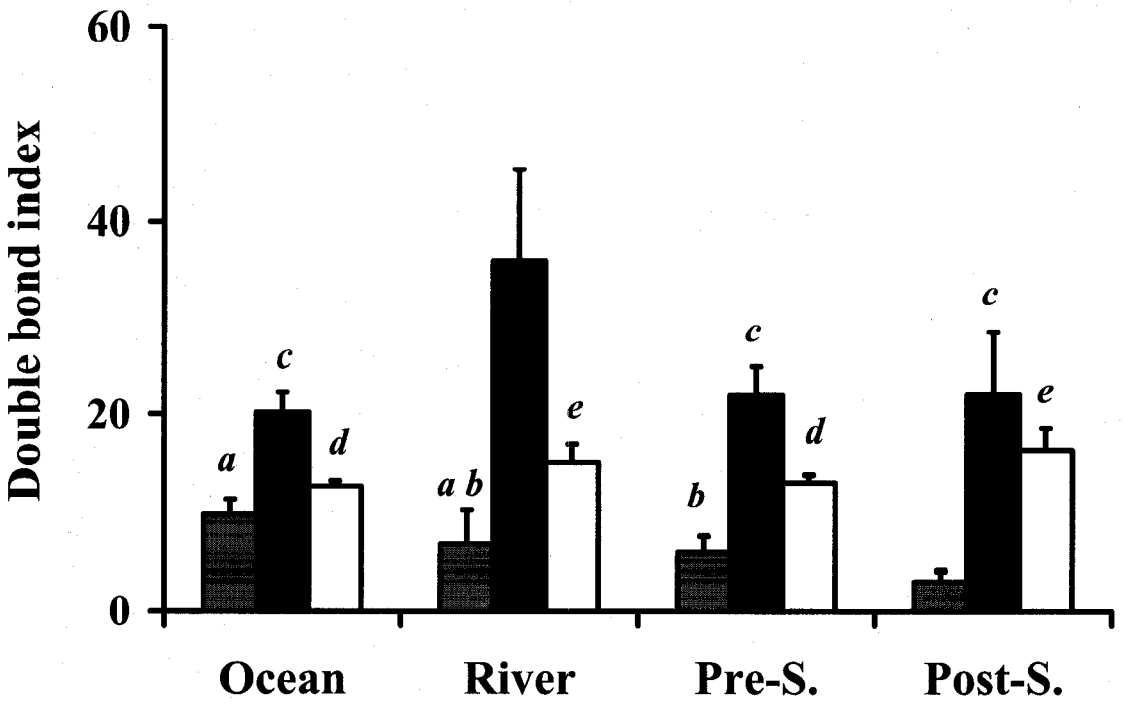

(a)
(b)
(c)
% Plasma fatty acids
80
60
40
20
0
Ocean
River
Pre-S.
Post-S.

Fig. 2.4. Double bond index (DBI) for non-esterified fatty acids (gray bars), triacylglycerol (black bars), and phospholipids (white bars) in the plasma of migrating sockeye salmon. River = River entry, Pre-S. = Pre-spawning, and Post-S. = Post-spawning stages. Values are means + SD for 10 fish, except for the ocean stage (n= 5). Letters indicate statistical comparisons between the 4 stages within each lipid class. Bars lacking letters or with different letters are different from each other ($P < 0.05$). Bars sharing the same letter are not statistically different.


Double bond index
60
40
20
0
a
c
d
a b
e
b
c
d
c
e
Ocean
River
Pre-S.
Post-S.

CHAPTER 3.

ENDURANCE SWIMMING ACTIVATES TROUT LIPOPROTEIN LIPASE: PLASMA LIPIDS AS A FUEL FOR MUSCLE

Based on

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Introduction

In fish, endurance swimming is primarily supported by lipids supplied to red muscle through the circulation, but the actual mechanism of delivery remains unknown (Moyes and West, 1995; Lauff and Wood, 1996; Rome, 1998; Richards *et al.*, 2002). By analogy with mammals, most fish studies have investigated the metabolic role of non-esterified fatty acids (NEFA), assuming that these lipids are responsible for transporting energy from adipose stores to locomotory muscles (Ballantyne *et al.*, 1996; Bernard *et al.*, 1999; Booth *et al.*, 1999; Weber *et al.*, 2002). However, this assumption is probably not justified because: 1) NEFA only account for a small percentage of the energy in plasma lipids (Plisetskaya, 1980; Babin and Vernier, 1989), and 2) endurance swimming has no effect on the turnover rate of NEFA (Bernard *et al.*, 1999). Although lipoproteins are used for transport to the gonads during egg production (Babin and Vernier, 1989), they have not been considered as a possible energy shuttle to working muscles. This is surprising because they carry most of the energy in the circulation (Moyes and West, 1995) and could theoretically play an important role in powering muscles during swimming. Lipoprotein lipase (LPL, EC 3.1.1.34) is the enzyme controlling the mobilization of lipoproteins and it has been well characterized in fish tissues (Lindberg and Olivecrona, 2002). Its potential role in orchestrating the supply of lipid fuel to working muscles has never been investigated.

In wild sockeye salmon, I previously proposed that lipoproteins are used to support swimming because their concentration changes dramatically during migration (Chapter 2). However, it is unclear whether this response to migration is only caused by exercise or by a combination of stresses including swimming, fasting, reproduction and

osmoregulation. To exclude confounding factors, this study investigates the effects of endurance swimming on lipoprotein metabolism of rainbow trout under controlled laboratory conditions. Previous studies have shown that the catabolism of large lipoproteins (VLDL) yields smaller particles such as VLDL-remnants or LDL (Havel, 1987; Zechner, 1997), and that monounsaturated fatty acids are a preferred substrate for oxidation (Henderson and Sargent, 1985; Kiessling and Kiessling, 1993; Sidell *et al.*, 1995; Weber *et al.*, 2002). I hypothesize that both, ***LPL*** (enzyme) and ***plasma lipoproteins*** (substrate) are modified by prolonged exercise. It is predicted that endurance swimming: 1) will activate LPL in red muscle, and 2) will alter circulating lipoprotein classes (high- , low- , and very low density lipoproteins: HDL, LDL, and VLDL), components (triacylglycerol, phospholipids and NEFA), and composition (individual fatty acids).

LPL is naturally bound to proteoglycans in the endothelium, but it can be released in the circulation by injecting heparin that has a higher affinity for the enzyme (Cryer, 1981). I was only able to find two studies where heparin who used as a tool to investigate LPL *in vitro* (Lindberg and Olivecrona, 1995) or *in vivo,* but in a single fish at rest (Skinner and Youssef, 1982). Here, my goals were to measure the effects of endurance exercise on LPL activity and on key characteristics of circulating lipoproteins. I have examined whether red muscle LPL and post-heparin plasma LPL are modified by several days of continuous swimming.

Methods

Animals

Female rainbow trout, *Oncorhynchus mykiss* (body mass 388 ± 85 g, total body length 31 ± 3 cm, N=54) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). They were kept in a 1,300 L flow-through holding tank in dechloraminated, well-oxygenated water at 13°C under 12 h:12 h L:D photoperiod. The same water quality and photoperiod were used during all the measurements, and the animals were acclimated to these conditions for at least one month before experiments. In the holding tank, routine swimming speed was <0.3 body length per second (BLs^{-1}). Trout were fed floating fish pellets (Martins Mills, Elmira, ON, Canada) five times a week until satiation. They were fasted for 72 h before measurements to eliminate circulating chylomicra (Wallaert and Babin, 1994b).

Exercise experiments

Fish were randomly divided in 2 groups: control and exercise. Pairs of animals were always measured simultaneously to be able to correct for potential effects of fasting. Exercising fish were placed in a modified Blazka-type swim tunnel (see Bernard *et al.*, 1999). Resting fish were measured in a tube with similar dimensions as the exercise chamber of the swim tunnel. For acclimation to the experimental setup, the animals were kept at rest in a weak water current (11 cm s^{-1}) for 24 h. For the following 4 days, the control fish was kept at rest, but the exercising fish had to swim at 1.5 BLs^{-1} (46 cm s^{-1}). At the end of the experiment, both fish were rapidly removed from the water and killed by a sharp blow to the head. Five ml of blood were sampled from the caudal artery

within 1 min after death using EDTA as anticoagulant (1 mg ml^{-1}). Plasma was separated by centrifugation (5,000 g for 10 min at 13°C) and used for the analysis of circulating lipoproteins. Red muscle (~2 g) from the caudal region of the lateral line was dissected in <2 min and stored at -80°C for analys of LPL activity.

Heparin experiments

A single catheter was surgically implanted in the dorsal aorta using buffered ethyl-N-aminobenzoate sulphonic acid as anaesthetic (MS-222, Sigma, St Louis, MO, USA) and sodium citrate as anticoagulant (13 μmol ml^{-1}). The animals were allowed to recover for 24 h in opaque plexiglass chambers (see Haman and Weber, 1996). Resting fish were injected with heparin (200, 600, or 1,000 U kg^{-1} body mass; Hepalean, Organon, Toronto, ON, Canada) and blood (0.5 ml) was collected before and after heparin injection. Plasma was immediately separated and stored at -80°C for subsequent analyses of LPL activity, as well as circulating TAG and glycerol concentrations (Sigma kits, St Louis, MO, USA).

Exercise + heparin experiments

Animals implanted with a dorsal aorta catheter were randomly divided in 2 groups: resting controls and swimming. Surgical procedures and swimming conditions were the same as above. After 4 days of rest or exercise, blood samples (0.5 ml) were obtained through the catheter before and 1 h after the administration of 600 U heparin kg^{-1} body mass to measure plasma LPL activity.

Lipoprotein lipase

LPL activity was measured in frozen samples within 4 weeks of sampling because preliminary experiments showed that it was not affected by freezing. For red muscle, 0.5 g tissue was homogenized in 9 vol buffer (10 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, and 5 U heparin ml^{-1} at pH 7.4) using a ground glass homogenizer on ice. Homogenates were centrifuged (20,000 g, 20 min at 4°C) and the clear phase between the top layer and the pellet was used for LPL analysis. A 20% lipid solution (Intralipid, Sigma, St Louis, MO, USA) was emulsified with tri [9,10(n)-^{3}H] oleate (Amersham, Buckinghamshire, UK). Preparation of the labelled substrate and assay conditions were described previously (Bengtsson-Olivecrona and Olivecrona, 1991) but were modified as follows. Radiolabeled trioleate (~45 μCi) was dried under N_2 and resuspended in 2 ml Intralipid solution and 8 ml Cortland saline. This mixture was sonicated for 5 min at 70% pulse mode and low setting (Branson Sonifier 450; Danbury, CT, USA). Each assay was carried out using a 50 μl aliquot of the emulsion as substrate, mixed with 50 μl preheated rat serum, 250 μl assay medium, and 100 μl plasma or red muscle homogenate. The reaction was stopped after 1 h incubation at 20°C by adding 3 ml methanol:chloroform:heptane (1.41:1.25:1 v/v/v) and 100 μl NaOH 0.1 N. After centrifugation (1,200 g, 20 min at 20°C), 1 ml of the upper phase was counted in 10 ml Safety Solve cocktail (Research Products, Mount Prospect, IL, USA) using a liquid scintillation counter (Beckman Coulter CS6500, Palo Alto, CA, USA). All LPL determinations were performed in triplicate.

Lipoprotein analysis

Lipoprotein classes were separated by ultracentrifugation (Beckman TL Optima; Palo Alto, CA, USA) using a self-generated gradient (Optiprep, Axis-Shield, Oslo, Norway) (see Graham *et al.*, 1996). Fresh plasma (3.2 ml), Optiprep gradient (0.8 ml) and buffered saline (0.7 ml) were layered in Optiseal ultracentrifuge tubes (Beckman Coulter, Palo Alto, CA, USA) before centrifugation (350,000 g, 3 h at 13°C). The different lipoprotein fractions were collected: high-density lipoproteins (1.6 ml), LDL (1.6 ml) and VLDL (1.5 ml). The exact nature of the 3-lipoprotein fractions was confirmed with agarose gels (Paragon electrophoresis system, Beckman Coulter, Fullerton, CA, USA). Fractions were stored at -80°C for subsequent analysis of protein content (Bradford reagent, Sigma, St Louis, MO, USA) and fatty acid composition by gas chromatography (Chapter 2).

Statistical analyses

A t-test was used to evaluate the effects of swimming on LPL activity in red muscle. In all other cases, statistical differences were assessed using analysis of variance (ANOVA), or Kruskal-Wallis analysis of variance on ranks when the assumption of normality or homoscedasticity was not met. When significant changes were detected by ANOVA, the Holm-Sidak method was used for pairwise comparisons. Percentages were transformed to the arcsine of their square root before statistical analysis and all values are given as means ± SE.

Results

Effects of sustained swimming

The effect of endurance swimming on the LPL activity of rainbow trout red muscle is presented in Fig. 3.1. Four days of continuous swimming at 1.5 BLs^{-1} caused a 2.7-fold increase in LPL activity (P= 0.009). The capacity of this enzyme to hydrolyze fatty acids from triacylglycerol went from 18 ± 5 nmol FA min^{-1} g^{-1} wet tissue to 49 ± 9 nmol FA min^{-1} g^{-1} wet tissue. The effects of prolonged swimming on circulating lipoproteins were examined in detail and this analysis is summarized in Figs. 3.2 and 3.3. Fatty acid content, protein content and the fatty acid/protein ratio of the three lipoprotein classes (HDL, LDL, or VLDL) are shown in Fig. 3.2. Endurance swimming had no measurable effect on these parameters (P>0.05). The relative fatty acid content of the different lipoprotein classes was the same (P>0.05), but their protein content (P=0.002) and their fatty acid/protein ratio (P<0.01) varied drastically. Protein content decreased in the following order: HDL>LDL>VLDL (Fig. 3.2B), whereas the opposite was true for the fatty acid to protein ratio (Fig. 3.2C).

Lipoproteins were also analyzed by separating their various components. The concentrations of PL, TAG, and NEFA in the three classes of lipoproteins are shown in Fig. 3.3. Endurance swimming had no measurable effect on these parameters (P>0.05). Phospholipid was the main lipid component of HDL (63% of total fatty acids in HDL) and LDL (59% of total fatty acids in LDL), whereas TAG was the main lipid component in VLDL (46% of total fatty acids in VLDL). NEFA only represented 6 to 11% of total FA in the various lipoprotein fractions. The detailed fatty acid composition of PL, TAG and NEFA in the three lipoprotein classes was also analyzed. Because no measurable

effect of endurance swimming was detected on fatty acid composition ($P>0.05$), Table 3.1 only presents pooled data for control and exercised fish, and groups individual fatty acids into major classes: saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA). In all lipoproteins, PUFA are the main fatty acids in PL (63-71%) and TAG (47-51 %), whereas SFA are dominant in NEFA (64-80%). MUFA are thought to provide the best fuel for oxidation (Sidell *et al.*, 1995) and they account for an important fraction of TAG fatty acids (34-40%).

Effects of heparin

The time course of changes in plasma LPL activity after injection of heparin is presented in Fig. 3.4. Baseline LPL activity was 0.04 ± 0.01 μmol FA released min^{-1} ml^{-1} plasma and it was strongly stimulated 30-60 min after heparin administration, when maximal values were recorded (1.07 ± 0.20 and 1.32 ± 0.67 μmol FA released min^{-1} ml^{-1} plasma for heparin doses of 200 and 600 U kg^{-1}, respectively) ($P<0.001$). Activity stayed elevated above baseline for 2 h after heparin injection, but values measured from 4 to 48 h after injection were not different from baseline ($P>0.05$). Maximal stimulation of plasma LPL was already obtained at 200 U heparin kg^{-1} because no significant difference between the two doses were detected ($P>0.05$). This was confirmed by administration of 1,000 U kg^{-1} in four additional fish (data not shown). Figure 3.5 shows the effects of heparin administration on the concentrations of plasma TAG and plasma glycerol. Injecting 200 or 600 U heparin kg^{-1} had no detectable effect on the concentrations of TAG or glycerol (Fig. 3.5) ($P>0.05$).

Combined effects of swimming and heparin on plasma LPL

The effects of prolonged swimming and heparin on plasma LPL activity are presented in Fig. 3.6. Heparin had a major stimulating effect on plasma LPL activity ($P<0.001$), but this strong response was not significantly different between resting fish and those that had been swimming for 4 days at 1.5 BL s^{-1} ($P>0.05$).

Discussion

This study shows that lipoprotein lipase of fish red muscle is strongly activated during prolonged exercise, implying that lipoproteins are used as an energy shuttle to working muscles (Fig. 3.1). In rainbow trout, the existence of this fuel supply mechanism is further supported by the high reserve capacity for lipoprotein hydrolysis demonstrated here with heparin-induced stimulation of plasma LPL (Figs. 3.4 and 3.6). Contrary to expectation, the large increases in LPL activity elicited by endurance exercise (three-fold) and heparin (27-fold) are not accompanied by changes in plasma lipoprotein concentration or composition (Figs. 3.3 and 3.5, Table 3.1).

Effects of swimming on red muscle LPL

Fish LPL has been characterized in most tissues (red and white muscle, mesenteric fat, gonads and liver) where it is strongly modulated by seasonal cycles associated with fasting and reproduction (Black and Skinner, 1986, 1987; Fremont *et al.*, 1987; Lindberg and Olivecrona, 1995; Ibáñez *et al.*, 2003; Saera-Vila *et al.*, 2005). My study is the first to examine the effects of sustained swimming on this enzyme, and it

shows that LPL is stimulated by exercise in lateral red muscle the engine for endurance swimming. The three-fold increase in LPL activity observed in trout muscle after prolonged exercise is consistent with the response reported for several mammals including rats (2 to 3-fold; Oscai *et al.*, 1982; Bagby *et al.*, 1986; Ladu *et al.*, 1991b), dogs (2-fold; Budohoski, 1985), and humans (3-fold; Lithell *et al.*, 1984). *In vitro* experiments show that the addition of LPL to co-culture systems containing lipoprotein-secreting hepatocytes and muscle cells of fish increases muscle FA uptake by 60% (Alam *et al.*, 2004).

Tissue uptake of fatty acids from circulating lipoproteins is thought to be limited by the rate of TAG hydrolysis and, therefore, it is mainly regulated by LPL activity (Nilsson-Ehle, 1980). Numerous hormones including insulin, catecholamines, glucocorticoids, and thyroxine are well known modulators of LPL. The relative distribution of their various receptors is responsible for tissue-specific responses (Mead *et al.*, 2002). The activation of mammalian LPL by exercise is linked to increased LPL mRNA levels in skeletal muscle (Kiens *et al.*, 2004), with catecholamines and insulin acting as the most probable hormonal modulators (Lithell *et al.*, 1981; Chernick *et al.*, 1986; Ladu *et al.*, 1991a; Enerback and Gimble, 1993; Seip *et al.*, 1997). In addition to systemic regulation by hormones, local signals associated with contractile activity have been implicated in LPL modulation. In rats, a significant role for contractile activity is supported by experiments in which electrical stimulation was performed unilaterally. Stimulated muscles showed a three-fold increase in LPL activity, whereas contralateral (rested) muscles did not respond (Hamilton *et al.*, 1998). Taken together, current data on the exercise-induced up-regulation of mammalian LPL do not allow establishing the

relative importance of these various mechanisms. For fish, even less information is available, but a significant role for insulin modulation of muscle LPL appears doubtful. Recent experiments show that *in vivo* administration of insulin causes the activation of adipose tissue LPL in rainbow trout, but has no measurable effect on red muscle (Albalat *et al.*, 2006). The exercise-induced activation of LPL observed in my study is therefore probably associated with changes in circulating catecholamines or contractile activity. For example, prolonged exercise causes a decrease in circulating epinephrine levels (Shanghavi and Weber, 1999) that may be involved in LPL activation. Even though further studies are needed to characterize exact regulatory mechanisms in fish, results clearly show that red muscle LPL is recruited during prolonged swimming, making lipoproteins available as a fuel for locomotion.

Effects of heparin on plasma LPL

This study characterizes the activation of plasma LPL by heparin in intact fish. It provides a time course of changes in circulating LPL activity for different doses (200-1,000 U heparin kg^{-1}) and shows that a maximal response is reached ~1 h after injecting 600 U kg^{-1}. I am aware of only one other study investigating this issue; it reports the partial response of an individual fish after injection of 100 U kg^{-1} (Skinner and Youssef, 1982). The maximal LPL activity measured here was much higher than in this previous study and it occurred later (60 *vs* 35 min after injection). Such discrepancy is not simply due to the higher doses used here, but mainly to the actual substrate for the LPL assay. Intralipid-based emulsions (this study) are considered a better imitation of real lipoproteins than those made with gum arabic (as in Skinner and Youssef, 1982) that

yield sub-optimal hydrolysis of radiolabeled trioleate (Bengtsson-Olivecrona and Olivecrona, 1991). This view is further supported by *in vitro* measurements of plasma LPL on intralipid emulsions that give values of up to 0.8 μmol FA min^{-1} ml^{-1} (Lindberg and Olivecrona, 2002), approaching maximal activities reported here (1.32 μmol FA min^{-1} ml^{-1}; Fig. 4). Under baseline conditions, LPL is mostly bound to the endothelium, and plasma LPL activity is therefore very low (here, only 3% of peak, post-heparin values; see Fig. 3.4). The injection of heparin causes a drastic increase in activity: 1) by releasing the enzyme into the plasma, and 2) by inhibiting its normal uptake by the liver for degradation (Chajek-Shaul *et al.*, 1988). The impressive 27-fold increase in activity observed here in plasma after release of the enzyme by heparin demonstrates that rainbow trout tissues have a remarkable reserve capacity for lipoprotein hydrolysis.

