

THE EVOLUTION OF GENOMIC AND NON-GENOMIC REGULATORY
MECHANISMS OF MITOCHONDRIAL LIPID OXIDATION IN FISH

THE EVOLUTION OF GENOMIC AND NON-GENOMIC REGULATORY
MECHANISMS OF MITOCHONDRIAL LIPID OXIDATION IN FISH

By

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TITLE: The evolution of genomic and non-genomic regulatory mechanisms of
mitochondrial lipid oxidation in fish

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ABSTRACT

Research in the field of lipid metabolism has blossomed in recent decades, particularly in terms of human metabolic disease. This has been studied mostly in mammals despite the importance of lipid metabolism on the energetics and life history of non-mammalian vertebrates such as fish. Fish have a large ecological and economic impact but have received little attention in the area of lipid metabolism. The main goal of my thesis is to understand the regulatory mechanisms which control lipid oxidation in fish under changing physiological conditions. In particular, I investigated the regulation of carnitine palmitoyltransferase (CPT) I, an enzyme which controls the entry of fatty acids into the mitochondria for oxidation. To accomplish this, I first examined the function and regulation of CPT I under routine conditions in liver and muscle tissue of rainbow trout. The results indicated that the expression, function and regulation of CPT I was variable across tissues. A phylogenetic reconstruction revealed multiple CPT I isoforms arising in fish after diverging from mammals highlighting the need for future tissue and species specific investigations.

With this fundamental knowledge, I was able to determine the effects of dietary manipulations and fasting on genomic and non-genomic CPT I regulation in rainbow trout. I found that changes in dietary fatty acids mainly effect the genomic regulation of CPT I through changes in the expression of CPT I and its transcription factor, PPAR. Fasting also induced increases in PPAR expression and further, caused a reduction in CPT I sensitivity to its inhibitor, malonyl-CoA, resulting in higher CPT I activity and rates of fatty acid oxidation.

In a final comparative study, I investigated the individual effects of endurance exercise on muscle fatty acid oxidation in rainbow trout and the combined effects of endurance exercise and fasting in a natural population of migrating pacific salmon. I found that changes in the activity of several lipid oxidation enzymes in red muscle during exercise were not as great as those found in the initial stages of migration in salmon. Similarly, changes in CPT I mRNA expression were greater in migrating salmon than in exercised trout. In contrast, changes in the mRNA expression of metabolic regulators and transcription factors were similar in both exercising trout and migrating salmon.

This thesis has significantly advanced our knowledge of lipid metabolism in fish and has highlighted the need to future research in this area.

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THESIS ORGANIZATION AND FORMAT

This thesis is organized in a sandwich format approved by McMaster University and with the recommendation of the supervisory committee. This thesis consists of seven chapters. Chapter one is a general introduction and summary of the objectives and completed work. Chapters two through six are discrete manuscripts that are published, accepted for publication or in preparation for submission to peer-reviewed scientific journals. Finally, chapter 7 summarizes the major findings of the thesis and indicates the significance and future directions of the research.

- Chapter 1:** **General introduction**
- Chapter 2:** **Intertissue regulation of carnitine palmitoyltransferase (CPT) I: Mitochondrial membrane properties and gene expression in rainbow trout (*Oncorhynchus mykiss*)**
Authors: Andrea J Morash, Makiko Kajimura and Grant B McClelland
Date accepted: February 20th, 2008
Journal: Biochimica et Biophysica Acta
Comments: This study was conducted by AJM under the supervision of GBM. MK provided spectrophotometric enzyme data.
- Chapter 3:** **Effects of dietary fatty acid composition on the regulation of carnitine palmitoyltransferase (CPT) I in rainbow trout (*Oncorhynchus mykiss*)**
Authors: Andrea J Morash, Dominique P. Bureau and Grant B McClelland
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Chapter 5: **The influence of exercise on gene expression and enzyme changes in migrating Pacific salmon**
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Chapter 6: **Regulation of carnitine palmitoyltransferase (CPT) I during fasting in rainbow trout (*Oncorhynchus mykiss*) promotes increased mitochondrial fatty acid oxidation**
Authors: Andrea J Morash and Grant B McClelland
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Journal: Journal of Experimental Biology
Comments: This study was conducted by AJM under the supervision of GBM.