Combined effects of swimming and heparin on plasma LPL

One goal of this study was to determine whether the changes in tissue LPL caused by exercise would be measurable in post-heparin plasma. Contrary to expectation, the observed increase in red muscle LPL (Fig. 3.1) was not mirrored by post-heparin plasma LPL (Fig. 3.6). However, close examination of the data reveals a non-significant trend towards an increase in the exercise group that would be consistent with my red muscle results. It is also conceivable that the activation of red muscle LPL is accompanied by inhibition of the enzyme in other tissues, yielding no overall change in post-heparin plasma LPL. Alternately, the effect of exercise on red muscle LPL may not be large enough to influence LPL activity in post-heparin plasma because trout red muscle only

represents 7% of total body mass. For example, rats using a larger muscle mass than the trout of my experiments show a significant increase in post-heparin plasma LPL after exercise (Hamilton *et al.*, 1998).

Lipoprotein concentration and composition

The exercise-induced increase in red muscle LPL activity is not accompanied by changes in the concentration or the composition of circulating lipoproteins. Therefore, the hypothesis that particular fatty acids are used preferentially (selectivity) is not supported by the results. The increase in muscle LPL activity implies that the rate of lipoprotein turnover is stimulated during swimming, and, therefore, that lipoprotein concentration is a poor indicator of TAG turnover rate. A mismatch between changes in flux and concentration is a common occurrence and has been demonstrated for various trout metabolites (Haman *et al.*, 1997). Unfortunately, no reliable method is currently available to measure TAG turnover rate directly.

The 27-fold increase in plasma LPL activity caused by heparin (Fig. 3.4) does not have any measurable effect on plasma TAG concentration (Fig. 3.5), the main substrate for the enzyme. In contrast, plasma TAG concentration of mammals is decreased by endurance exercise (Hardman, 1998; Ensign *et al.*, 2002), and *in vivo* heparin administration has a marked lipolytic effect on circulating TAG (Skoglund-Andersson *et al.*, 2003). The high lipoprotein concentrations of fish compared to mammals (Babin and Vernier, 1989) may be responsible for the concentration inertia observed here in trout. Because concentration stays constant, tissue uptake of lipoprotein-derived fatty acids

must be regulated by changes in LPL activity rather than by a mass action effect of its substrate (Nilsson-Ehle, 1980).

Most lipoprotein studies report concentrations in weight % (mg/100 ml). However, this unit fails to reveal quantitative differences in energy content because lipoprotein classes contain different amounts of protein, as well as cholesterol. To avoid this problem, Fig. 3.3 expresses concentrations of the different lipid components in μmol fatty acid ml^{-1} plasma (because fatty acids contain most of the energy in lipoproteins). This figure shows that the great majority of the energy circulating in the plasma lipids of rainbow trout resides in PL (55%) and TAG (37%), whereas NEFA only make a minor contribution (8% of the energy). Within each lipoprotein class, fatty acid content is similar, but protein content is highly variable (HDL>LDL>VLDL). Therefore, the FA/protein ratio ranges between ~0.2 for HDL and ~2.0 for VLDL (Fig. 3.2). Using protein content from Fig. 3.2B (in mg protein ml^{-1} plasma: HDL= 33, LDL= 5, and VLDL= 0.7) and published values for % protein in each lipoprotein class by weight (% protein: HDL= 45, LDL= 30, and VLDL= 13; Babin and Vernier, 1989), I can estimate that 70% of all trout lipoproteins are HDL, 21% are LDL, and the remaining 9% are VLDL.

Conclusion

Red muscle LPL is activated by endurance swimming and rainbow trout show a very high reserve capacity for the hydrolysis of circulating lipoproteins. These novel characteristics of trout LPL and the fact that lipoproteins contain 92% of the energy in

plasma lipids imply that lipoproteins are used as an energy shuttle between fat reserves and working muscles. Such a mechanism contrasts with the classic mammalian strategy where lipid fuel is supplied by NEFA-albumin complexes. Because lipoprotein concentration does not reflect changes in flux, direct measurements of lipoprotein kinetics will be needed as soon as adequate methods are developed.

Table 3.1. Percentages of total fatty acids per lipid class (PL, TAG and NEFA) transported in High density (HDL), Low density (LDL) and Very low density lipoproteins (VLDL) in rainbow trout plasma.

	HDL			**LDL**			**VLDL**		
Fatty acids	PL	TAG	NEFA	PL	TAG	NEFA	PL	TAG	NEFA
SFA	19.2 (2.1)	15.0 (2.3)	63.6 (9.4)	18.8 (2.4)	17.4 (4.3)	69.7 (6.6)	16.2 (3.5)	11.3 (4.3)	80.4 (6.9)
MUFA	17.5 (0.9)	34.4 (2.1)	22.4 (6.6)	17.4 (0.7)	35.2 (2.7)	16.5 (3.6)	12.9 (2.1)	39.8 (4.3)	16.8 (7.0)
PUFA	63.3 (2.6)	50.6 (3.1)	14.0 (2.9)	63.7 (2.9)	47.4 (4.6)	13.7 (4.0)	70.9 (5.1)	48.9 (5.8)	2.8 (1.1)

Measurements were made on two groups of 7 fish after 4 days of resting or swimming. Because exercise had no significant effect, pooled values are reported as means (SE) (N=14). Abbreviations: phospholipids (PL), triacylglycerol (TAG), Non-esterified fatty acids (NEFA); Saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA).

Fig. 3.1. Effect of endurance swimming (4 days at 1.5 BLs^{-1}) on lipoprotein lipase activity (LPL) in red muscle of rainbow trout. Values are mean ± SE (N=8). Letter *a* denotes a significant difference from the resting control ($p<0.01$).

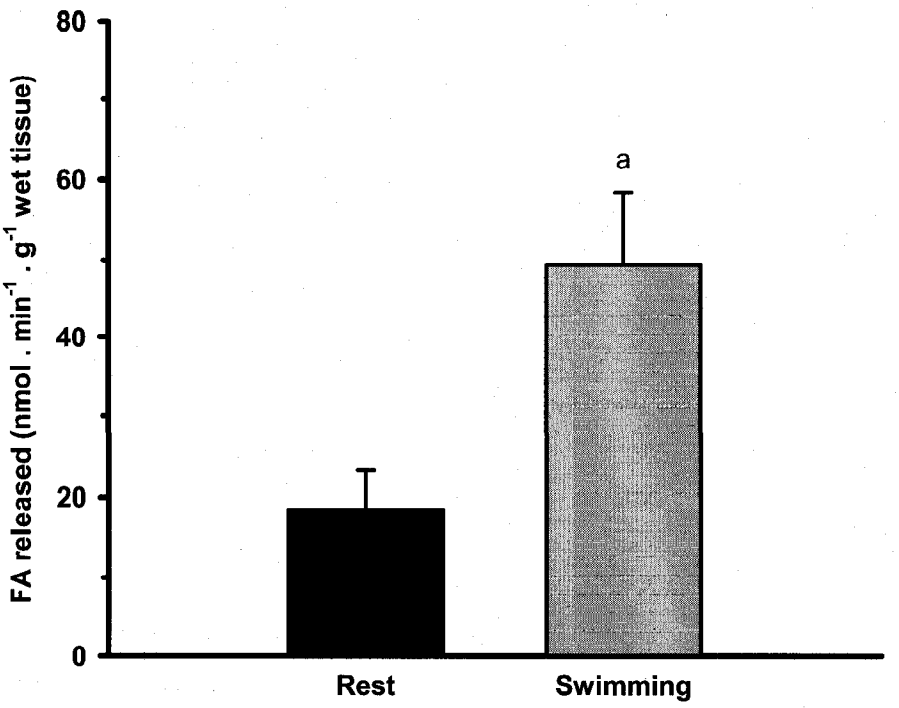

80
60
40
20
0
FA released (nmol . min-1 . g-1 wet tissue)
a
Rest
Swimming

Fig. 3.2. Concentrations of fatty acids (FA) (A), proteins (B), and FA to protein ratio (C) in the plasma lipoproteins of rainbow trout after 4 days of resting or 4 days of sustained swimming at 1.5 BLs^{-1}. Values are mean ± SE (N=7). HDL, LDL, and VLDL stand for high density, low density and very low density lipoproteins, respectively.

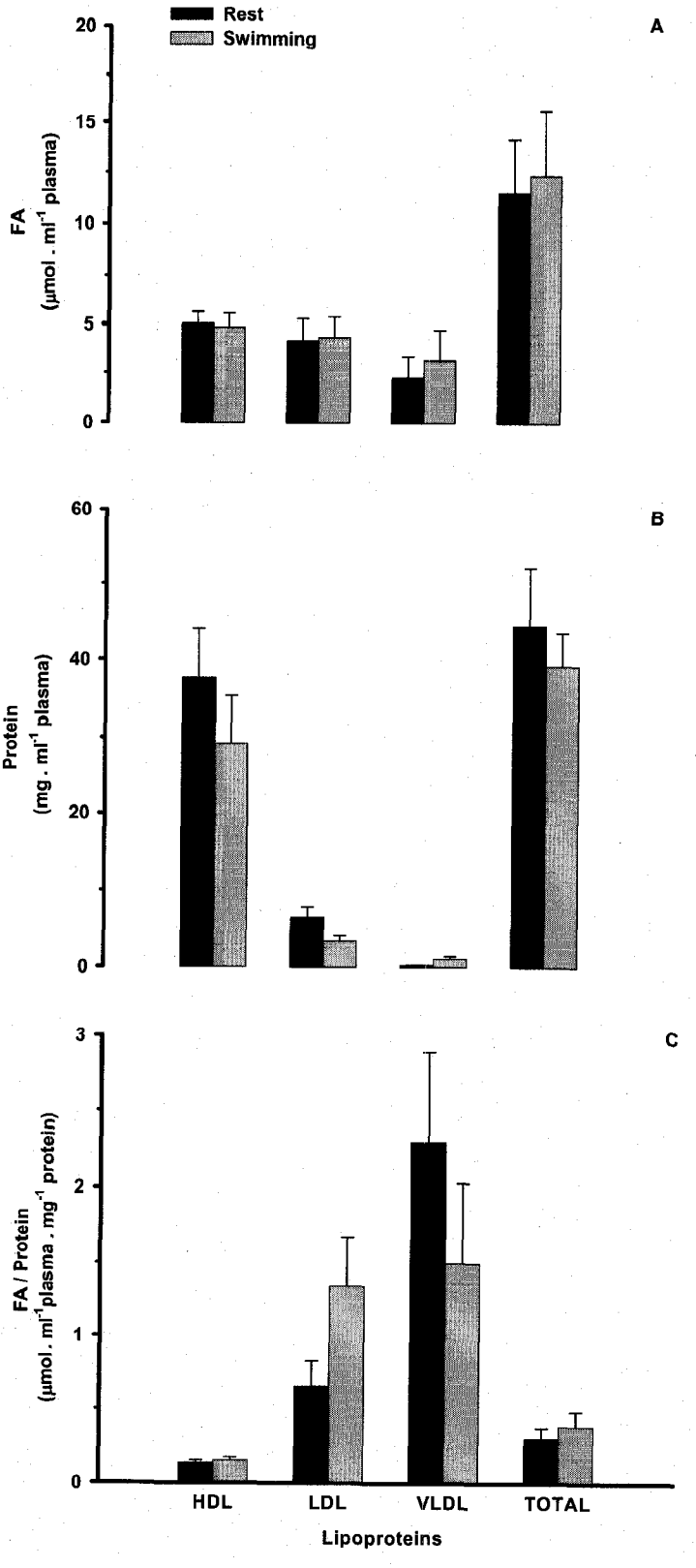

Rest
Swimming
A
FA
(µmol . ml-1 plasma)
20
15
10
5
0
B
Protein
(mg . ml-1 plasma)
60
40
20
0
C
FA / Protein
(µmol . ml-1plasma . mg-1 protein)
3
2
1
0
HDL
LDL
VLDL
TOTAL
Lipoproteins

Fig. 3.3. Concentrations of phospholipids (PL) (A), triacylglycerol (TAG) (B), and non-esterified fatty acids (NEFA) (C) in the plasma lipoproteins of rainbow trout after 4 days of rest or swimming at 1.5 BLs^{-1}. To allow comparisons between lipid classes, all concentrations are given in μmol fatty acids per ml plasma. Absolute PL and TAG concentrations can be obtained by dividing the values presented in (A) by 2 (2 fatty acids per PL) and in (B) by 3 (3 fatty acids per TAG). Values are mean ± SE (N=7). HDL, LDL, and VLDL stand for high density, low density and very low density lipoproteins.

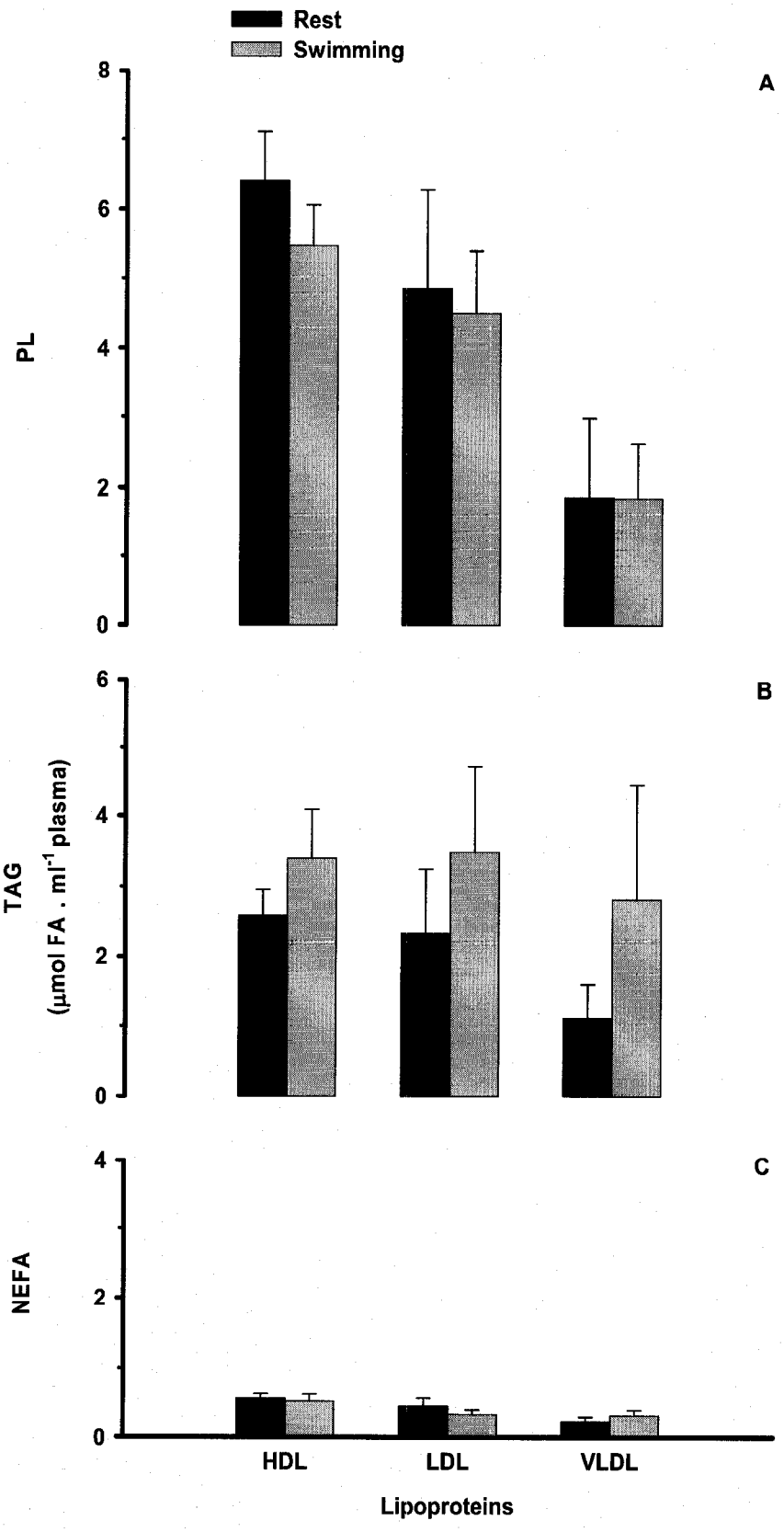

Rest
Swimming
A
PL
8
6
4
2
0
B
TAG
(µmol FA . ml-1 plasma)
6
4
2
0
C
NEFA
4
2
0
HDL
LDL
VLDL
Lipoproteins

Fig. 3.4. Time course of changes in lipoprotein lipase activity of rainbow trout plasma after injection of 200 and 600 U heparin kg^{-1} body mass. Values are mean ± SE (N=8). Letters show significant differences from control values at time 0 (before heparin administration).

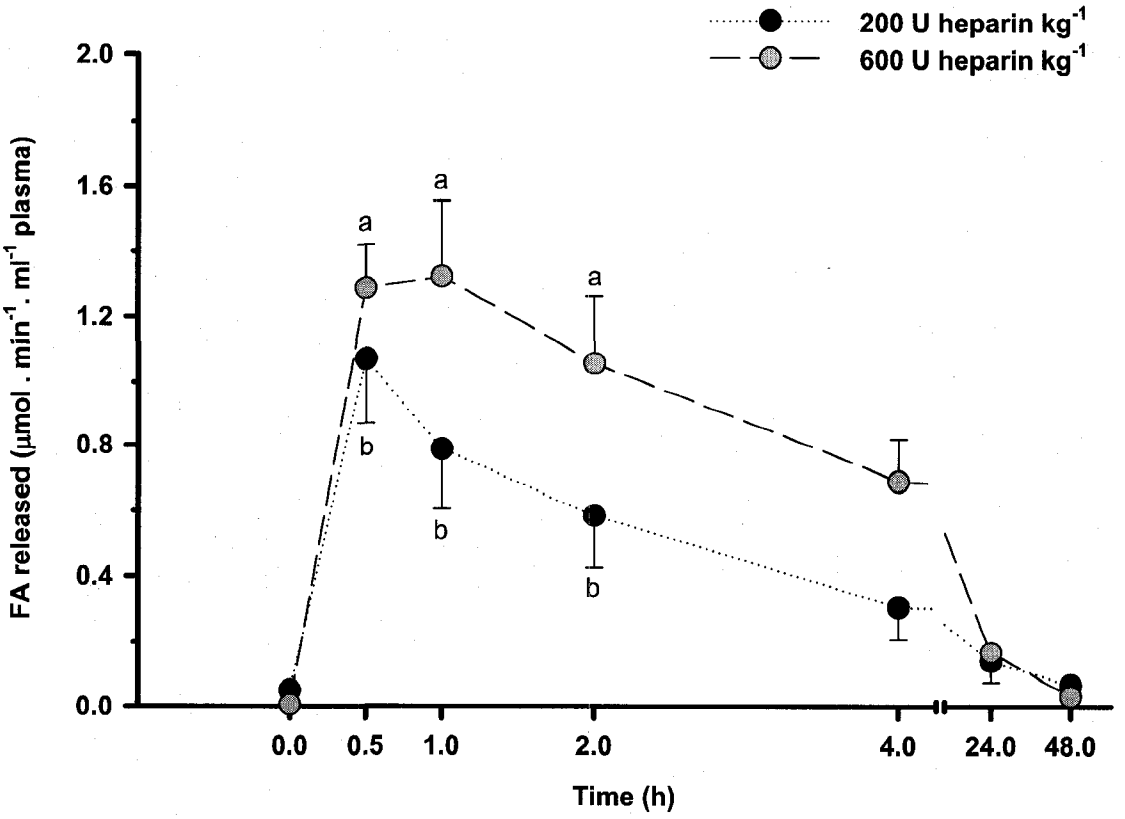

200 U heparin kg-1
600 U heparin kg-1
FA released (µmol . min-1 . ml-1 plasma)
2.0
1.6
1.2
0.8
0.4
0.0
a
a
a
b
b
b
0.0
0.5
1.0
2.0
4.0
24.0
48.0
Time (h)

Fig. 3.5. Concentrations of triacylglycerol (TAG) (A), and glycerol (B) in rainbow trout plasma after injection of 200 and 600 U heparin kg^{-1} body mass. Values are means ± SE (N=8).

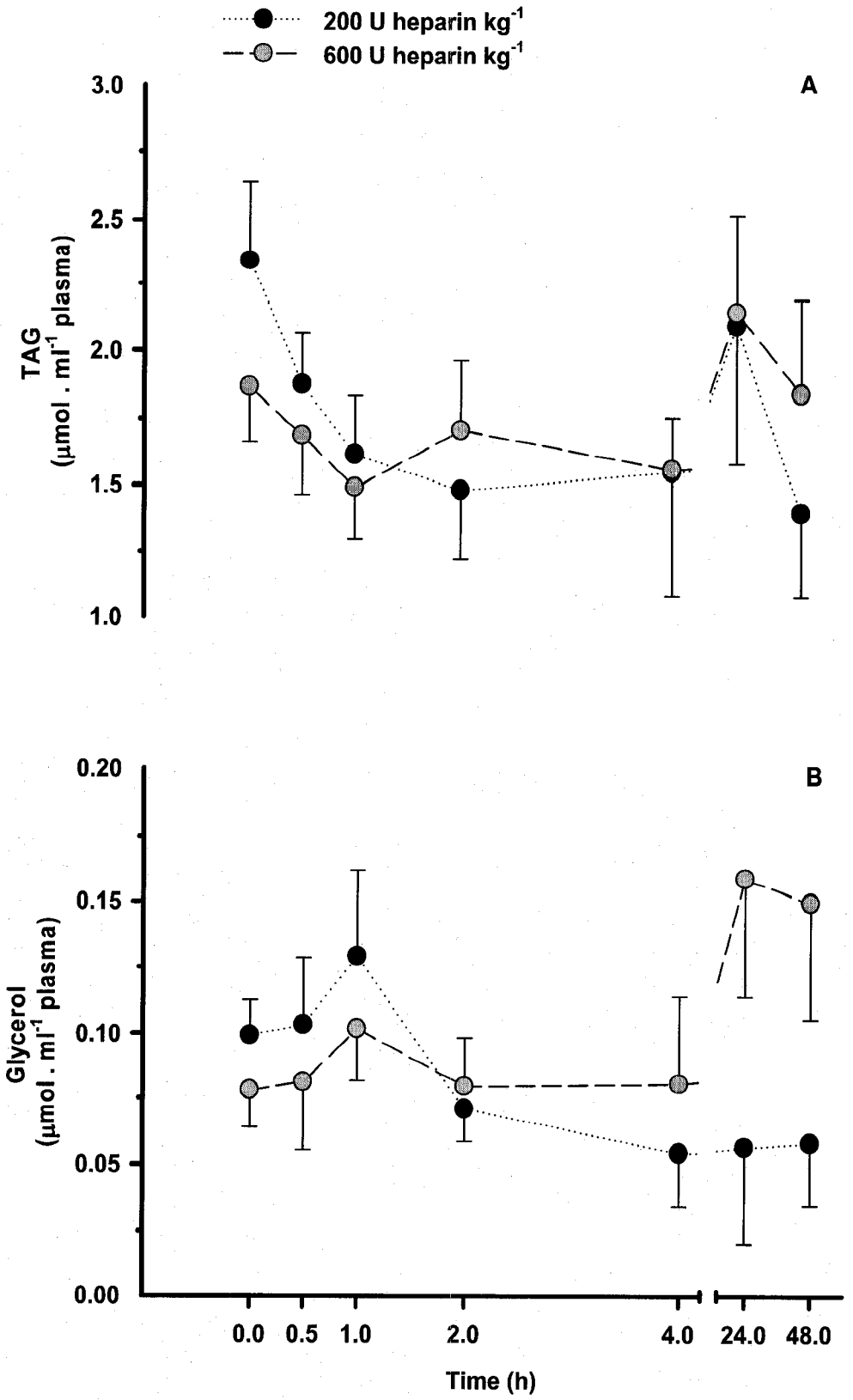
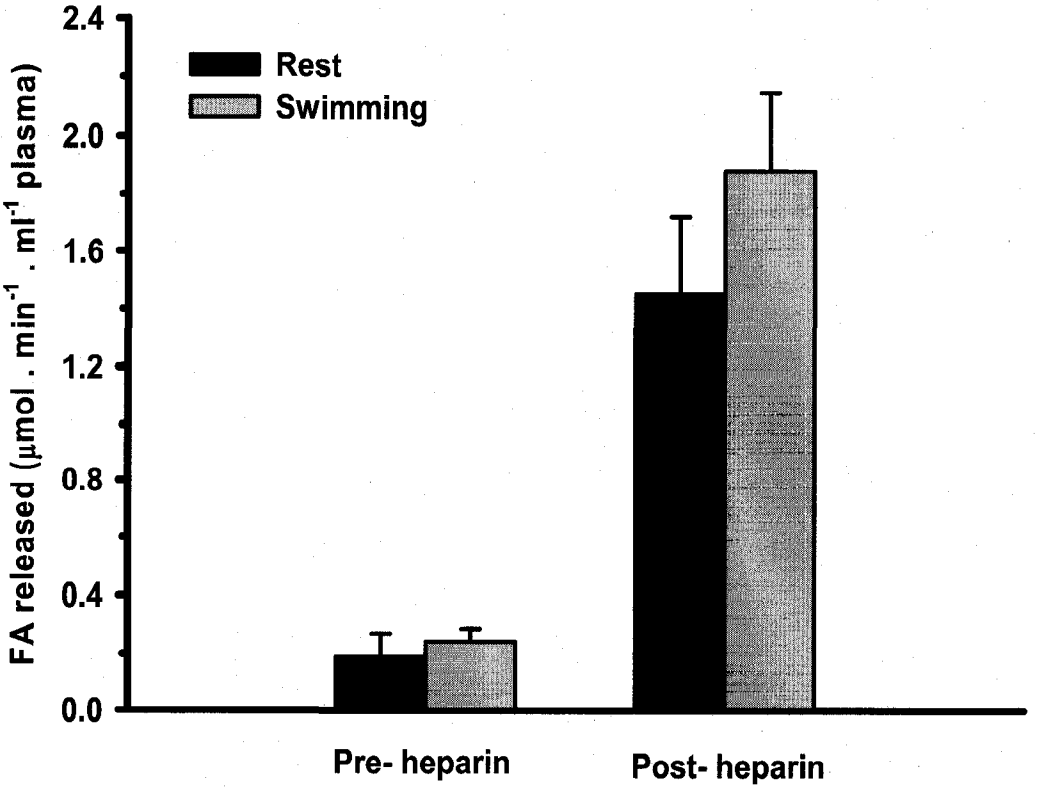

200 U heparin kg-1
600 U heparin kg-1
A
TAG
(µmol . ml-1 plasma)
3.0
2.5
2.0
1.5
1.0
B
Glycerol
(µmol . ml-1 plasma)
0.20
0.15
0.10
0.05
0.00
0.0
0.5
1.0
2.0
4.0
24.0
48.0
Time (h)

Fig. 3.6. Plasma lipoprotein lipase activity before and 1 h after administration of 600 U heparin kg^{-1} body mass in resting (control) and swimming rainbow trout (4 days at 1.5 BLs^{-1}). Heparin increased LPL activity in both groups ($P<0.001$), but this effect was not different between resting and swimming fish ($P>0.05$). Values are means ± SE ($N=9$).


2.4
2.0
1.6
1.2
0.8
0.4
0.0
FA released (µmol . min-1 . ml-1 plasma)
Rest
Swimming
Pre- heparin
Post- heparin

CHAPTER 4.

HIGH RESTING TRIACYLGLYCEROL TURNOVER OF RAINBOW TROUT EXCEEDS THE ENERGY REQUIREMENTS OF ENDURANCE SWIMMING

Based on

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Introduction

Circulating lipids play a critical role in energy distribution during sustained exercise in vertebrates (Moyes and West, 1995; van Loon *et al.*, 2001; McWilliams *et al.*, 2004). They are transported as non-esterified fatty acids (NEFA) or esterified fatty acids in triacylglycerol (TAG) and phospholipids (PL), two critical components of lipoproteins. Because the relative abundance of nonesterified and esterified fatty acids differs among vertebrates, different species may show a variety of strategies to shuttle energy from lipid reserves to working muscles. Even though mammals can use significant amounts of lipoproteins (Helge *et al.*, 2001; Morio *et al.*, 2004), they transport most lipids to working muscles as NEFA-albumin complexes (Terjung and Kaciuba-Usciłko, 1986) and their NEFA flux is strongly stimulated by exercise (Wolfe *et al.*, 1990; Brooks, 1998). In contrast, fish do not increase NEFA flux during prolonged swimming (Bernard *et al.*, 1999), possibly because they use lipoproteins to fuel locomotory muscles. Recent evidence supporting this view includes: (i) plasma lipoprotein concentration of sockeye salmon changes dramatically over the course of migration (Chapter 2); (ii) rainbow trout have particularly high lipoprotein levels (~4 times mammalian values; Babin and Vernier, 1989) that account for 92% of total plasma lipids; and, (iii) endurance swimming activates lipoprotein lipase (LPL) in trout red muscle (Chapter 3).

Paradoxically, the strong activation of trout LPL elicited by prolonged swimming (in red muscle) or by heparin (in plasma) is not accompanied by significant changes in lipoprotein concentration or composition (Chapter 3). However, this does not mean that the TAG turnover rate of fish is unaffected by such treatments, because flux and

concentration often vary independently (Haman *et al.*, 1997). TAG turnover rate has never been measured in fish, and only rarely in mammals (Havel and Kane, 1975; Bagby *et al.*, 1987; Hirano *et al.*, 1988; Mittendorfer *et al.*, 2003; O'Donnell *et al.*, 2006). This is probably because lipoproteins are normally not a major fuel for working mammalian muscles (Terjung and Kaciuba-Usciłko, 1986) (although their role can increase after a high fat diet; (Helge *et al.*, 2001)), and the *in vivo* measurement of TAG turnover rate by tracer kinetics is technically challenging (Berman *et al.*, 1982). Exercise (Moyes and West, 1995) and heparin administration (Chapter 3) have numerous metabolic consequences in fish, but their effects on TAG turnover rate are unknown. In mammals, heparin frees LPL from its active sites on endothelial cells to plasma. In this study, I have developed novel *in vivo* tracer methods for measuring TAG turnover rate in fish. The first approach uses bolus injection of very low density lipoproteins (VLDL) labeled *in vivo* from donor fish, a natural substrate for fish LPL, but impossible to produce in large quantities. The second approach uses continuous infusion of an exogenously labeled Intralipid emulsion, an easy to prepare, artificial substrate hydrolyzed by trout LPL. Both substrates were labeled with tri-[^{3}H]-oleate and successfully used as tracers in previous mammalian studies of lipoprotein kinetics (Mackie *et al.*, 1980; Redgrave and Maranhao, 1985; Nakandakare *et al.*, 1994; Karpe and Hultin, 1995; Teusink *et al.*, 2003). My goals were to characterize the effects of endurance swimming and heparin administration on the TAG turnover rate of rainbow trout. I hypothesized that endurance swimming would stimulate TAG turnover rate to provide more fuel to working muscles, and that heparin would reduce it by releasing LPL in plasma, thereby preventing normal lipoprotein uptake.

Methods

Animals

Adult, sexually immature female rainbow trout, *Oncorhynchus mykiss*, were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) in September 2006. They were kept in a 1,300 L flow-through holding tank in dechloraminated, well-oxygenated water at 13 °C under a 12 h: 12 h L: D photoperiod. The same water quality and photoperiod were used during all the measurements. Animals were acclimated to these conditions for at least 55 days before the experiments that were carried out between November 2006 and March 2007. In the holding tank, routine swimming speed was <0.3 body length per second (BL s^{-1}). Trout were fed floating fish pellets (Martins Mills, Elmira, ON, Canada; see fatty acid composition of the food in Table 4.1) once a day for five days per week (or an average of ~0.45% of body weight per day). They were fasted for 72 h before measurements to eliminate circulating chylomicra (Wallaert and Babin, 1994b).

Preparation of tri-[^{3}H]-oleate emulsion for measurement of TAG kinetics

An Intralipid emulsion (Sigma, St Louis, MO, USA) labeled with tri-[^{3}H]-oleate was used to prepare the tracer for *bolus injection* and *continuous infusion* experiments. A stock solution of labeled TAG was made by drying 450 μCi tri-[9,10(n)-^{3}H] oleate (Amersham, Buckinghamshire, UK) (22.5 μCi ml^{-1}) under N_2 and resuspending in heptane. It was stored anaerobically at -20°C for up to 2 months. Before each

experiment, an emulsion was freshly prepared by sonicating an aliquot of the tri-[^{3}H]-oleate stock solution with Intralipid (20%) and Cortland saline as described previously (Augustus *et al.*, 2003). The fatty acid composition and ^{3}H content of the different lipid classes in the emulsion are presented in Table 4.2.

Bolus injections

These experiments were designed to measure baseline TAG kinetics in resting rainbow trout, using VLDL labeled *in vivo* from donor fish as tracer. A batch of donor fish (body mass 462 ± 49 g, N=9) was implanted with single dorsal aorta catheters and was used to produce the tracer for bolus injections. Surgery was performed under buffered ethyl-N-aminobenzoate sulphonic acid anaesthesia (MS- 222, Sigma, St Louis, MO, USA) following the procedure of Haman and Weber (1996), but using 13 mM sodium citrate as anticoagulant (< 0.2 ml, pH 7.7). The animals were allowed to recover for 24 h in opaque Plexiglas^{TM} chambers (Chapter 3). They were then infused with 8 ml tri-[^{3}H]-oleate emulsion at 1 ml h^{-1}. Twelve hours after the end of infusion, plasma was harvested from the donor animals and the different lipoprotein fractions (HDL, LDL, and VLDL; see Table 4.4) were separated following the procedure described in Chapter 3. This allowed the collection of enough labeled VLDL to perform four bolus injection experiments. The nature of the labeled VLDL particles was assessed by agarose gel electrophoresis (Paragon system, Beckman Coulter, Fullerton, CA, USA). This procedure showed that VLDL migration was unaffected by label incorporation and that 92% of the activity was in TAG (see Table 4.4).

Animals used for bolus injections were implanted with single catheters as described above (body mass 410 ± 14 g, N=4). At time 0, a bolus of radiolabeled VLDL (total activity of ~ 500,000 dpm in 4 ml or ~ 60,000 dpm/ μmol TAG) was injected through the catheter in resting fish and blood samples were collected at different times after injection. Plasma was immediately separated and stored at -80°C for subsequent determination of radioactivity (Beckman Coulter CS6500 scintillation counter, Palo Alto, CA, USA) and TAG concentration. Concentration was measured by spectrophotometry using a kit (TR0 100; Sigma, St Louis, MO, USA) after validation by gas chromatography. All TAG measurements with the kit were corrected for free glycerol in plasma.

Continuous infusion experiments

The purpose of these experiments was twofold: 1) to measure baseline TAG kinetics using an alternative tracer method to bolus injection; and, 2) to quantify the effects of heparin and endurance swimming on TAG kinetics (bolus injection is not suited to follow changes in flux over time). Animals were implanted with two dorsal aorta catheters (Haman and Weber, 1996) and allowed to recover at rest for 24 h, either in opaque Plexiglas chambers (heparin group, body mass 374 ± 35 g, N=8), or in a swim tunnel (swimming group, body mass 435 ± 54 g, N=8) (masses were not different between heparin and swimming groups: t-test P=0.32). A priming dose of tri-[^{3}H]-oleate equivalent to 3 h of infusion was administered before starting infusions to ensure that isotopic steady state was reached in less than 2 h. For all blood samples collected

during continuous infusions, hematocrit did not decrease significantly throughout the experiments (25.4±2.0% before, and 21.6±1.9% at the end of the experiments, P=0.322).

For heparin experiments, the labeled emulsion was infused at 1 ml h^{-1} (206 dpm min^{-1} g^{-1}) in resting fish for 4 h, using a calibrated syringe pump (Harvard Apparatus, South Natick, MA, USA). A bolus of 600 U heparin kg^{-1} (Hepalean, Organon, Toronto, ON, Canada) was injected 2 h after starting infusions. Blood samples (0.3 ml each) were drawn every 30 min throughout the heparin experiments. For swimming experiments, the emulsion was infused at 1 ml h^{-1} for 10 h in animals placed in a modified Blazka-type swim tunnel: for 2 h at rest, 6 h swimming at 1.5 BLs^{-1} (~70 % U_{crit}, or moderate exercise intensity), and 2 h of recovery from exercise. Characteristics of the swim tunnel and environmental conditions during exercise were previously described in Chapter 3. Blood samples were collected in 1 mg ml^{-1} EDTA, centrifuged at 5,000 g for 10 min at 13°C, and separated plasma was stored at -80°C before analyses.

Intralipid emulsion and plasma lipoprotein analyses

Fatty acid composition was determined 1) for the different lipid classes of the Intralipid emulsion (TAG, NEFA and PL), and 2) for each lipid class of the different plasma lipoproteins (HDL, LDL, and VLDL). Due to blood volume restrictions, plasma fatty acids were analyzed in a separate group of fish (458 ± 25 g, N=12) than used for flux measurements by bolus injection and continuous infusion. The different plasma lipoprotein fractions were first separated by ultracentrifugation as described in Chapter 3. Lipids of all samples (emulsion and plasma lipoproteins) were extracted with 2:1 chloroform:methanol (v/v) (Folch *et al.*, 1957) and centrifuged (2,000 g for 10 min).

Pellets were discarded and supernatants filtered before adding 0.25% KCl. After shaking, the mixture was centrifuged (2,000 g for 10 min) before discarding the aqueous phase and drying the organic phase under N_2. Lipids extracted from the Intralipid emulsion and from the plasma lipoprotein fractions were loaded on solid-phase extraction columns (Supelclean, Sigma, St. Louis, Missouri, USA) to separate TAG, NEFA, and PL by sequential elution. Fatty acid composition was measured by gas chromatography using heptadecanoic acid (17:0) as an internal standard as detailed previously in Chapter 3. Individual fatty acids were identified by determining exact retention times with authentic standards (Sigma-Aldrich, St. Louis).

Calculations and statistical analyses

For *bolus injections*, the turnover rate of TAG was calculated as the dose injected divided by the surface area under the specific activity decay curve (see Fig. 4.1). To calculate this surface area, the decay curve was fitted with a multiexponential function using nonlinear regression (SigmaPlot 9.0). The fitted function was integrated between time 0 and the time when 5 or 10% of maximum activity was reached: a procedure commonly used because it corrects for label recycling (Weber *et al.*, 1986; Weber *et al.*, 1987). Maximum activity was calculated as the dose injected divided by the volume of the rapidly mixing pool estimated at 5% of total body volume. For *continuous infusions*, the turnover rate of TAG was calculated as TAG infusion rate divided by TAG specific activity (Steele, 1959).

Statistical differences were assessed using one-way analysis of variance (ANOVA) or one-way analyses of variance with repeated measures (RM ANOVA).

When significant changes were detected by ANOVA, the Holm-Sidak method was used to determine which means were different from baseline. Values given are means ± SE. A level of significance of $P<0.05$ was used in all tests.

Results

Fatty acid composition of Intralipid emulsion and plasma

Characteristics of the ^{3}H-labeled Intralipid emulsion used for measuring TAG kinetics by continuous infusion are presented in Table 4.2. It shows the fatty acid composition and relative distribution of total radioactivity in different lipid classes. The vast majority of fatty acids (95%) and radioactivity (90%) were found in TAG. Together, PL and NEFA only accounted for 5% of all the fatty acids and 10% of all the activity in the emulsion. The lipid composition of trout plasma is presented in Table 4.3. The great majority of fatty acids in rainbow trout plasma were found in PL and TAG (58% and 37%, respectively), particularly as PUFA (66% for PL and 49% for TAG). The fatty acid concentration for the three lipid classes and the ratio between phospholipids and TAG within each lipoprotein fraction are presented in Table 4.5. Results show that 50% of the fatty acids in trout VLDL are found in TAG, but that this percentage is smaller for other lipoproteins. The PL/ TAG ratio is inversely proportional to lipoprotein size, and, therefore, VLDL has the lowest ratio among all lipoprotein fractions (0.9).

TAG kinetics measured by bolus injection of tri-[^{3}H]-oleate VLDL

Intralipid tri-[^{3}H]-oleate emulsion was infused in donor fish to produce labeled lipoproteins for the measurement of TAG kinetics by bolus injection in other fish. Label incorporation in lipoproteins is presented in Table 4.4. Twelve hours after infusion, most of the tri-[^{3}H]-oleate activity was incorporated into VLDL (77%), while the remaining activity was equally shared between HDL and LDL. Moreover, 92% of the activity incorporated in VLDL was found in the TAG of this lipoprotein fraction, with minor amounts in PL and NEFA. Therefore, labeled VLDL from the donor fish were used for bolus injection experiments.