Chapter 7: **General summary and significance of the thesis**

Chapter 8: **References**

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GENERAL INTRODUCTION

Lipid metabolism is a fundamental component of most living organisms and plays a role not only in energy homeostasis, but also in molecular signaling and cell structure. Lipids are a primary form of energy storage for most vertebrates and also fuel the majority of aerobic ATP production to support ATP consuming reactions. Defects in these pathways contribute to the root cause of many known human diseases such as obesity, diabetes mellitus and a variety of circulatory system diseases (Lee et al., 2003). Consequently, there has been a surge in research about the fundamental aspects of the physiology of lipid metabolism. Most of what is known about lipid metabolism has stemmed from humans and rodent models as well as *in vitro* and cell culture research. Due to its important role in overall metabolism, the liver has been the focus of much of this research. However, muscle has been receiving more attention due to its high oxidative capacity and prominent use of lipids as fuel (Leary et al., 2003; McClelland, 2004). Furthermore, muscle makes up a large proportion of the total body mass and plays an important role in metabolic homeostasis (Froyland et al., 1998; Hochachka and McClelland, 1997). Of key importance to this research will be the understanding of the regulatory mechanisms controlling lipid use in these tissues.

The majority of the main lipid metabolism pathways have been conserved across most vertebrate taxa including mammals and fish, although their distinct regulatory mechanisms have not been the subject of cross-taxa investigation. Fish have become an important model for the study of the regulation of lipid metabolism for a number of reasons. First, fish have anatomically distinct groups of muscles composed of single fiber types, making it easy to discern the relative contribution of both red and white muscle to lipid metabolism (Leary et al., 2003); a process not easily accomplished using mammalian models. Secondly, fish, particularly salmonids, are an economically important group of animals. Wild salmonid populations have been declining over the last several decades to the point where they have been added to the endangered species list (NOAA, 2010). To accommodate for this decline there has been a significant increase in the aquaculture of several important salmonid species. In Canada, salmon and rainbow trout compose approximately 70% of the total finfish aquaculture industry (DFO, 2009). The largest expense to any aquaculture facility is the production of high energy fish food allowing the quick production of large, lean fish. Therefore, the fundamental aspects of lipid oxidation and the dietary effects on lipid oxidation have become extremely important. Lastly, many salmonid species naturally experience a range of environmental challenges which make them particularly useful for metabolic research. For example, several species of salmonids migrate thousands of kilometers to spawn, during which time they engage in endurance exercise and fasting (Gilhousen, 1980). Fish also experience natural changes in ambient temperature and oxygen concentration as well as changes in food availability and composition. Therefore, the focus of this thesis will be to

investigate the effects of physiological state on the regulatory mechanisms of mitochondrial lipid oxidation using salmonids as a model animal.

Lipid metabolism

Lipid metabolism is composed of two opposing forces; fat synthesis (lipogenesis) and fatty acid oxidation (lipolysis). The balance of these two pathways determines a net gain or loss of lipid stores. This balance is highly dependent on the energetic demands of the tissues, the nutritional status of the animal and many environmental cues. It is controlled mainly through nutritional, hormonal and molecular regulation.

Lipogenesis

The synthesis of fatty acids is important not only for energy balance, but also for membrane biosynthesis and metabolic signaling pathways (Lee et al., 2003; Price et al., 2000). In most animals, the liver is the primary site of lipogenesis. When the energy balance is shifted towards energy storage, the product of glycolysis, pyruvate, is converted to acetyl-CoA by pyruvate dehydrogenase. Acetyl-CoA is then carboxylated to form malonyl-CoA (M-CoA) by acetyl-CoA carboxylase (ACC) in the cytoplasm. Fatty acid synthases then use acetyl-CoA, M-CoA, NADPH and protons to form palmitate. Palmitate can then be elongated in the endoplasmic reticulum to form fatty acids 18-24 carbons in length (For review see Glatz et al., 2010). When conditions favoring fat storage occur, these long-chain fatty acids will combine with glycerol to form triacylglycerol (TAG), the primary lipid storage molecule. Most TAGs are stored in adipose tissue, which is the primary storage tissue for lipids, but also to some extent in other tissues such as the liver and muscle (McClelland, 2004). TAGs are the preferred form of energy storage because gram for gram, they store more calories when compared to carbohydrates, are highly reduced and do not require hydration for storage (Hillgartner et al., 1995). Furthermore, the ability of tissues to store glycogen is rather low, while lipids can be stored in large quantities.

Lipolysis

When the energy balance shifts away from storage to a greater demand for energy, TAGs stored in the adipose tissue must be mobilized (Fig. 1.1). As a first step, prior to their entry into circulation, TAGs must be broken down into glycerol and non-esterified fatty acids (NEFAs) by hormone sensitive lipase (Holm, 2003). Both glycerol and NEFAs can then enter the circulation for transport to the tissues. Glycerol is highly soluble and can therefore be easily transported in the blood. In contrast, NEFAs require a transport protein due to their highly insoluble nature. In most vertebrates, this protein is albumin, which binds NEFAs upon release from the adipocytes and carries them to their tissue destination (Glatz et al., 2010). Alternatively, TAGs may remain intact and be transported by lipoproteins, which are assemblies of both lipids and proteins designed to transport lipids in the blood (Kwiterovich, 2000). At the tissue, both glycerol and NEFAs are taken up into the cell. There has been considerable debate as to how NEFAs cross the plasma

membrane and whether this process is carrier mediated. One hypothesis suggest that NEFAs can "flip-flop" across the membrane (Hamilton, 1998) while others suggests that this movement is facilitated by transport proteins (Schaffer, 2002). The proteins implicated in this process are FAT/CD36, fatty acid binding protein plasma membrane (FABPpm) and fatty acid transport protein (FATP) (Luiken et al., 2002; Schaffer, 2002; Stahl et al., 2001).

Once inside the cell, glycerol immediately enters the glycolytic pathway to be used for ATP production. NEFAs are carried to the mitochondria by fatty acid binding proteins (FABPs) for oxidation and converted to fatty acyl-CoAs by acyl-CoA synthase. Alternatively, NEFAs may be liberated from the small intracellular fat stores. Carnitine palmitoyltransferase (CPT) I lies in the outer mitochondrial membrane and is responsible for converting fatty acyl-CoAs to fatty acyl-carnitine, a rate-limiting process required for entry into the mitochondria (McGarry and Brown, 1997). Fatty acyl-carnitine is transported across the inner mitochondrial membrane by carnitine acyl transferase where they are met by CPT II which converts fatty acyl-carnitines back to fatty acyl-CoAs prior to their oxidation (McGarry and Brown, 1997). Inside the mitochondria, the fatty acyl-CoA enters the β -oxidation spiral which cleaves two carbons in the form of acetyl-CoA from the fatty acid tail during each passage. This process is catalyzed by four enzymes; 1) acyl dehydrogenase which dehydrogenates the bond between C2 and C3, 2) enoyl-CoA hydratase which hydrates the double bond between C2 and C3, 3) β -hydroxy-CoA dehydrogenase (HOAD) which dehydrogenates the newly formed alcohol group, and 4) thiolase which cleaves the thioester bond releasing a two-carbon acetyl-CoA molecule from the fatty acid, and adds a new CoA molecule to the tail. This new shorter fatty acid then continues to cycle through this process to release acetyl-CoA. Acetyl-CoA enters into the tricarboxylic acid cycle to eventually generate ATP.

Nutritional, hormonal and molecular control of lipolysis

The rate of lipolysis is greatly dependent on the energetic and nutritional state of the tissue and can be regulated through hormonal and molecular signaling. The volume of consumption (feeding or fasting) and composition of carbohydrates and lipids in the diet will greatly determine the rates of lipid oxidation. For example, during fasting many of the enzymes involved in lipogenesis, such as ACC and pyruvate dehydrogenase become downregulated when compared to a fed animal (Nepokroeff et al., 1974). Concurrently, the activities of many lipid oxidizing enzymes such as CPT I are increased (Drynan et al., 1996). Diets high in carbohydrates will stimulate lipogenesis while the addition of polyunsaturated fats (PUFAs) to the diet will reduce the rate of many lipogenic enzymes in the liver (Hillgartner et al., 1995). This phenomenon occurs even if the PUFAs are added to a high carbohydrate diet, indicating that it is a specific inhibition by the PUFAs and not related to a reduction in carbohydrates in the diet.