Figure 4.1 shows the decay curve for mean triacylglycerol specific activity (TAG SA) after a bolus injection of labeled VLDL. It was fitted with the sum of three exponential functions ($R^2 = 0.999$) to calculate the rate of appearance of TAG by integrating the surface area under the curve. Specific activity decreased sharply for 4 h after injection and then more gradually. Plasma TAG concentration remained stable throughout the experiments (see inset Fig. 4.1 where time had no effect, $P > 0.05$). The rate of appearance of TAG calculated from the decay curve was 24.0 ± 7.5 μmol kg^{-1} min^{-1} (when the curve was integrated until specific activity reached 10% of the maximum) and 49.1 ± 17.8 μmol kg^{-1} min^{-1} (when the curve was integrated until specific activity reached 5% of the maximum).

Effect of heparin on TAG turnover

Figure 4.2 shows the effect of heparin administration on TAG metabolism measured by continuous infusion of a tri-[^{3}H]-oleate emulsion in resting trout. Plasma

TAG concentration decreased from a baseline value of 1.3 ± 0.1 to 0.9 ± 0.1 μmol ml^{-1} ~1- 2 h after heparin injection ($P< 0.001$) (Fig. 4.2 A). Heparin caused a significant increase in TAG specific activity from 5,777 to 11,832 dpm μmol^{-1} after 2 h ($P< 0.001$) (Fig. 4.2 B). The higher variability in TAG specific activity compared to TAG concentration and flux reflects differences in body size because the same infusion rate (40,207 dpm min^{-1}) was used for all fish. The rate of appearance of TAG was significantly decreased from a baseline value of 24.4 ± 5.5 μmol kg^{-1} min^{-1} 1 hour after heparin administration ($P< 0.001$) (Fig. 4.2 C).

Effect of swimming on TAG turnover

Figure 4.3 shows the effect of swimming on TAG kinetics measured by continuous infusion of a tri-[^{3}H]-oleate emulsion in trout. Six hours of sustained swimming at 1.5 BLs^{-1} did not alter plasma TAG concentration from resting values (Fig. 4.2 A; $P>0.05$) or TAG specific activity (Fig. 4.2 B; $P> 0.05$). Therefore, the high rate of appearance of plasma TAG measured at rest was not significantly affected by prolonged exercise, even after 6 h of endurance swimming (Fig. 4.2 C; $P> 0.05$).

Bolus injection vs continuous infusion

The first TAG turnover rates reported for fish are summarized in Fig. 4.4 in the resting state. To increase confidence in the estimates of TAG kinetics, two separate tracer methods were used: bolus injection and continuous infusion. Both methods yielded similar values ranging from 24 to 49 μmol kg^{-1} min^{-1}. For the group of fish

measured by bolus injection of tri-[^{3}H]-oleate VLDL, values of 24.0 ± 7.5 and 49.1 ± 17.8 μmol kg^{-1} min^{-1} were obtained (depending on the recycling correction applied). Both estimates obtained by bolus injection fell within the range of values measured by continuous infusion. The two groups of resting animals measured by continuous infusion of tri-[^{3}H]-oleate emulsion (pre-heparin and pre-exercise) had TAG turnover rates of 25.0 ± 3.3 and 42.6 ± 2.6 μmol kg^{-1} min^{-1}, respectively.

TAG turnover rate over metabolic rate ratios in vertebrates

Figure 4.5 summarizes TAG turnover rates and the ratios between this parameter and oxygen consumption for vertebrates measured to date at rest or during exercise. The TAG turnover rates measured in trout are within the same range to values found for endotherm vertebrates (5-21μmol kg^{-1} min^{-1}). However, TAG turnover rates can be divided by the oxygen consumption of the animal, in order to allow correction for differences in metabolic rate between different vertebrates. Such calculated ratios show that all values are quite similar for vertebrates, ranging from 0.009 (exercising dog) to 0.089 (resting human), with the exception of fish, that show extreme ratios of 0.52 at rest, and 0.22 during sustained swimming.

Discussion

This study provides the first measurements of TAG turnover rate in fish and shows that baseline lipoprotein metabolism is particularly active in this group of vertebrates (Fig. 4.5). Both tracer methods reveal that rainbow trout support very high TAG

turnover rates, even at rest (Fig. 4.4). Such high baseline turnover rates can cover all the fuel requirements of locomotion (see calculations below) and they are not stimulated by endurance swimming (Fig. 4.3). Results also show that heparin-induced release of LPL into the circulation causes a 50% inhibition of TAG turnover rate (Fig. 4.2). The *continuous infusion* method (using an easily prepared, artificial substrate) and *bolus injection* method (using a difficult to produce, natural substrate) provide similar estimates of flux (Fig. 4.4). The continuous Intralipid infusion method implemented here is a new tool for *in vivo* studies of fish lipoproteins that allows flux measurements under non-steady state conditions.

Endurance swimming does not stimulate high resting TAG turnover rate

The baseline TAG turnover rates of rainbow trout range from 25 to 42 μmol TAG kg^{-1} min^{-1} (Fig. 4.4). This study characterizes the response of female trout, but the changes in lipid metabolism reported here probably also reflect those of males because sexually-immature animals were measured. However, further studies will be needed to confirm this. Unexpectedly, the resting values of this ectothermic animal (after 72 h of fasting) exceed all fluxes measured to date in endotherms (fed, fasting or exercising). Previous studies report post-prandial TAG turnover rates of 18 μmol kg^{-1} min^{-1} in resting or exercising dogs (Terjung *et al.*, 1982), 13 μmol kg^{-1} min^{-1} in resting humans (Terjung *et al.*, 1983), and lower fluxes of 5 and 1.5 μmol TAG kg^{-1} min^{-1} in rats fasted for 12 or 42 h, respectively (Bagby *et al.*, 1987; Teusink *et al.*, 2003) (Fig. 4.5A). Taken together, these mammalian results suggest that the effects of mass-specific metabolic rate on TAG

turnover rate are dwarfed by those of fasting (body mass ratio of dog/rat=60, human/rat=220).

Label recycling is difficult to estimate experimentally and I did not attempt to quantify it in this study. However, it is important to note that any recycling would lead to underestimating true TAG turnover rate (because it would erroneously increase surface area under the specific activity decay curve for bolus injection experiments and increase plateau specific activity in continuous infusion experiments). For bolus injection, I have applied a commonly used recycling correction by interrupting curve integration when specific activity reaches 5% of its maximal value (Weber *et al.*, 1986; 1987). I have repeated calculations using a value of 10% that would assume unusually high recycling rates.

To determine whether circulating lipoproteins could fuel the locomotory muscles of swimming trout, I have calculated the theoretical metabolic rate needed to oxidize all fatty acids supplied by the TAG turnover rate measured in this study (24 μmol TAG kg^{-1} min^{-1} or 72 μmol FA kg^{-1} min^{-1} = lowest value from Fig. 4.4). Assuming that energy metabolism is only supported by lipid oxidation and that TAG is entirely made of trioleate, this theoretical metabolic rate would be 1,872 μmol O_2 kg^{-1} min^{-1} (if each oleate requires 26 O_2 for oxidation and if the contribution of glycerol is ignored). Because the real metabolic rate of a swimming trout is ~109 μmol O_2 kg^{-1} min^{-1} (Burgetz *et al.*, 1998), I can determine that only 6% of TAG turnover rate is necessary to support exercise. Therefore, it is clear that resting TAG turnover rate is high enough to provide several times the energy needed by working muscles, and this explains why TAG

turnover is not stimulated during swimming (Fig. 4.3). Why then does lipoprotein metabolism remain so active in a resting animal? I propose that high TAG turnover rates (this Chapter) and high lipolytic rates (Bernard *et al.*, 1999) (Chapter 5) are fundamental features of ectotherm metabolism that allow the restructuring of membrane phospholipids to be synchronized with frequent changes in body temperature. To preserve normal membrane fluidity (Hazel, 1984), adequate homeoviscous adaptation may depend on the rapid supply of lipoprotein-bound fatty acids with different chain lengths and degrees of saturation.

TAG turnover rate is reduced by heparin

What are the mechanisms responsible for the decrease in TAG turnover rate observed after heparin administration? Heparin is known to release lipoprotein lipase (LPL) into plasma from its natural location, bound to endothelial proteoglycans, because of heparin's very high affinity for this enzyme (Olivecrona and Bengston-Olivecrona, 1999). This effect of heparin could therefore explain the decrease in TAG turnover rate (Fig. 4.2), because tissue uptake of fatty acids from lipoproteins mainly relies on bound LPL (Goldberg *et al.*, 1991). In mammals, this effect of heparin on LPL has been previously invoked to explain declines in fatty acid supply from lipoproteins (Chevreuil *et al.*, 1993), and in the uptake of a TAG-rich emulsion by the heart (Augustus *et al.*, 2003). Therefore, both fish and mammals seem to depend on the natural presence of LPL bound to the vascular endothelium for proper delivery of lipoprotein fatty acids to tissues. Even in mammals, the exact mechanism of action for LPL is still controversial

and several models have been proposed (Merkel *et al.*, 2002). In addition, LPL-independent mechanisms of TAG uptake by tissues cannot be excluded.

Measuring TAG turnover rate in fish: bolus injection vs continuous infusion

Investigating the metabolism of circulating lipids has been hindered by the lack of suitable methods to measure TAG turnover rate. The limited information presently available has only been obtained for mammals, and almost exclusively by *bolus injection*, a method requiring a single catheter and small amounts of labeled substrate (Mackie *et al.*, 1980; Wallinder *et al.*, 1984; Peterson *et al.*, 1990; Magkos and Sidossis, 2004). Unfortunately, these advantages come with significant limitations. Bolus injection *must* be used under steady state conditions and only provides a single value of flux per experiment. In addition, flux calculations from bolus injection experiments depend on the surface area under the specific activity decay curve, a value difficult to estimate accurately because of label recycling. The more versatile method of *continuous infusion* can provide multiple flux measurements under non-steady state conditions, but requires the surgical placement of two catheters and larger amounts of labeled substrate. Such technical difficulties explain why I was only able to find two studies of lipoprotein kinetics that rely on continuous infusion in mammals (Bagby *et al.*, 1987; Teusink *et al.*, 2003) and none in fish. For this first investigation of TAG turnover rate in fish, I used both methods in an attempt to exploit their respective advantages. Developing a new continuous infusion technique for fish lipoproteins was greatly simplified by adapting a known double cannulation procedure specifically designed for trout (Haman and Weber, 1996).

Results show that *bolus injection* of endogenously-labeled trout VLDL (Table 4.4) and *continuous infusion* of a labeled Intralipid emulsion provide similar high estimates of TAG turnover rates in trout, using two different substrates (Fig. 4.4). With a 400 g fish having a blood volume of 20 ml and a hematocrit of 25%, I can calculate that the TAG pool in VLDL is 10 μmol (for a VLDL-TAG concentration of 2 μmol ml plasma^{-1}). With an average TAG turnover rate of 36 μmol kg^{-1} min^{-1} (Fig. 4), this trout has a clearance rate of 18 ml plasma kg^{-1} min^{-1}.

A comparison of Tables 4.2 and 4.3 reveals similarities and differences in the fatty acid composition of TAG between the Intralipid emulsion and trout plasma. Both had the same percentage of saturated fatty acids (15%), but their largest difference was probably the presence of more linoleic acid in the emulsion (55%) than in plasma (7%). Another important similarity relevant to my experiment was that emulsion and plasma showed no significant difference in TAG concentration (9 vs 8 μmol FA ml^{-1}). However, the emulsion had a much higher % total FA in TAG than trout plasma (95% vs 37%). Nevertheless, Intralipid particles appear to mimic the metabolic behaviour of trout VLDL (Table 4.5), even though the PL/TAG ratios of these two substrates are different. This may be possible because artificial Intralipid emulsions are known to acquire natural apolipoproteins from circulating HDL and VLDL, thereby becoming suitable substrates for LPL *in vivo* (Robinson and Quarfordt, 1979; Hultin *et al.*, 1995; Callow *et al.*, 1998; Ferezou and Bach, 1999; Augustus *et al.*, 2003). Finally, the significant decrease in TAG turnover rate induced by heparin (Fig. 4.2) shows that the

continuous infusion method proposed here is sensitive enough to monitor biologically relevant changes in the TAG turnover rate of fish.

Perspectives and significance

This first study of lipoprotein kinetics in an ectotherm reveals that baseline TAG turnover rate is higher in rainbow trout than in any endotherm measured to date (Fig. 4.5). It shows that resting TAG turnover rate is not stimulated by endurance swimming because it is already high enough to cover all the fuel requirements of exercise. Results suggest that rainbow trout need to maintain a high TAG turnover rate at all times to cope with fluctuations in environmental temperature by rapid restructuring of membrane phospholipids. In fish, the inhibition of TAG turnover by heparin suggests that LPL must be bound to the endothelium for normal tissue uptake of fatty acids from lipoproteins. The continuous infusion method implemented here is a new versatile tool to investigate the potential role of lipoproteins in homeoviscous adaptation.

Table 4.1. Fatty acid composition of the food expressed as a percentage of total fatty acids.

Fatty acids	%
14:0	5.0 ± 0.2
16:0	12.9 ± 0.8
16:1 (n-7)	5.9 ± 0.4
18:0	1.8 ± 0.2
18:1 (n-7 + n-9)	13.7 ± 0.9
18:2 (n-6)	8.9 ± 0.8
18:3 (n-3 + n-6)	1.1 ± 0.1
18:4 (n-3)	1.3 ± 0.0
20:1 (n-11)	15.0 ± 1.1
20:5 (n-3)	4.4 ± 0.6
22:1 (n-11)	25.9 ± 2.4
22:6 (n-3)	4.1 ± 0.9
SFA	19.7 ± 0.5
MUFA	60.6 ± 1.2
PUFA	19.7 ± 0.6

Values are means ± SE (N=3). Saturated fatty acids: SFA; monounsaturated fatty acids: MUFA; polyunsaturated fatty acids: PUFA.

Table 4.2. Fatty acid composition of the three lipid classes (NEFA, PL and TAG) from the Intralipid emulsion used to measure TAG kinetics in rainbow trout. Composition is expressed as a percentage of total fatty acids within each class. Fatty acid concentration and ^{3}H content of the three classes are also presented. ^{3}H content is expressed as % total activity in the emulsion lipids.

Fatty acids	**NEFA**	**PL**	**TAG**
16:0	33.0 ± 2.3	25.7 ± 1.6	10.4 ± 1.5
18:0	32.6 ± 2.4	19.3 ± 1.3	4.5 ± 0.1
18:1 (n-7 + n-9)	13.7 ± 0.8	24.5 ± 1.5	21.6 ± 2.2
18:2 (n-6)	17.0 ± 0.7	14.8 ± 1.0	54.8 ± 2.7
18:3 (n-3 + n-6)	1.8 ± 0.1	-	7.3 ± 0.4
20:4 (n-6)	1.9 ± 0.2	4.8 ± 0.5	-
22:5 (n-3)	-	4.6 ± 0.4	-
22:6 (n-3)	-	6.3 ± 0.8	-
Others	-	-	1.4 ± 0.1
SFA	65.7 ± 2.2	45.0 ± 1.5	15.3 ± 1.1
MUFA	13.7 ± 0.7	24.5 ± 1.1	22.0 ± 2.0
PUFA	20.6 ± 0.2	30.5 ± 0.7	62.7 ± 2.2
Total [FA] (μmol ml^{-1})	0.1 ± 0.0	0.4 ± 0.0	9.1 ± 0.5
^{3}H content (%)	7.8 ± 1.5	2.4 ± 0.4	89.8 ± 1.5

Values are means ± SE (N=3). Non-esterified fatty acids: NEFA; phospholipids: PL; Triacylglycerol: TAG; Saturated fatty acids: SFA; monounsaturated fatty acids: MUFA; polyunsaturated fatty acids: PUFA. Others: sum of minor fatty acids individually accounting for less than 1% (20:0, 20:1 n-9, 20:2 n-6, and 20:5 n-3).

Table 4.3. Fatty acid composition of the three lipid classes (NEFA, PL and TAG) from trout plasma expressed as a percentage of total fatty acids within each class. Fatty acid concentration of each class is also presented.

Fatty acids	NEFA	PL	TAG
14:0	1.2 ± 0.2	0.2 ± 0.1	-
16:0	28.3 ± 1.2	14.2 ± 1.2	9.2 ± 1.4
16:1 (n-7 + n-9)	3.7 ± 2.5	0.5 ± 0.1	1.6 ± 0.3
18:0	8.8 ± 1.6	3.6 ± 0.6	3.4 ± 0.9
18:1 (n-7 + n-9)	42.0 ± 3.8	10.2 ± 0.7	18.1 ± 1.6
18:2 (n-6)	2.0 ± 0.3	2.3 ± 0.2	6.9 ± 1.9
18:4 (n-3)	2.3 ± 0.8	-	-
20:1 (n-11)	2.8 ± 0.8	3.7 ± 0.3	7.9 ± 0.6
20:3 (n-3 + n-6)	-	0.5 ± 0.1	-
20:4 (n-3 + n-6)	1.0 ± 0.7	1.5 ± 0.4	1.3 ± 0.4
22:0	-	-	1.9 ± 0.6
20:5 (n-3)	1.6 ± 0.5	7.3 ± 0.5	9.5 ± 0.7
22:1 (n-11)	3.2 ± 0.9	1.5 ± 0.4	8.9 ± 1.2
22:3 (n-3)	0.6 ± 0.4	-	2.5 ± 0.6
22:5 (n-3)	-	2.1 ± 0.2	1.4 ± 0.3
22:6 (n-3)	2.5 ± 0.7	52.4 ± 2.3	27.4 ± 2.2
SFA	38.3 ± 2.1	18.1 ± 1.6	14.5 ± 2.2
MUFA	51.6 ± 3.0	16.0 ± 0.8	36.5 ± 1.8
PUFA	10.1 ± 1.9	65.9 ± 2.2	49.0 ± 2.6
Total [FA] (μmol ml^{-1} plasma)	1.2 ± 0.2	12.5 ± 2.3	7.9 ± 1.3

Values are means ± SE (N=12). Non-esterified fatty acids: NEFA; phospholipids: PL; Triacylglycerol: TAG; Saturated fatty acids: SFA; monounsaturated fatty acids: MUFA; polyunsaturated fatty acids: PUFA.

Table 4.4. Total absolute ^{3}H activity within each lipoprotein fraction (HDL, LDL and VLDL) for three lipid classes (NEFA, PL and TAG) from trout plasma, 12 h after administration of labeled Intralipid emulsion. Percent total ^{3}H activity in the different lipoprotein fractions is also presented. VLDL of these donor animals were separated from other lipoproteins and used for measuring TAG kinetics by bolus injection in other fish.

	HDL	**LDL**	**VLDL**
Total activity (dpm ml^{-1})	18,477 ± 11,571	19,111 ± 11,564	124,042 ± 54,953
NEFA	28.6 ± 3.9	19.4 ± 1.0	3.1 ± 0.9
PL	22.8 ± 7.8	14.7 ± 0.6	5.1 ± 0.1
TAG	48.6 ± 11.6	65.9 ± 0.8	91.8 ± 0.8

Values are means ± SE (N=7 for total activity and 3 for percentages). High density: HDL; low density: LDL; and very low density: VLDL; Non-esterified fatty acids: NEFA; phospholipids: PL; Triacylglycerol: TAG.

Table 4.5. Fatty acid concentration (μmol FA ml^{-1} plasma) of trout plasma for three lipid classes (PL, TAG and NEFA), and PL to TAG ratio within each lipoprotein fraction (HDL, LDL and VLDL).

	PL	TAG	NEFA	Ratio PL/TAG
HDL	5.9 ± 0.5	3.0 ± 0.4	0.5 ± 0.1	1.9
LDL	4.7 ± 0.8	2.9 ± 0.7	0.4 ± 0.1	1.6
VLDL	1.9 ± 0.7	2.0 ± 0.8	0.3 ± 0.1	0.9

Values are means ± SE (N=12). High density lipoproteins: HDL; low density lipoproteins: LDL; and very low density lipoproteins: VLDL; Non-esterified fatty acids: NEFA; phospholipids: PL; Triacylglycerol: TAG. Values for PL and TAG concentrations are expressed in μmol fatty acids ml^{-1} plasma. Absolute PL and TAG concentrations can be obtained by dividing table values by 2 (PL) and 3 (TAG).

Fig. 4.1. Specific activity decay curve for plasma triacylglycerol (TAG) in resting trout after bolus injection of VLDL pre-labeled with tri-[^{3}H]-oleate in donor fish. Each bolus was injected at time 0 (shaded vertical line) and the curve was fitted by regression with 3 exponentials (dotted line). Inset shows plasma TAG concentration during the sampling period. Values are means ± SE (N=4).

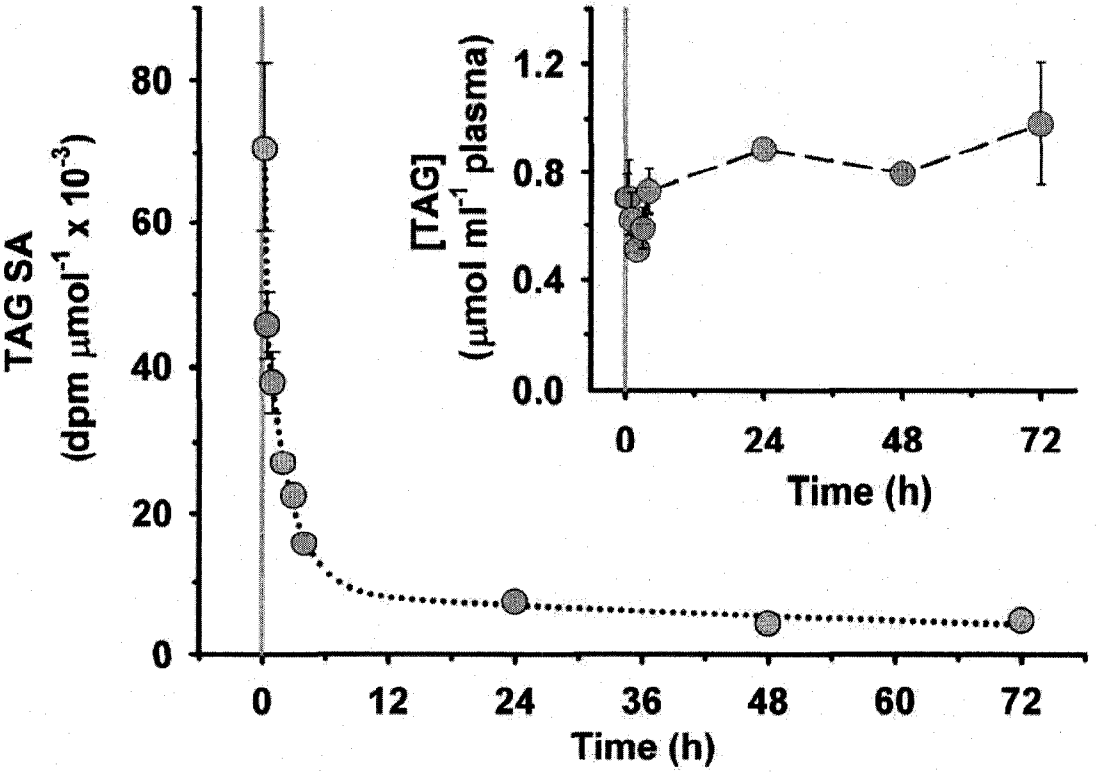

TAG SA
(dpm µmol^{-1} x 10^{-3})
80
60
40
20
0
0
12
24
36
48
60
72
Time (h)
[TAG]
(µmol ml^{-1} plasma)
1.2
0.8
0.4
0.0
0
24
48
72
Time (h)

Fig. 4.2. Effects of heparin on: (A) plasma triacylglycerol (TAG) concentration, (B) TAG specific activity (SA), and (C) TAG rate of appearance (R_a) in resting trout. Measurements were made by continuous infusion of an Intralipid emulsion labeled with tri-[^{3}H]-oleate. Arrow and vertical line indicate the injection of 600 U heparin kg^{-1} body mass in the circulation. Asterisks (*) show significant differences from control at time 0. Values are means ± SE (N=8).

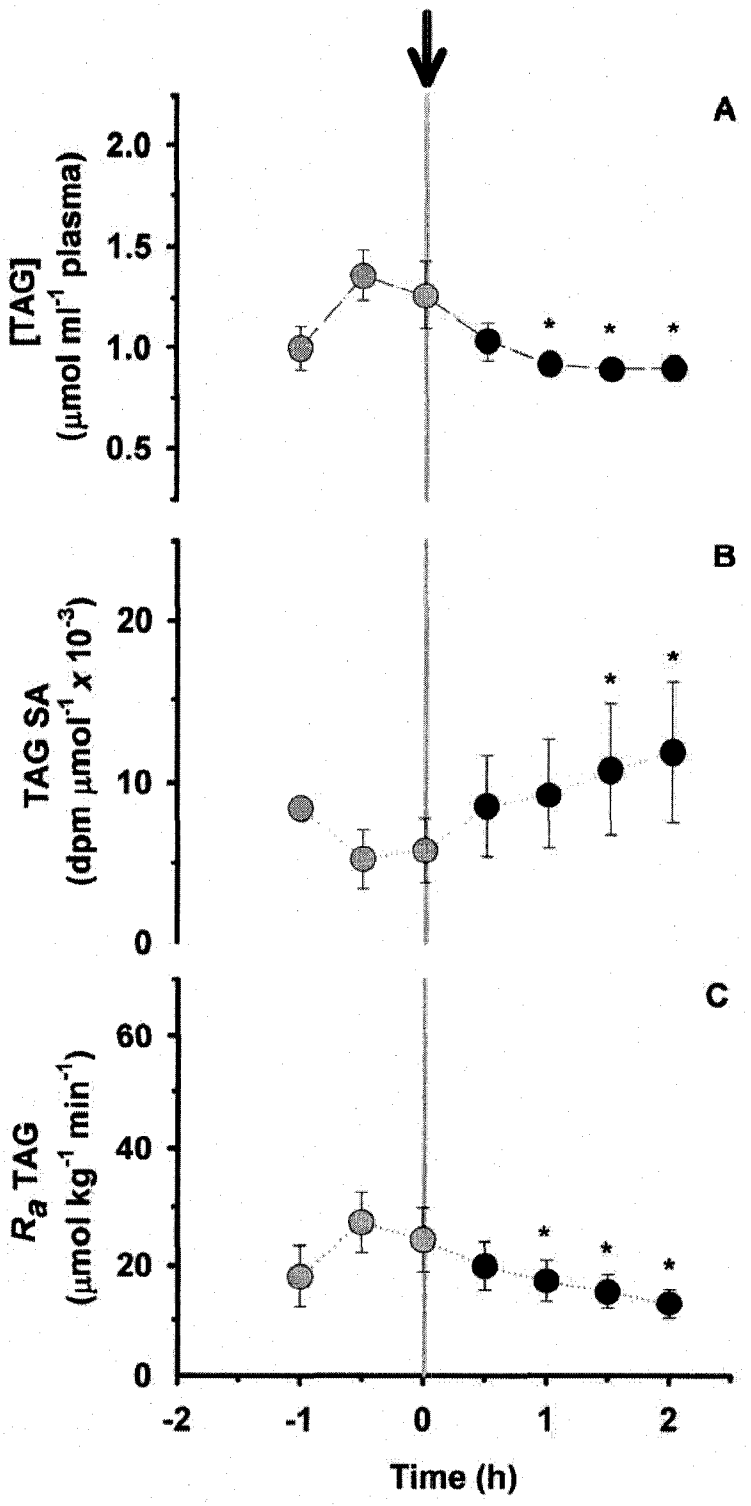

A
[TAG]
(μmol ml-1 plasma)
2.0
1.5
1.0
0.5
B
TAG SA
(dpm μmol-1 x 10-3)
20
10
0
C
Ra TAG
(μmol kg-1 min-1)
60
40
20
0
-2
-1
0
1
2
Time (h)

Fig. 4.3. Effects of endurance swimming (6 h at 1.5 BLs^{-1}) on: (A) plasma triacylglycerol (TAG) concentration, (B) TAG specific activity (SA), and (C) TAG rate of appearance (R_a) in rainbow trout. Measurements were made by continuous infusion of an Intralipid emulsion labeled with tri-[^{3}H]-oleate, before, during and for 2 h after exercise (recovery). Values are means ± SE (N=8).

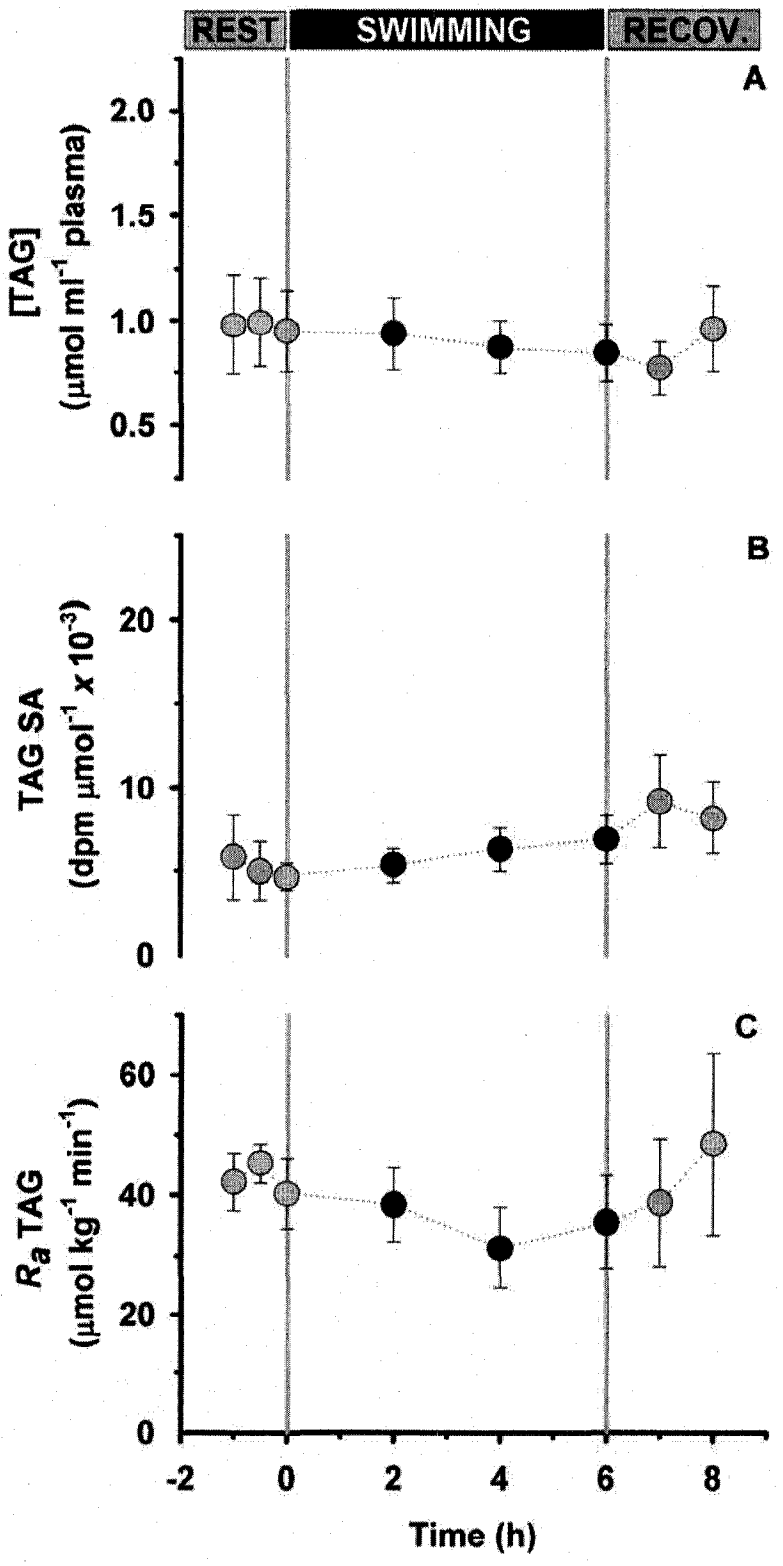

REST
SWIMMING
RECOV.
A
[TAG]
(μmol ml-1 plasma)
2.0
1.5
1.0
0.5
B
TAG SA
(dpm μmol-1 x 10-3)
20
10
0
C
Ra TAG
(μmol kg-1 min-1)
60
40
20
0
-2
0
2
4
6
8
Time (h)

Fig. 4.4. Mean rates of appearance of triacylglycerol (R_a TAG) in resting rainbow trout measured by bolus injection of ^{3}H-labeled trout VLDL (left panel), or continuous infusion of an Intralipid emulsion labeled with tri-[^{3}H]-oleate (right panel). For bolus injection, R_a TAG was calculated by dividing the dose injected by the surface area under the specific activity decay curve fitted on mean values from 4 experiments. Surface area was calculated by integrating the decay curve from time 0 to the time when 10 or 5% of maximal specific activity (SA_{max}) was reached (see Methods for details). For continuous infusions, mean baseline values (± SE) measured before heparin injection (Group A; N=8) and before swimming (Group B; N=8) are presented.

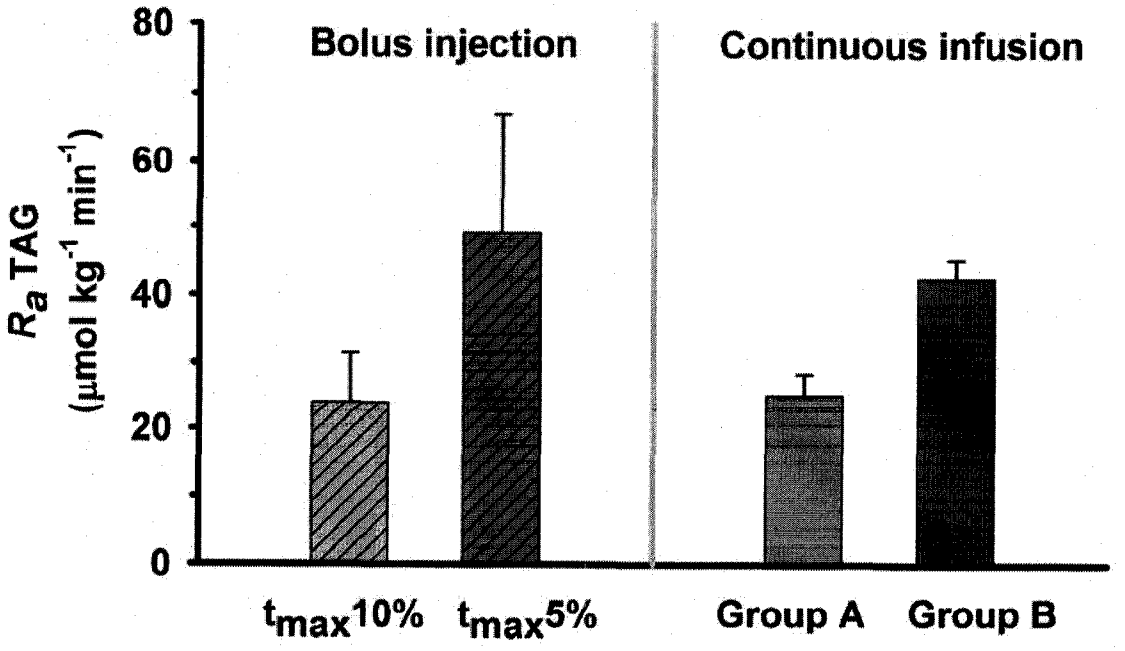
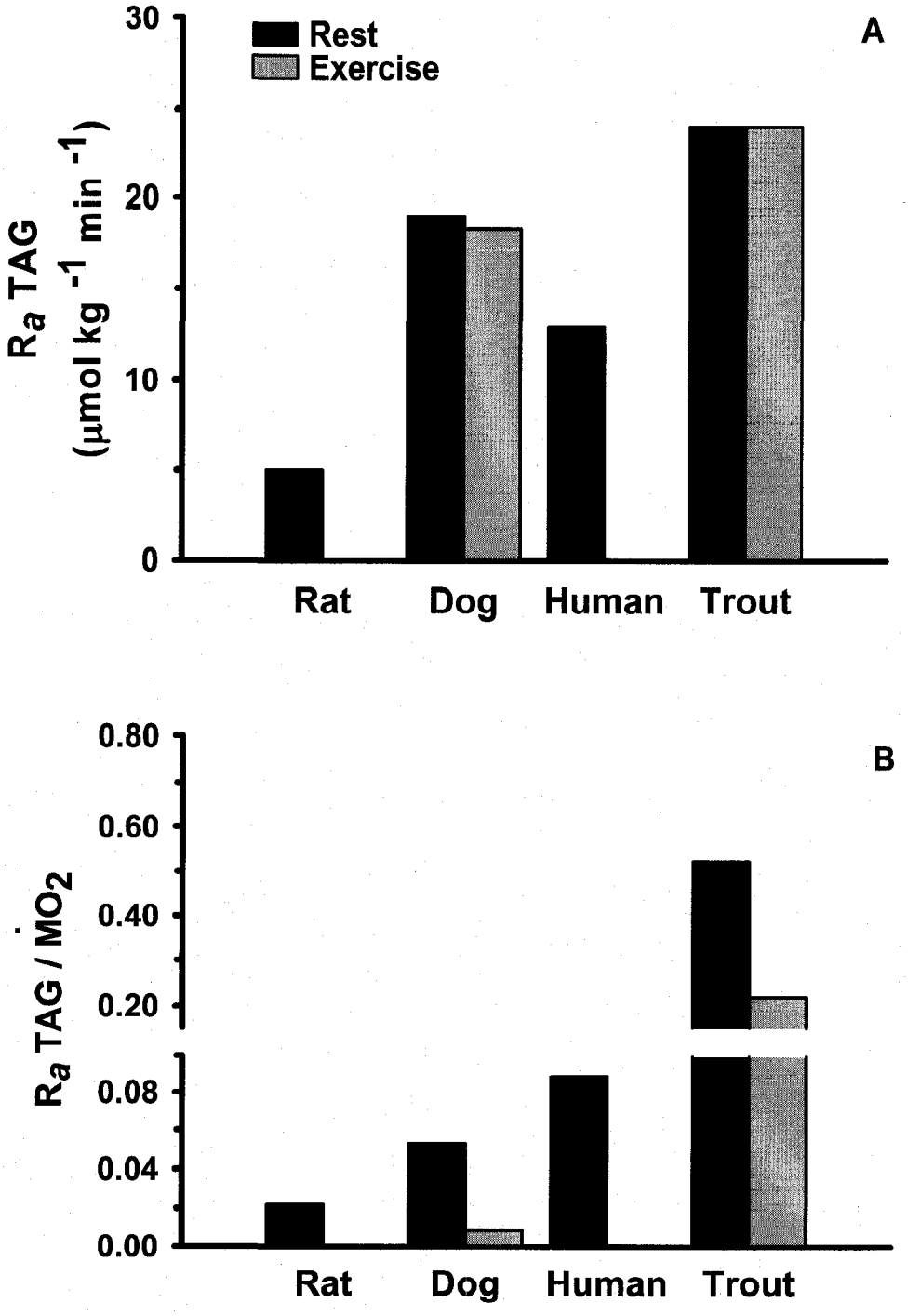

80
60
40
20
0
R_a TAG
(μmol kg^{-1} min^{-1})
Bolus injection
Continuous infusion
t_{max}10%
t_{max}5%
Group A
Group B

Fig. 4.5 TAG turnover rate (R_a TAG) (A), and the ratio obtained when divided by the metabolic rate (MO_2) (B) for different vertebrates measured to date. Values correspond to vertebrates at rest and during exercise at equivalent intensities (40-70% $MO_{2\ max}$). Ratios were calculated from the following studies: rat (McClelland *et al.*, 2001), dogs (Issekutz *et al.*, 1967; Issekutz *et al.*, 1975), human (Wolfe *et al.*, 1990), and trout (this study using MO_2 values from Burgetz *et al.*, 1998).


A
Rest
Exercise
R_a TAG
(μmol kg $^{-1}$ min $^{-1}$)
30
20
10
0
Rat
Dog
Human
Trout
B
R_a TAG / $\dot{M}O_2$
0.80
0.60
0.40
0.20
0.08
0.04
0.00
Rat
Dog
Human
Trout

CHAPTER 5.

IN VIVO REGULATION OF RAINBOW TROUT LIPOLYSIS BY CATECHOLAMINES

Based on

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Introduction

Fatty acids play key physiological roles as membrane components, oxidative fuels and metabolic signals. Therefore, the regulation of fatty acid supply, or lipolysis, affects many fundamental life processes (Clarke *et al.*, 1997; Raclot and Oudart, 1999; Hulbert *et al.*, 2007). In the intact organism, lipolysis is measured as the rate of appearance of glycerol (R_a glycerol) (Wolfe, 1992) and it has been thoroughly investigated in mammals, mainly humans (Klein *et al.*, 1989; Wolfe *et al.*, 1990; Klein *et al.*, 1995; Quisth *et al.*, 2005). Unfortunately, the information available for other vertebrates is restricted to two *in vivo* studies on birds (Bernard *et al.*, 2002; Vaillancourt and Weber, 2007) and one on fish (Bernard *et al.*, 1999). The lack of data for ectotherms is particularly unfortunate because adequate supply of fatty acids is crucial to restructure membrane phospholipids during homeoviscous adaptation (Hazel and Williams, 1990) and to fuel endurance swimming in fish (Moyes and West, 1995).