These changes in diet are thought to be communicated to tissues either through the circulating levels of the fuels themselves (glucose/fructose or PUFAs) or through the stimulatory effect of hormones activated by the diet. Two of the main target hormones are insulin and glucagon. Insulin levels in the blood are higher during the fed state,

particularly if the diet is high in carbohydrates, and the level of insulin correlates positively with the rate of fatty acid synthesis (Cahill et al., 1966). Conversely, in the starved state, or after meals with little to no carbohydrates, glucagon is elevated in the blood and there is an increase in lipolysis. Much of the evidence for the role of insulin and glucagon as hormonal regulators of lipid metabolism has come from research on type II diabetes mellitus, a condition characterized by decreased insulin and increased glucagon in the blood. In this state, as well as during starvation, lipogenesis and the activity of many lipogenic enzymes are markedly reduced.

Recently, metabolites such as fatty acids have also been postulated to act as signaling molecules within the tissues. Increased fatty acids in the blood, an indicator of starvation or exercise, will inhibit many lipogenic enzymes (Clarke et al., 1976). This process appears to be mediated partly by the inhibition of ACC and fatty acid synthase which greatly reduces the rate of fatty acid synthesis. Concomitantly, fatty acids will also act to stimulate the expression of enzymes in the lipid oxidation pathway (Price et al., 2000).

The cellular and molecular regulation of lipid oxidation can be both rapid and gradual depending on the duration of the metabolic disturbance (McClelland, 2004). Acutely, it can be regulated by adjusting the flux through the lipid oxidation pathway. This can be achieved through either covalent modulation such as phosphorylation/dephosphorylation or through allosteric modulation. Many of the enzymes involved in lipid metabolism are subject to phosphorylation/dephosphorylation and this is one of the main mechanisms to regulate lipid oxidation. For example, during starvation, many of the important enzymes involved in lipogenesis (ACC, pyruvate dehydrogenase) are phosphorylated causing their inhibition, while feeding will cause their dephosphorylation and an increase in activity (Hillgartner et al., 1995). FAT/CD36 which controls entry of fatty acids into the cell and possibly the mitochondrial outer membrane is covalently modulated by AMP kinase (AMPK) (Chabowski et al., 2006). AMPK has also recently been found to modulate a variety of other enzymes and transcription factors and has been termed one of the cell's major controls for energy homeostasis (Hardie, 2008). Additionally, there are many metabolic intermediates which act as either positive or negative allosteric modulators of enzymes along lipid metabolism pathways. For example, citrate acts positively on ACC during the production of lipids, while malonyl-CoA, the product of ACC activity, acts negatively on CPT I to prevent the oxidation of newly formed fatty acids (McGarry et al., 1978).

A chronic demand for an increase in the rate of lipolysis can result in regulation via changes in mRNA or protein expression when allosteric or covalent modulation alone cannot meet the demand. For some lipid metabolizing proteins, particularly in fish, major changes tend to take place at the transcriptional level via changes in mRNA expression versus changes in protein activity (Hughes, 1994; Robinson-Rechavi et al., 2001a; Robinson-Rechavi et al., 2001b). These changes are generally stimulated by hormones (as indicated above) and facilitated through transcription factors which can bind to regulatory elements of the genes. Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins which act as transcription factors for many metabolic genes, specifically lipid metabolism genes. For example, during fasting or intake of diets high in

PUFAs, PPARs will induce mRNA expression of a host of genes involved in lipid oxidation such as FAT/CD36, CPT I, HOAD, etc. (Price et al., 2000). Alternatively, there may be a host of post-transcriptional modifications such as splice variants, changes in translation and cellular trafficking of enzymes which may also mediate rates of lipid metabolism.