Lipolytic rate is modulated by several hormones including catecholamines, insulin and glucagon (Coppack *et al.*, 1994; Londos *et al.*, 1999). In mammals, lipolysis is stimulated by norepinephrine (NE) as well as epinephrine (Epi) (Fain and Garcia-Sainz, 1983; Nonogaki, 2000), but these hormones have controversial effects in fish (Table 5.1). Various species show different responses *in vitro,* and the effects of catecholamines on fish lipolysis have not been measured *in vivo* using tracer methods. Overall, however, NE appears to inhibit lipolysis in fish adipose tissue (Farkas, 1967a, 1967b; Vianen *et al.*, 2002), although not in all species (Farkas, 1967b). NE-induced inhibition of fish lipolysis also appears to be supported by *in vivo* observations that the hormone decreases plasma non-esterified fatty acids (NEFA) in many species (Farkas,

1967b; Minick and Chavin, 1973; Ince and Thorpe, 1975; Van Raaij *et al.*, 1995), with a few exceptions (Leisbon *et al.*, 1968; Plisetskaya, 1980). However, assuming that plasma NEFA concentration reflects changes in lipolytic rate may lead to erroneous conclusions (Haman *et al.*, 1997). Even for Epi, the response may be very different between fish and mammals. Some studies report *in vivo* inhibition of lipolysis by Epi for some fish species (Minick and Chavin, 1973; Ince and Thorpe, 1975), or no local effect on adipose tissue (Migliorini *et al.*, 1992). The only information available for rainbow trout shows activation of lipolysis by both catecholamines in isolated hepatocytes (Van Heeswijk *et al.*, 2006), but no effect of Epi on red muscle or on plasma NEFA concentration *in vivo* (Bilinski and Lau, 1969; Perrier *et al.*, 1972).

The goal of this study was to investigate the effects of catecholamines on R_a glycerol in intact fish to obtain an integrated hormonal response rather than tissue- or cell-specific contribution to total fatty acid supply. My aim was to start quantifying the effects of supranormal concentrations of NE (~170 nM) and Epi (~500 nM) on the lipolytic rate of rainbow trout using *in vivo* tracer kinetics. Based on the balance of evidence presently available, it was hypothesized that trout lipolysis would be inhibited by NE and stimulated by Epi. I also examined the relationship between R_a glycerol and glycerol concentration to determine whether glycerol concentration could be used as a reliable index of fish lipolysis.

Methods

Animals

Adult rainbow trout, *Oncorhynchus mykiss*, were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). They were kept in a 1,300 L flow-through holding tanks in dechloraminated, well-oxygenated water at 13 °C under a 12 h: 12 h L: D photoperiod. Animals were acclimated to these conditions for at least one month before experiments. The same water quality and photoperiod were used during the measurements. Trout were fed floating fish pellets (Martins Mills, Elmira, ON, Canada) until satiation five times a week and were randomly assigned to three groups: saline control (body mass 460 ± 52 g; N=4), epinephrine (485 ± 35 g; N=8), and norepinephrine (514 ± 40 g; N= 8).

Surgery

Fish were fasted for 48 h before surgery and did not feed during recovery or measurements. Double cannulation was performed as previously reported (Haman and Weber, 1996). The animals were anaesthetized in a solution of 0.1 g L^{-1} ethyl-N-aminobenzoate sulphonic acid (MS-222) buffered with 0.2 g L^{-1} sodium carbonate, and two PE-50 catheters (Intramedic, Clay-Adams) were implanted in the dorsal aorta. Both catheters were filled with Cortland saline and sutured to the roof of the buccal cavity. After surgery, the animals were placed in opaque Plexiglas™ chambers (60 x 16 x 18 cm) supplied with the same quality water as the acclimation tank at a rate of 5-6 L min^{-1}. This low rate was selected to maintain normoxic conditions without causing swimming. During recovery from surgery, the catheters were regularly checked for patency and

flushed with Cortland saline containing sodium citrate as anticoagulant (13 μmol ml^{-1}). Only animals with hematocrits >20% after recovery from surgery were used in experiments. Glycerol kinetics were then measured in the same chambers 24 h after surgery.

Continuous tracer infusions

The rate of appearance of glycerol (R_a glycerol), also commonly called lipolytic rate (Wolfe, 1992), was measured by continuous infusion of 2-[^{3}H] glycerol (37 GBq $mmol^{-1}$; Amersham, Buckinghamshire, UK) as previously reported (Bernard *et al.*, 1999). The infusate was prepared daily by drying 25 μCi of the radioisotope under N_2 and resuspending in Cortland saline. This solution was infused for 3 h at 1 ml h^{-1} through one of the catheters using a calibrated syringe pump (Harvard Apparatus, South Natick, MA, USA). Tracer infusion rates ranged between 375 x 10^3 and 1480 x 10^3 dpm kg^{-1} min^{-1}. Because the infusate had a high specific activity, the total amount of glycerol administered (labeled and unlabeled) was < 0.5 nmol kg^{-1} min^{-1}. Isotopic steady state in the blood was reached in < 1 h. Baseline glycerol kinetics was quantified between 60 and 90 min of infusion before investigating the effects of catecholamines.

Catecholamine administration

A second calibrated syringe pump was activated after 90 min of tracer infusion to administer saline (control), Epi or NE for 30 min at 1 ml h^{-1}. To avoid light-induced breakdown of catecholamines, hormone solutions were prepared under red, low-intensity

light, and similar light conditions were used during administration. Rates of catecholamine administration were 0.45 nmol kg^{-1} min^{-1} for NE and 1.34 nmol kg^{-1} min^{-1} for Epi. This lower rate of administration used for norepinephrine was chosen to preserve the proportions between Epi and NE observed after intense stress in trout (Ristori and Laurent, 1985; Tang and Boutilier, 1988; Fievet *et al.*, 19990; Wood *et al.*, 1990). A previous study shows that the rate of Epi administration selected leads to plasma levels of ~500 nM in trout (Weber and Shanghavi, 2000). Recovery was monitored by prolonging tracer infusions for 60 min after saline, Epi, or NE administration.

Blood sampling and analysis

Eight 0.3 ml-blood samples were drawn in each experiment. Plasma was immediately separated by centrifugation and stored at -80°C until analyses. Each plasma sample was divided in two aliquots to measure glycerol concentration by spectrophotometry (Weber *et al.*, 1993) and glycerol activity by scintillation counting (Beckman Coulter CS6500, Palo Alto, CA, USA). Infusion of 2-[^{3}H] glycerol only leads to significant radioactivity in plasma H_2O, glucose and glycerol. Glycerol specific activity was determined after drying plasma to eliminate labeled water and after correction for glucose activity, using thin layer chromatography as previously described (Bernard *et al.*, 1999).

Calculations and statistical analyses

The rate of appearance of glycerol or lipolytic rate was calculated as glycerol infusion rate divided by glycerol specific activity (Bernard *et al.*, 1999). The effects of hormone administration over time were assessed by one-way analyses of variance with repeated measures (RM ANOVA). When significant changes were detected, the Holm-Sidak method was used to determine which means were different from baseline (Figs. 5.1-3). Differences in mean glycerol concentrations and R_a glycerol between control and catecholamine-treated animals were evaluated with unpaired t-tests (Table 5.2). Values presented are means ± SE. A level of significance of $P<0.05$ was used in all tests.

Results

Mean hematocrit was not affected by saline, epinephrine (Epi) or norepinephrine (NE) administration and averaged 24 ± 2% (saline), 25 ± 4% (Epi), and 24 ± 3% (NE). Figure 5.1 show that saline administration has no effect on plasma glycerol concentration, specific activity, or R_a glycerol ($P > 0.05$). These control experiments demonstrate that isotopic and concentration steady-state of glycerol can be maintained for 3 h.

Effects of norepinephrine

Figure 5.2 shows the effects of norepinephrine administration on plasma glycerol concentration, specific activity, and R_a glycerol. Baseline values for glycerol concentration and R_a glycerol averaged 0.20 ± 0.05 μmol ml^{-1} and 4.4 ± 0.6 μmol kg^{-1}

min^{-1}. Glycerol concentration and R_a glycerol were both decreased by norepinephrine ($P<0.05$). Concentration reached a minimal value of 0.10 ± 0.03 μmol ml^{-1} and R_a glycerol a minimal value of 2.3 ± 0.7 μmol kg^{-1} min^{-1} after 30 min of NE administration and both returned to baseline during recovery ($P>0.05$).

Effects of epinephrine

Figure 5.3 shows the effects of epinephrine administration on plasma glycerol concentration, specific activity, and R_a glycerol. Baseline values for glycerol concentration and R_a glycerol averaged 0.22 ± 0.05 μmol ml^{-1} and 4.4 ± 0.5 μmol kg^{-1} min^{-1}. Glycerol concentration and R_a glycerol were both stimulated by epinephrine ($P< 0.001$). Concentration reached a maximal value of 0.55 ± 0.07 μmol ml^{-1} and R_a glycerol a maximal value of 8.2 ± 0.8 μmol kg^{-1} min^{-1} after 20 min of Epi administration. Concentration and R_a glycerol stayed elevated until the end of Epi administration ($P< 0.001$), but returned to baseline during recovery ($P>0.05$). Table 5.2 summarizes average values for plasma glycerol concentration and R_a glycerol during the last 10 min of saline, Epi and NE administration. Both parameters were decreased by NE ($P<0.05$) and increased by Epi ($P<0.001$).

Relationship between glycerol concentration and rate of appearance

Figure 5.4 plots plasma glycerol concentration vs R_a glycerol (or lipolytic rate) during the last 10 min of saline (stars), Epi (closed circles), and NE administration (open circles). Separate linear regressions were fitted for the three groups. For epinephrine

the relationship had a positive slope different from 0 (R=0.48, P<0.05). Concentration and rate of appearance were not significantly related for saline (R=0.03, slope not different from 0, P=0.905) or for NE (R =0.05, slope not different from 0, P=0.830).

Lipolytic rate over metabolic rate ratios in vertebrates

Figure 5.5 summarizes R_a glycerol and the ratios between this parameter and oxygen consumption for vertebrates measured to date at rest or during exercise. Although there exists high variation in the lipolytic rates, trout lipolytic rate (~5 μmol kg^{-1} min^{-1}) is within the same range of the values reported for endotherms (4-56 μmol kg^{-1} min^{-1}). However, lipolytic rate can be divided by the oxygen consumption of the animal, to allow correcting for differences in metabolic rate between different vertebrates. Such calculated ratios show that all values are very similar for vertebrates, ranging from 0.009- 0.031, with the exception of resting fish that show an extreme ratio of 0.115.

Discussion

This study investigates glycerol kinetics in rainbow trout and provides the first *in vivo* measurements of the effects of catecholamines on the lipolytic rate of an ectotherm. It shows that NE (~170 nM) inhibits lipolysis in trout instead of stimulating it like in mammals. Epinephrine (~500 nM), on the other hand, has the same activating effect in both groups of vertebrates. Changes in plasma glycerol concentration are weakly correlated with R_a glycerol (Epi stimulation), or not correlated at all (NE inhibition),

making glycerol concentration a very poor predictor of lipolysis. Comparing all *in vivo* lipolytic rates measured to date reveals that trout maintain a disproportionately high resting R_a glycerol for their low metabolic rate compared to endotherms. No demonstrated functional explanation for maintaining elevated lipolytic rates in the resting state is available. However, results suggest that ectotherms may always need high rates of fatty acid supply for rapid remodeling of membrane phospholipids as they cope with changes in body temperature.

Opposing effects of NE and Epi on trout lipolysis

At the whole-organism level and at the concentrations selected, this study shows that NE (inhibition) and Epi (activation) have opposite effects on the lipolytic rate of rainbow trout (Figs. 5.2 and 5.3). Both catecholamines are released during hypoxia (see Reid *et al.*, 1998 for an extensive review), with NE preceeding Epi release, at least in cod (Fritsche and Nilsson, 1990). In trout, it has been shown that NE concentration only reaches ~20 nM after exposure to hypoxia (Ristori and Laurent, 1989), and ~85 nM after extreme stress (Butler *et al.*, 1986). Therefore, the effects of the supranormal concentrations selected for the present experiments should be interpreted with caution. If physiological NE concentrations also cause the inhibition of lipolysis, this hormone could play an interesting role during hypoxia and may be reponsible for the observed depression of lipid metabolism. This mechanism may explain the decreases in plasma NEFA concentration (Van Raaij *et al.*, 1996) and NEFA turnover rate (Haman *et al.*, 1997) previously observed when trout are exposed to hypoxic conditions. The inhibition of lipolysis is also probably responsible for the decreases in plasma NEFA concentration

reported after NE administration in bream and pike-perch (Farkas, 1967b), as well as in goldfish (Minick and Chavin, 1973), pike (Ince and Thorpe, 1975), and carp (Van Raaij *et al.*, 1995). These *in vivo* effects are consistent with the inhibition of lipolysis demonstrated *in vitro* for adipose tissue in carp, pike-perch (Farkas, 1967b) and tilapia (Vianen *et al.*, 2002). However, more experiments are needed to test these notions.

Epi administration (~ 500 nM) causes the stimulation of lipolysis (Fig. 5.3), but the only other *in vivo* trout study addressing this issue failed to demonstrate an increase in plasma NEFA concentration (Perrier *et al.*, 1972). In these previous experiments, however, lipolysis may have already been stimulated before Epi administration because measurements were not made on cannulated trout. Therefore, the injection of Epi in animals with high endogenous Epi levels potentially caused by handling stress may not have shown further activation of lipolysis. In several other fish species, significant increases in plasma NEFA concentration were reported after Epi injection and these observations are consistent with present results [scorpion fish (Leisbon *et al.*, 1968), eel (Larsson, 1973), lamprey (Plisetskaya, 1980), plaice (White and Fletcher, 1989), carp (Van Raaij *et al.*, 1995)].

Possible mechanisms of action for catecholamines

In mammals, NE and Epi increase fatty acid mobilization from adipose tissue and muscle by activating hormone-sensitive lipase (HSL). This involves the stimulation of G_s proteins by β-adrenergic receptors causing the subsequent activation of adenylate cyclase and protein kinase A (Holm, 2003). The presence of one or more lipases also

activated by hormones, homologous or not to mammalian HSL, has been reported in the liver and adipose tissue of rainbow trout (Albalat *et al.*, 2005a; Van Heeswijk *et al.*, 2006). In trout hepatocytes, both catecholamines activate lipolysis and appear to act through β_2-adrenergic receptors (Van Heeswijk *et al.*, 2006). However, no Epi-induced stimulation of lipolysis was detected in red muscle slices from trout (Bilinski and Lau, 1969). Taken together, these *in vitro* studies suggest that the increase in lipolytic rate observed here after *in vivo* administration of Epi was caused by the activation of HSL-like lipases in liver and adipose tissue, but not in red muscle. Furthermore, the potential implication of other lipases such as lipoprotein lipase (LPL), or other circulating hormones acting indirectly like glucagon, insulin or cortisol cannot be eliminated. In mammalian heart and adipose tissue, for example, LPL is activated by Epi and several other hormones (Zechner, 1997; Merkel *et al.*, 2002; An *et al.*, 2005). In trout, a homolog of mammalian LPL sensitive to insulin has also been characterized in several tissues (Lindberg and Olivecrona, 2002; Albalat *et al.*, 2006). Therefore, further studies will be needed to separate the effects of HSL-like lipases and LPL, and to characterize potential interactions of catecholamines with other hormones.

Even though NE causes the stimulation of mammalian lipolysis *in vivo*, it also triggers local inhibition when activating G_i proteins *via* α_2-adrenergic receptors in adipocytes (Holm, 2003). However, another mechanism inhibiting lipolysis appears to operate in tilapia, the activation of G_i proteins *via* β_3-adrenergic receptors (Vianen *et al.*, 2002). The exact nature of the receptors mediating the decrease in lipolytic rate observed here in intact rainbow trout (Fig. 5.2) remains to be established.

Plasma glycerol concentration is not an index of lipolytic rate

The observed changes in R_a glycerol caused by Epi were weakly correlated with changes in plasma glycerol concentration, but no such correlation was apparent for NE (Fig. 5.4). Because Epi causes a much stronger stimulation of cardiac output than NE, the relationship between flux and concentration could be influenced by the differential effects of catecholamines on the cardiovascular system (Gannon and Brunstock, 1969; Wood and Shelton, 1980; Randall and Perry, 1992). Previous studies have shown that plasma metabolite fluxes can be increased in two ways: 1) by augmenting concentration in plasma (mass action effect), or 2) by increasing blood flow (perfusion effect) (Weber *et al.*, 1987). Therefore, the stimulating effect of Epi on cardiac output may have amplified the slope of the relationship between glycerol concentration and R_a glycerol. Interestingly, the stimulating effect of NE on cardiac output appears to have been sufficient to offset the negative mass action effect of a decrease in plasma glycerol concentration (thereby eliminating a potential relationship between flux and concentration in the NE experiments). A detailed analysis of trout glucose and NEFA metabolism also showed that the plasma concentration of these metabolites does not reflect changes in their flux (Haman *et al.*, 1997). The present study reinforces the idea that changes in metabolite concentration cannot be used to speculate on possible changes in metabolite fluxes, unless a clear relationship between the two parameters has been formally established under specified conditions.

High resting lipolytic rates in trout

In previous studies, lipolytic rate has almost exclusively been measured in warm-blooded animals. The rates reported here for trout (2-8 μmol kg^{-1} min^{-1}) fit within the endotherm range (2 to 72 μmol kg^{-1} min^{-1}) (Fig. 5.5A), but relevant comparisons can only be made if differences in metabolic rate are taken into account. Therefore, ratios between lipolytic rate and metabolic rate were plotted as well for all vertebrates measured to date (Fig. 5.5B). In endotherms, the ratio varies between 0.01 and 0.03, and it does not vary much between rest and exercise because lipolytic rate is stimulated in parallel with metabolic rate. This analysis reveals that swimming trout fall within the normal vertebrate range (0.03), but that resting trout have a drastically different ratio of 0.10, more than 5 times the average (~0.02). What is the physiological significance of this unexpected observation? I have calculated the theoretical metabolic rate needed to oxidize all fatty acids supplied by lipolysis at rest (4.6 μmol glycerol kg^{-1} min^{-1} or ~14 μmol FA kg^{-1} min^{-1}). Assuming that energy metabolism is only supported by lipids, this theoretical metabolic rate would be 360 μmol O_2 kg^{-1} min^{-1} (if oleate is considered as an average fatty acid requiring 26 O_2 for oxidation and if the contribution of glycerol oxidation is ignored). Because the real metabolic rate of a resting trout is only 46 μmol O_2 kg^{-1} min^{-1} (Burgetz *et al.*, 1998), I calculate that 13% of their lipolytic rate can fuel resting energy metabolism entirely. Therefore, 87% of the fatty acids released by trout lipolysis have to undergo reesterification, a much higher proportion than in endotherms (<70%; see Fig. 5.5 for references). Such high lipolytic rates (this study) and TAG turnover rates (Chapter 4) demonstrate that fatty acid mobilization and reesterification

are particularly active in trout. I propose that this rapid cycling of fatty acids may be crucial for restructuring membrane phospholipids and, therefore, necessary in all ectotherms for adequate homeoviscous adaptation.

Conclusion and perspectives

Results show for the first time that NE (~ 170 nM) inhibits whole-organism lipolysis in trout rather than stimulating it as in mammals. It supports previous *in vitro* studies suggesting that fish and mammals regulate lipid mobilization differently. In trout, changes in lipolytic rate are not well reflected by changes in the concentration of plasma glycerol. Therefore, glycerol concentration cannot be used as an index of glycerol flux (R_a glycerol), a recommendation previously made for other key intermediates of energy metabolism (Haman *et al.*, 1997). Finally, this study demonstrates that resting trout maintain a disproportionately high lipolytic rate, because 13% of the fatty acids supplied by lipolysis are sufficient to support total energy expenditure. Therefore, most mobilized fatty acids must undergo reesterification (87%). Such cycling implies rapid exchange between fatty acid pools, a metabolic adaptation improving the capacity for remodeling membrane phospholipids with fatty acids of different chain length and degree of saturation. Determining whether such an adaptation is ubiquitous among ectotherms strikes me as a fascinating avenue for future research.

Table 5.1. Changes in fatty acids concentration, as an index for lipolysis, measured *in vivo* and *in vitro* after catecholamine treatment (epinephrine, Epi or norepinephrine, NE) in different species of fish and mammals.

Group	Epi	NE
Fish		
In vivo		
1) Trout	NC (Perrier *et al.*, 1972)	
2) Carp	+ (Van Raaij *et al.*, 1995) NC (Farkas, 1969)	- (Farkas, 1967a; Van Raaij *et al.*, 1995) NC (Farkas, 1969)
3) Goldfish	- (Minick and Chavin, 1973)	- (Minick and Chavin, 1973)
4) Plaice	+ (White and Fletcher, 1989)	
5) Lamprey	+ (Plisetskaya, 1980)	+ (Plisetskaya, 1980)
6) Scorpion fish	+ (Leisbon *et al.*, 1968)	+ (Leisbon *et al.*, 1968)
7) Eel	+ (Larsson, 1973)	
8) Pike	- (Ince and Thorpe, 1975)	- (Ince and Thorpe, 1975)
9) Bream		- (Farkas, 1967b)
10) Pike perch		- (Farkas, 1967b)
In vitro		
Adipocytes		
1) Tilapia		- (Vianen *et al.*, 2002)
2) Carp		- (Farkas, 1967b)
3) Bream		+ (Farkas, 1967b)
4) Pike perch		- (Farkas, 1967b)
5) Tiger fish	NC (Migliorini *et al.*, 1992)	
Hepatocytes		
1) Trout	+ (Van Heeswijk *et al.*, 2006)	+ (Van Heeswijk *et al.*, 2006)
2) Brook charr	NC (Scott-Thomas *et al.*, 1992)	+ (Scott-Thomas *et al.*, 1992)
3) Coho salmon (slices)	NC (Sheridan, 1987)	+ (Sheridan, 1987)
Mammals (Rat, Human)		
In vivo	+ (Saleh *et al.*, 1999)	+ (Saleh *et al.*, 1999)
In vitro		
Adipocytes (white)	+ (Saleh *et al.*, 1999)	+ - (Saleh *et al.*, 1999)
Hepatocytes		
Muscle	+ (Donsmark *et al.*, 2005)	+ (Nonogaki, 2000)

Symbols +, -, and NC denotes increase, decrease or no change in plasma fatty acid concentration after catecholamine administration, respectively.

Table 5.2 Mean plasma glycerol concentration (μmol ml^{-1} plasma) and rate of appearance (R_a glycerol in μmol kg^{-1} min^{-1}) of rainbow trout during the last 10 min of saline (control), norepinephrine (NE), or epinephrine (Epi) administration.

Infusion	[Glycerol]	R_a Glycerol
Saline	0.22 ± 0.03	4.6 ± 0.4
NE	0.12 ± 0.02 *	2.6 ± 0.5 *
Epi	0.52 ± 0.04 *	7.7 ± 0.6 *

Values are means ± SE (N=4 for saline and N=8 for NE and Epi). * indicate significant differences from saline control ($P < 0.05$).

Fig. 5.1. Control experiments showing the effect of vehicle saline infusion (shaded area) on glycerol concentration in plasma (A), specific activity (SA) (B), and rate of appearance (R_a glycerol) (C) in resting trout. Measurements were made by continuous infusion of ^{3}H-glycerol. No significant differences were found ($P > 0.05$). Values are means ± SE (N=4).

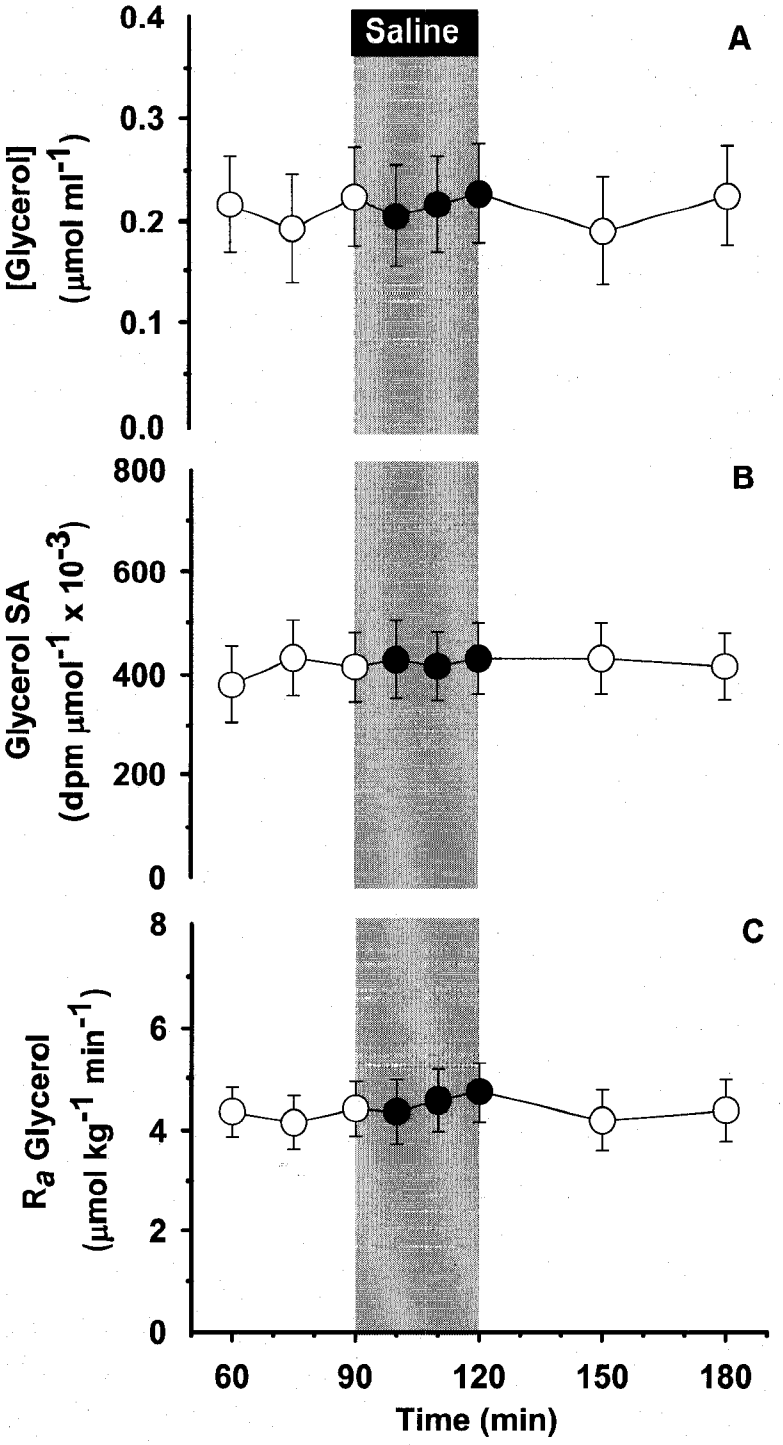

Saline
A
B
C
[Glycerol] (μmol ml-1)
Glycerol SA (dpm μmol-1 x 10-3)
Ra Glycerol (μmol kg-1 min-1)
0.4
0.3
0.2
0.1
0.0
800
600
400
200
0
8
6
4
2
0
60
90
120
150
180
Time (min)

Fig. 5.2. Effects of exogenous norepinephrine (NE) infusion (shaded area) on glycerol concentration in plasma (A), specific activity (SA) (B), and rate of appearance (R_a glycerol) (C) in resting trout. * indicate significant differences ($P< 0.001$) from baseline values at 90 min. Values are means ± SE (N=8).

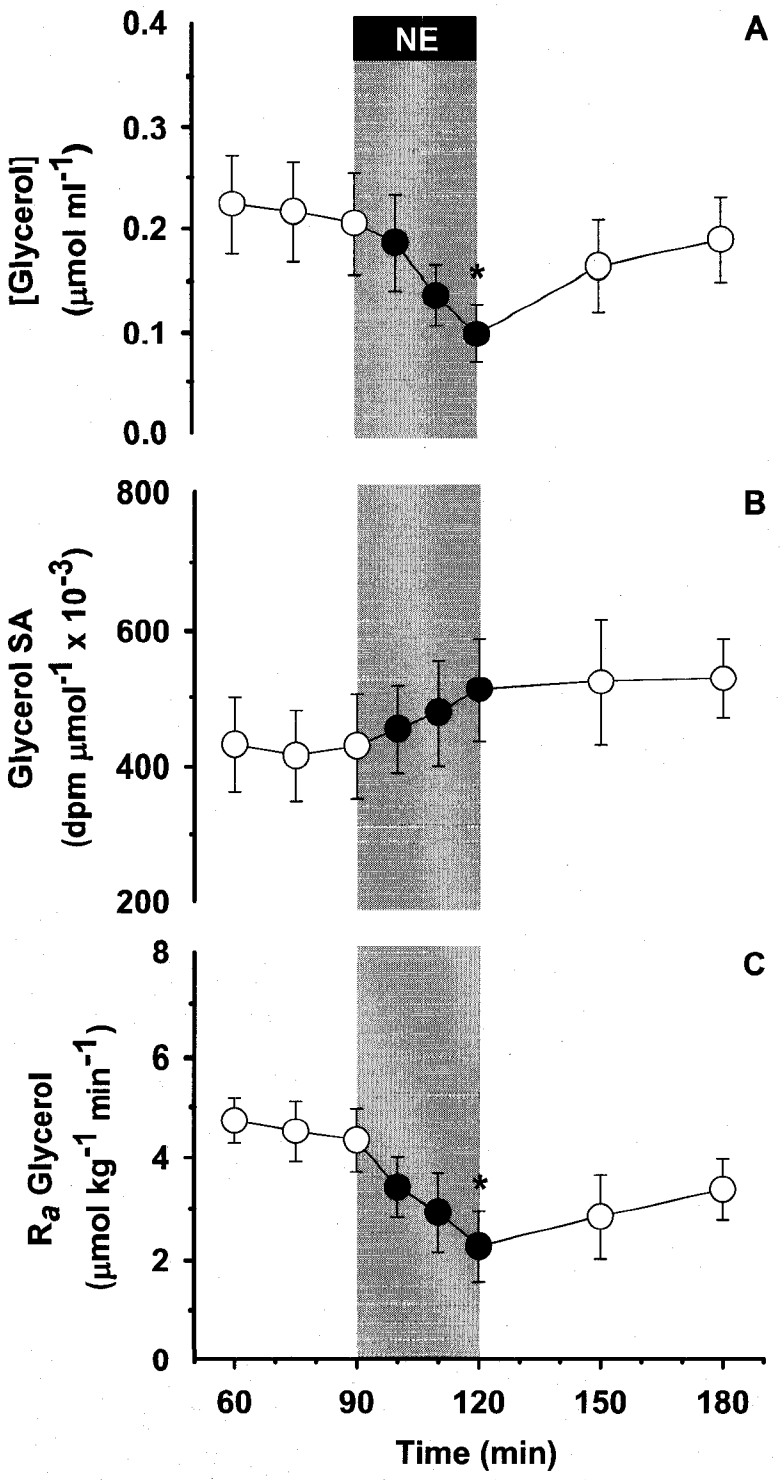

NE
A
B
C
[Glycerol] (μmol ml-1)
Glycerol SA (dpm μmol-1 x 10-3)
Ra Glycerol (μmol kg-1 min-1)
0.4
0.3
0.2
0.1
0.0
800
600
400
200
8
6
4
2
0
*
*
60
90
120
150
180
Time (min)

Fig. 5.3. Effects of exogenous epinephrine (Epi) infusion (shaded area) on glycerol concentration in plasma (A), specific activity (SA) (B), and rate of appearance (R_a glycerol) (C) in resting trout. * indicate significant differences ($P< 0.001$) from baseline values at 90 min. Values are means ± SE (N=8).

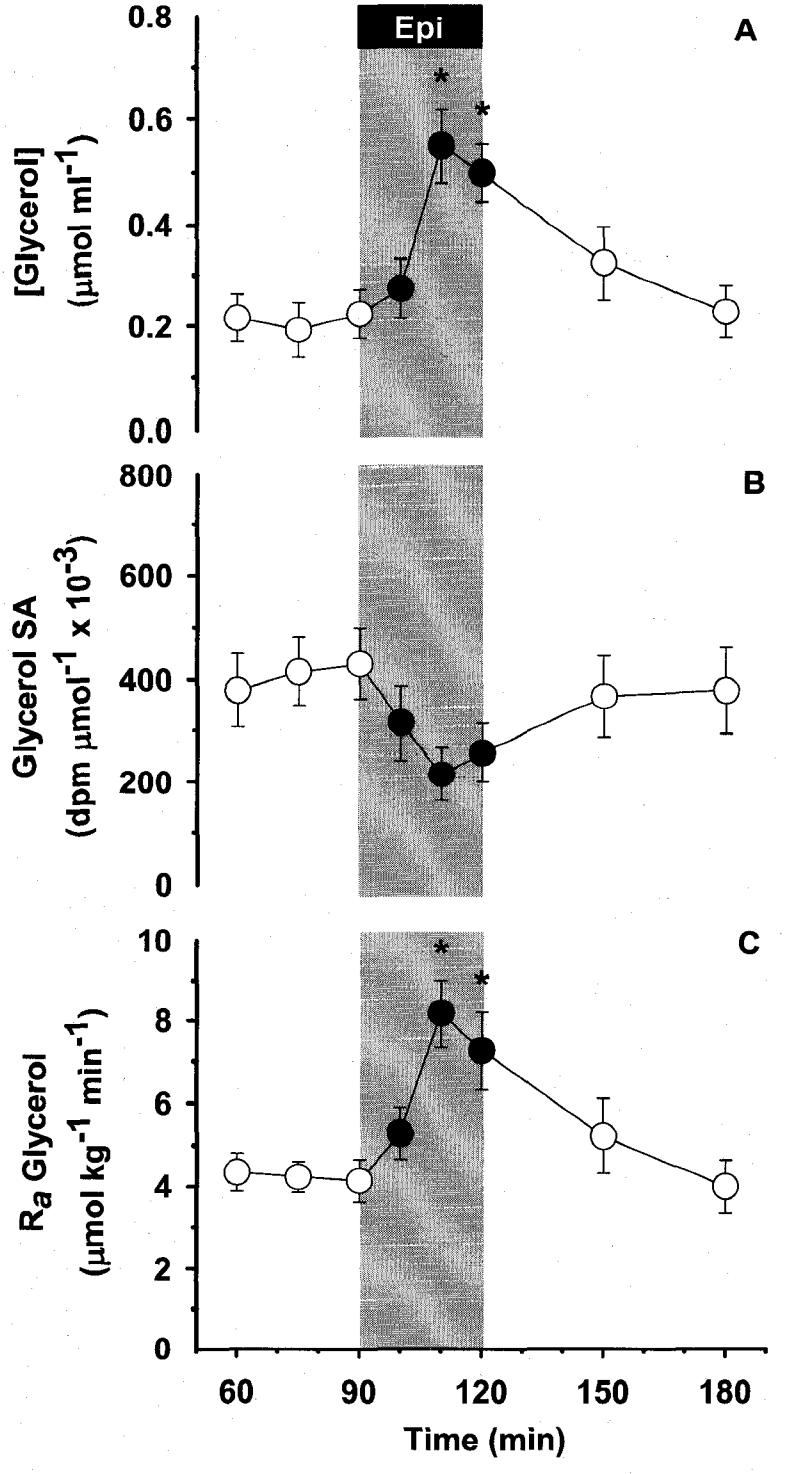

Epi
A
B
C
[Glycerol] (μmol ml-1)
Glycerol SA (dpm μmol-1 x 10-3)
Ra Glycerol (μmol kg-1 min-1)
0.0
0.2
0.4
0.6
0.8
0
200
400
600
800
0
2
4
6
8
10
60
90
120
150
180
Time (min)

Fig. 5.4. Relationship between glycerol turnover (R_a glycerol) and glycerol concentration in resting trout during saline (stars), norepinephrine (open circles) or epinephrine administration (closed circles). Only values measured during the last 10 min of saline, epinephrine and norepinephrine administration are included (110-120 min in Figs. 5.1-3). Lines were fitted by linear regression for saline [(y= 4.326 - 0.375 x) continuous line; R=0.03; slope not different from 0 (P=0.905)], norepinephrine [(y= 2.750 + 0.860 x) broken line; R=0.05; slope not different from 0 (P=0.830)], and epinephrine [(y= 4.286 + 5.944 x) dotted line; R=0.48; slope different from 0 (P<0.05)]. Values are means ± SE (N=4 for saline and N=8 for catecholamines).

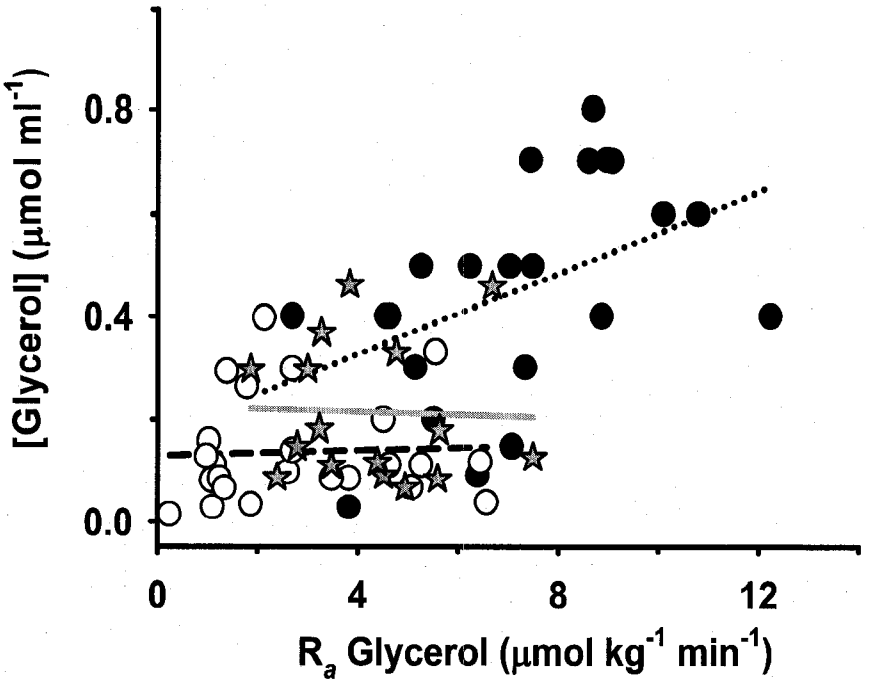
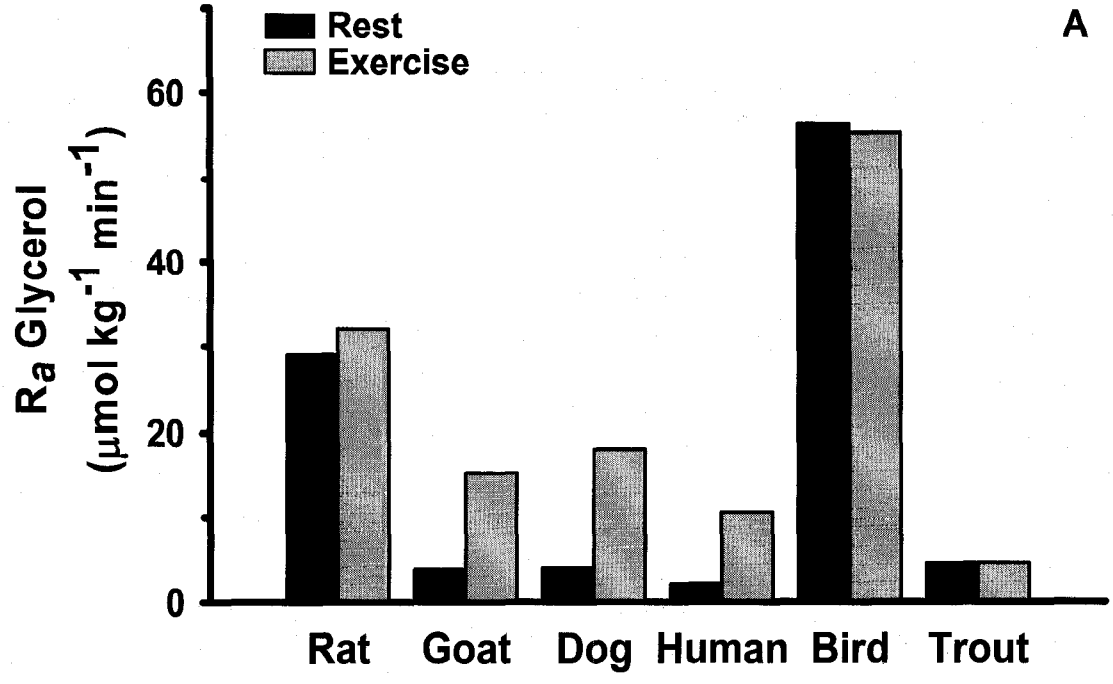
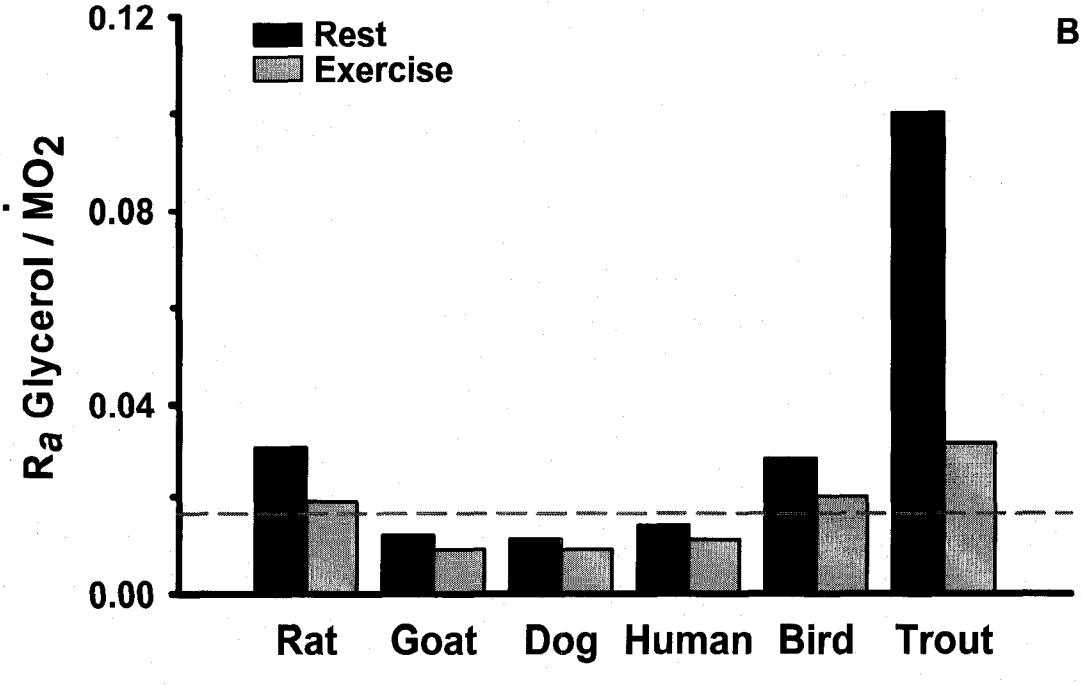

[Glycerol] (μmol ml-1)
0.8
0.4
0.0
0
4
8
12
Ra Glycerol (μmol kg-1 min-1)

Fig. 5.5. Lipolytic rate (R_a glycerol) (A) and the ratio obtained when divided by the metabolic rate (MO_2) (B) for different vertebrates measured to date. Values correspond to vertebrates at rest and during exercise at equivalent intensities (40-70% $MO_{2\ max}$), except for birds, where only shivering data is available (Vaillancourt and Weber, 2007). Ratios were calculated from the following studies: rat (McClelland *et al.*, 2001), goat (Weber *et al.*, 1993), dogs (Issekutz *et al.*, 1967; Issekutz *et al.*, 1975), human (Wolfe *et al.*, 1990), ruff sandpiper (Vaillancourt and Weber, 2007) and trout (this study and Bernard et al, 1999 using MO_2 values from Burgetz *et al.*, 1998). Broken line shows the calculated average ratio for resting and exercising endotherms (0.016).


Rest
Exercise
A
R_a Glycerol
(μmol kg^{-1} min^{-1})
60
40
20
0
Rat
Goat
Dog
Human
Bird
Trout


Rest
Exercise
B
R_a Glycerol / $\dot{M}O_2$
0.12
0.08
0.04
0.00
Rat
Goat
Dog
Human
Bird
Trout

CHAPTER 6.

GENERAL DISCUSSION AND CONCLUSIONS

THESIS OVERVIEW

The main purpose of this thesis was to investigate the possible role of lipoproteins in supplying fatty acids to red muscle during prolonged exercise in fish. Additional objectives were to clarify the mechanisms of action of lipoprotein lipase on circulating lipids and to study how lipolytic rate may be regulated *in vivo* by catecholamines. All experiments were conducted in salmonids (sockeye salmon, *Oncorhynchus nerka*, and rainbow trout, *Oncorhynchus mykiss*), a group of fish extensively used as experimental models in biology, and that commonly swim at sustainable speeds in nature.

By analogy with mammals, previous research in this field has focused on the metabolic role of non-esterified fatty acids (NEFA), assuming that these lipids are responsible for transporting energy from adipose stores to locomotory muscles (Ballantyne *et al.*, 1996; Bernard *et al.*, 1999; Booth *et al.*, 1999; Weber *et al.*, 2002). However, this assumption is probably not justified because endurance swimming has no effect on the turnover rate of NEFA (Bernard *et al.*, 1999). Although lipoproteins are known to be used for lipid transport to the gonads during egg production (Babin and Vernier, 1989), they have not been considered as a possible energy shuttle to working muscles. This is surprising because they carry most of the energy in the circulation of fish (Moyes and West, 1995) and could theoretically play an important role in powering muscles during swimming.

This work was subdivided into four studies (CHAPTERS 2-5). In chapter 2, the effects that prolonged swimming have on plasma lipids containing fatty acids (NEFA, TAG and PL) were quantified during the long-distance migration of wild sockeye salmon. In CHAPTER 3, the effects of endurance swimming on circulating lipids of

rainbow trout were measured under controlled laboratory conditions. In CHAPTER 4, the effects of endurance swimming and heparin administration on TAG turnover rate were investigated *in vivo* in rainbow trout. Finally, the goal of CHAPTER 5 was to measure lipolytic rate (R_a glycerol) *in vivo* and to investigate the effects of catecholamines on this metabolic parameter in intact fish to obtain an integrated hormonal response of total fatty acid supply. The following conclusions can be drawn from this thesis.

SUMMARY OF PRINCIPAL FINDINGS

CHAPTER 2. EFFECTS OF LONG-DISTANCE MIGRATION ON CIRCULATING LIPIDS OF SOCKEYE SALMON (ONCORHYNCHUS NERKA)

1. I found that lipids transported as NEFA represent < 7% of total plasma fatty acids and show only a minor decrease during a 500 km migration.

2. Lipoproteins account for > 93% of all the energy of circulating lipids and their main constituents show a 27-fold decrease (TAG) and a 6-fold decrease (PL) during migration.

3. The decline in PL and TAG levels do not proceed at the same pace. The PL/TAG ratio increases along the migration path, suggesting a decrease in the average size of circulating lipoproteins from large TAG-rich to smaller TAG-poor particles.

CHAPTER 3. ENDURANCE SWIMMING ACTIVATES TROUT LIPOPROTEIN LIPASE: PLASMA LIPIDS AS A FUEL FOR MUSCLE

1. Sustained swimming for 4 days at 1.5 body lengths/s caused a 2.7-fold increase in lipoprotein lipase (LPL) activity in lateral red muscle, the engine for endurance swimming.

2. The same duration and intensity of swimming (~ 150 km) that affected red muscle LPL had no measurable effect on the fatty acid content, protein content or fatty acid/protein ratio of the 3 lipoprotein classes (HDL, LDL, or VLDL).

3. *In vivo* heparin administration causes a 27-fold increase in LPL activity in plasma, demonstrating that rainbow trout tissues have a remarkable reserve capacity for lipoprotein hydrolysis.

CHAPTER 4. HIGH RESTING TRIACYLGLYCEROL TURNOVER OF RAINBOW TROUT EXCEEDS THE ENERGY REQUIREMENTS OF ENDURANCE SWIMMING

1. The high baseline TAG turnover rate measured in fish reveals a particularly active lipoprotein metabolism in this group of vertebrates.

2. TAG turnover rate is not stimulated by endurance swimming.

3. The heparin-induced release of LPL in the circulation causes a 50% inhibition of TAG turnover rate.

CHAPTER 5. IN VIVO REGULATION OF RAINBOW TROUT LIPOLYSIS BY CATECHOLAMINES

1. Norepinephrine (~ 170 nM) inhibits lipolytic rate (R_a glycerol) in trout instead of stimulating it as it does in mammals.

2. Epinephrine (~ 500 nM) activates R_a glycerol as in mammals.

3. Changes in plasma glycerol concentration are weakly correlated with lipolytic rate (Epi stimulation) or not correlated at all (NE inhibition), making glycerol concentration a poor predictor of lipolysis.

4. Trout maintain a disproportionately high resting lipolytic rate for their low metabolic rate compared to endotherms.

GENERAL DISCUSSION

I. The modulation of lipoprotein metabolism by exercise

There is a great deal of interest in the study of lipoprotein metabolism, particularly in how it may be regulated during exercise, when the demand for metabolic fuels is increased, because this knowledge may help to understand how energy homeostasis can be reached in animals (Zechner, 1997). LPL is the rate-limiting enzyme for the catabolism of TAG-rich lipoproteins, and local regulation of its activity in vascular beds can be a means to generate FA and other lipoprotein-derived lipids. Although, the regulation on LPL during exercise has been partially characterized in endotherms, this thesis constitutes the first effort to examine the effects of endurance swimming on this enzyme in fish muscle. I showed that fish LPL is stimulated by exercise in lateral red muscle, the engine for endurance swimming (Chapter 3). The three-fold increase in LPL activity for trout muscle after prolonged exercise (Fig. 3.1) is consistent with the response reported for several mammals including rats (2 to 3-fold; Oscai *et al.*, 1982; Bagby *et al.*, 1986; Ladu *et al.*, 1991b), dogs (2-fold; Budohoski, 1985), and humans (3-fold; Lithell *et al.*, 1984). This exercise-induced activation of LPL is probably mediated by contractile activity *in situ*, as shown in isolated muscle fibers of rats (Hamilton *et al.*, 1998). Additionally, a number of hormones including insulin, catecholamines and cortisol, have been proposed as possible modulators of LPL during exercise in mammals (Lithell *et al.*, 1981; Ladu *et al.*, 1991a; Enerback and Gimble, 1993; Seip *et al.*, 1997). Regulation of LPL may occur through transcriptional control

mechanisms (Ladu *et al.*, 1991b, 1991a; Seip *et al.*, 1997; Hildebrandt *et al.*, 2003), but also at the post-transcriptional level (Bergo *et al.*, 1996; Mead *et al.*, 2002). Therefore, future studies should investigate the existence of similar control mechanisms of LPL in fish red muscle, as it has been shown for the skeletal muscle of mammals.

This thesis provides the first *in vivo* measurements of TAG turnover rate in an ectothermic animal (Chapter 4). Contrary to expectation, this thesis has established that rainbow trout do not increase TAG turnover rate beyond resting levels even if they swim continuously for several hours (Fig 4.3). However, the resting values of TAG turnover rate for this ectotherm exceed all fluxes measured to date in endotherms (Terjung *et al.*, 1982; Bagby *et al.*, 1987; Teusink *et al.*, 2003) (Fig. 4.5). Moreover, the basal TAG turnover rate measured in trout (25 μmol TAG kg^{-1} min^{-1}) is already high enough to cover all the fuel requirements of exercise. Only 6% of the TAG turnover rate measured in trout is necessary to support exercise, which may explain why I did not detect an increase during endurance swimming.

Why doesn't the exercise-induced activation of LPL found in red muscle of trout appear to alter TAG turnover rate? Clearly, up regulation of LPL activity in red muscle would enhance hydrolysis of circulating TAG-rich lipoproteins, thus increasing TAG turnover rate. However, I established in Chapter 3 that the increase in red muscle LPL activity (Fig. 3.1) was not mirrored by a similar increase in post-heparin plasma LPL (Fig. 3.6). Therefore, the activation of red muscle LPL could be accompanied by inhibition of the enzyme in other tissues (such as adipose tissue, heart, and liver), yielding no overall change in TAG turnover rate. In this regard, it has been shown that exercise

produces a decrease of LPL activity in adipose tissue, as uptake of FA for the synthesis of TAG is reduced (Nikkila, 1987). In particular, Ladu *et al.* (1991a) confirm a 43% decrease in LPL activity of adipose tissue in rats after 2 h of swimming. It is also possible that the 3-fold increase of muscle LPL activity may not be large enough to influence total LPL activity at the whole organism level, as trout red muscle represents only 7% of total body mass. In support of this idea, previous trout studies by Lindberg *et al.* (1995) showed high LPL activities in adipose tissue (300 nmol FA min^{-1} g^{-1}) when compared to red muscle (18-50 nmol FA min^{-1} g^{-1}; this thesis).

Prolonged exercise is, in fact, associated with a small decrease in plasma TAG concentration of mammals, which may reflect use of TAG-rich lipoproteins as a fuel for exercise (Nagel *et al.*, 1989). The decrease in plasma lipid concentration measured in sockeye salmon during the spawning migration is in accordance with this observation (Chapter 2). However, such changes in plasma lipid concentration in salmon could not only be explained by the 500 km swim, but also by other physiological changes occurring simultaneously during migration (e.g. fasting, egg production, and osmoregulatory adjustments). Whether an increase in activity of LPL induced by exercise in working muscle of vertebrates should or should not affect plasma TAG concentration remains under debate. A number of studies measured plasma TAG uptake by locomotory muscle during exercise in fasting mammals and found no significant extraction of FA from VLDL-TAG (Olsson *et al.*, 1975; Havel, 1987; Kiens *et al.*, 1993), despite the increase of LPL activity during similar exercise conditions (Oscai *et al.*, 1982; Lithell *et al.*, 1984; Bagby *et al.*, 1986; Ladu *et al.*, 1991b). In this thesis (Chapter 3), four days of sustained swimming in trout at 1.5 body lengths per second

(~150 km) did not have any measurable effect on plasma TAG concentration (Fig. 3.2-3). The high lipoprotein concentrations of fish compared to mammals (Babin and Vernier, 1989) and the high TAG turnover rate in trout (Chapter 4) may be responsible for the concentration inertia observed in trout lipoproteins.

II. Lipoprotein lipase: mechanism of action

Under baseline conditions, LPL is entirely bound to the endothelium, and plasma LPL activity is therefore very low (Fig. 3.4). The injection of heparin causes a drastic increase in activity, 1) by releasing the enzyme into the plasma (Olivecrona and Bengston-Olivecrona, 1999), and 2) by inhibiting its normal uptake by the liver for degradation (Chajek-Shaul *et al.*, 1988). The remarkable 27-fold increase in LPL activity observed here in plasma after the release of the enzyme produced by heparin demonstrates that rainbow trout tissues have an extraordinary reserve capacity for lipoprotein hydrolysis (Chapter 3). The release of lipoprotein lipase (LPL) into plasma by heparin could therefore explain the decrease in TAG turnover rate measured in Chapter 4 (Fig. 4.2), because tissue uptake of fatty acids from lipoproteins mainly relies on bound LPL (Goldberg *et al.*, 1991). In mammals, this effect of heparin on LPL has been previously cited to explain declines in fatty acid supply from lipoproteins (Chevreuil *et al.*, 1993), and in the uptake of a TAG-rich emulsion by the heart (Augustus *et al.*, 2003). Therefore, both fish and mammals seem to depend on the natural presence of LPL bound to the vascular endothelium for proper delivery of lipoprotein fatty acids to tissues.

The precise mechanisms for LPL action are still under discussion, and several models have been proposed (Merkel *et al.*, 2002). Tissue-specific regulation of LPL (Fig. 1.2) would allow for the production of local increases in FA concentration close to the endothelium that drives the diffusion (or transport) of these molecules into the underlying cells (Hamilton and Kamp, 1999). In mammals, muscle contraction increases the transport of fatty acids into this tissue (Turcotte *et al.*, 1992; Dyck and Bonen, 1998), and it is believed that such an effect is mediated by an increase in the translocation of specific transporters (FAT/ CD36) to the sarcolemma (Bonen *et al.*, 2000). In contrast, the transport process of FA across the muscle membrane does not contribute to the overall regulation of lipid oxidation by muscle in trout (Richards *et al.*, 2003). Taken together these data suggest that a different control of FA availability may be operating in fish muscle.

III. Lipolytic rate and TAG turnover rate

Upon hydrolysis, each molecule of TAG yields three molecules of NEFA and one of glycerol. Lipolysis in adipocytes and muscle release glycerol that cannot be reesterified *in situ* because these tissues contain an extremely low amount of the enzyme necessary for this re-synthesis: glycerokinase (Newsholme and Taylor, 1969; Brooks *et al.*, 1982; Reshef *et al.*, 2003). Consequently, TAG hydrolysis results in the quantitative release of glycerol (R_a glycerol) in the circulation (Wolfe *et al.*, 1990). In mammals, glycerol is derived primarily from lipolysis in adipose tissue during endurance exercise, and muscle only becomes a significant source after strenuous exercise (Romijn *et al.*,

1993). However, glycerol can be generated from circulating lipoproteins, and lipoprotein-derived glycerol can contribute to the lipolytic rate measured in Chapter 5 (Fig. 5.5). The hydrolysis of lipoproteins by LPL also releases FA that may be in rapid exchange with NEFA. Therefore both pools of FA may contribute to the rate of appearance of FA in plasma (R_a NEFA), and they may serve to maintain the driving force for facilitated diffusion of FA (Teusink *et al.*, 2003). Regardless of the exact contribution that lipoprotein hydrolysis may have on the release of glycerol and FA to plasma, trout still maintain high NEFA (Bernard *et al.*, 1999) and TAG turnover rates (Chapter 4), and the FA generated from these sources are well in excess of needs for oxidative fuel during exercise. What is the physiological significance of this observation? The measurement of such high lipid fluxes indicate a rapid cycling of fatty acids in trout, implying a fast exchange between fatty acid pools, a metabolic adaptation providing a high capacity for remodeling membrane phospholipids with fatty acids of different chain length and degree of saturation (Hazel and Williams, 1990). I propose that this could be a common feature of lipid metabolism in fish that may be necessary for adequate homeoviscous adaptation. Determining whether such an adaptation is ubiquitous among ectotherms strikes me as a fascinating avenue for future research.

IV. Regulation of lipolysis by catecholamines

Epinephrine has the same activating effect on lipolysis in rainbow trout (Fig. 5.3) and in mammals (Fain and Garcia-Sainz, 1983). Endurance exercise is associated with a

rise in the release of both catecholamines, Epi and NE, in mammals. Such response is frequently associated with an increased demand for metabolic fuels required by the working muscle (Arner, 1995; Horowitz *et al.*, 1999). However, Epi release probably does not play an important role in the long term regulation of fatty acid supply in trout, because it decreases during endurance swimming (Shanghavi and Weber, 1999), and only increases during burst swimming (Randall and Perry, 1992). Therefore, the regulation of lipolysis by Epi can be relevant during the onset of swimming, and during short term acceleration. The increase in lipolytic rate observed here after *in vivo* administration of Epi probably was caused by the activation of HSL-like lipases in liver and adipose tissue. Furthermore, the potential implication of other lipases such as lipoprotein lipase (LPL), or other circulating hormones acting indirectly like glucagon, insulin or cortisol cannot be eliminated. In mammalian heart and adipose tissue, for example, LPL is activated by Epi and several other hormones (Zechner, 1997; Merkel *et al.*, 2002; An *et al.*, 2005). In trout, a homolog of mammalian LPL sensitive to insulin has also been characterized in several tissues (Lindberg and Olivecrona, 2002; Albalat *et al.*, 2006) (Chapter 3). Therefore, further studies will be needed to separate the effects of HSL-like lipases and LPL, and to characterize potential interactions of catecholamines with other hormones.

In contrast, norepinephrine (~170 nM) inhibits whole-organism lipolysis in trout (Fig. 5.2) rather than stimulating it as in mammals (Fain and Garcia-Sainz, 1983; Horowitz and Klein, 2000; Nonogaki, 2000). It has been implied that the enzymatic machinery responsible for mobilization of lipids in fish is similar to that found in mammalian (Sheridan, 1994). Here, I show that lipolysis in trout is modulated differently

by both catecholamines. The exact nature of those different responses and their ubiquity in other species of fish remains to be established.

GENERAL CONCLUSIONS

Animals must match metabolic fuel supply with demand. This is achieved by constantly adjusting rates of metabolite turnover, particularly in response to environmental stressors. This thesis investigates how circulating lipids can contribute to metabolic fuel supply to the working muscle of swimming trout. Experimental evidence demonstrates that lipoproteins account for more than 93% of all the energy of circulating lipids, and that LPL is strongly activated during prolonged swimming in red muscle. The large amount of FA derived from the hydrolysis of circulating lipoproteins is a substantial source of energy available for locomotion, although TAG turnover rate is not altered during exercise. In rainbow trout, TAG turnover rate is affected by heparin administration, that causes a 50% decrease in baseline TAG turnover rate. This suggests that fish LPL must be bound to the endothelium for normal tissue uptake of fatty acids supplied by lipoproteins, as in mammals. Trout maintain particularly high baseline lipolytic rates, because only 13% of it is needed to fuel resting energy metabolism entirely (87% reesterification). Baseline glycerol turnover rate is inhibited by 170 nM norepinephrine (-56%), instead of being stimulated as in mammals, whereas epinephrine (~ 500 nM) has the same activating effect in both groups of vertebrates (+167%). High TAG turnover rates and lipolytic rates indicate that fatty acid mobilization and re-esterification are particularly active in fish. These characteristics of

lipid metabolism allow for the rapid cycling of fatty acids and may be crucial for restructuring membrane phospholipids.

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