Carnitine palmitoyltransferase (CPT) system

While there are many regulatory sites along the pathway to oxidation, the CPT system is one of the most highly regulated and rate-limiting aspects. As mentioned earlier, the CPT system controls the entry of fatty acids into the mitochondria for oxidation (Fig. 1.2). In the intermembrane space, CPT I converts fatty acyl-CoA into fatty acyl-carnitine which can then be transported across the inner mitochondrial membrane by carnitine acyl-transferase (CAT). Once inside the mitochondria, CPT II converts the fatty acyl-carnitine back to fatty acyl-CoA which can proceed into the β -oxidation cycle. This system has received a great deal of attention due to its regulatory role in lipid metabolism, however, much of this research has come from humans and small mammals. In particular, CPT I is unique not only in its structure and function, but also how it is regulated and how it responds to physiological stressors and changes in fuel demand. In contrast, the role of CPT II is not rate-limiting and is not subjected to strict regulation like CPT I.

CPT I Structure and Function

CPT I is an 88kDa membrane bound enzyme which is located on the outer mitochondrial membrane. It has two transmembrane domains within the first 130 amino acids and both the amino and cytosolic termini protrude into the cytosol (Fig. 1.2). The catalytic site of CPT I is located on the inner side of the protein facing the intermembrane space (Murthy and Pande, 1987). CPT I catalyzes the rate-limiting formation of acyl-carnitines in the intermembrane space. There are two allosteric inhibitor binding sites on CPT I; a low affinity site near the catalytic core which does not have a large impact on regulation, and a high affinity site facing the cytosol, which is involved in CPT I regulation by M-CoA (Morillas et al., 2002). In mammals, there are two CPT I isoforms; CPT I α which is expressed in the liver and CPT I β which is expressed in the heart and skeletal muscle (McGarry et al., 1983). These two isoforms differ greatly in their kinetic properties. Most notably, the K_m for carnitine in liver CPT I is $\approx 30\mu\text{M}$ while muscle is $\approx 500\mu\text{M}$ (McGarry and Brown, 1997). These two isoforms also differ in their sensitivity to M-CoA where the muscle is ~ 100 times more sensitive than the liver (McGarry and Brown, 1997). In fish, there has only been one CPT I gene identified which appears to be similar to CPT I α in mammals (Gutières et al., 2003). CPT I activity in fish was higher in red muscle and heart than in liver or white muscle and sensitive to M-CoA in all tissues tested (Gutières et al., 2003). The limited data available on kinetics or inhibitory properties of CPT I in fish suggest that the IC_{50} in red muscle is $\sim 1\mu\text{M}$ (Rodnick and Sidell, 1994). However, there are no data available for other tissues.

CPT I regulation

The regulation of CPT I activity is complex and involves both genomic and non-genomic mechanisms. Not only is CPT I transcriptionally regulated, it is also allosterically modulated by M-CoA (Murthy and Pande, 1987), sensitive to changes in pH (Bezaire et al., 2004) and membrane fluidity (Zammit et al., 1997) and may also undergo covalent modulation (Harano et al., 1985). The diversity of regulatory mechanisms allows for precise control of CPT I activity within tissues.

In mammals, CPT I α and β expression is restricted to liver and muscle tissues, respectively, with the exception of the heart during development. This expression pattern automatically dictates overall function of CPT I within a given tissue. While the isoform expression does not change within the tissue, there is a possibility that there are splice variants or post-translational modifications taking place. For example, rat red muscle expresses a M-CoA insensitive splice variant, however, the control of this expression has yet to be elucidated (Kim et al., 2002). Total CPT I mRNA expression is partly controlled by PPARs. In mammals, CPT I contains a PPAR response element (PPRE). There are three identified PPAR isoforms in mammals; α , β and γ . Of these three, PPAR α and PPAR β are involved in lipolysis, while PPAR γ is involved in adipocyte differentiation. PPAR α is expressed in highly oxidative tissues such as liver, heart and skeletal muscle, while PPAR β is expressed ubiquitously in all tissues (Batista-Pinto et al., 2005; Braissant and Wahli, 1998; Kliewer et al., 1994). PPAR α can be activated by a wide array of fatty acid ligands to induce the expression of several key metabolic genes involved in lipid metabolism including CPT I (Desvergne and Wahli, 1999). Much less is known about PPAR β , however, there is evidence that PPAR β promotes lipid storage (Peters et al., 2000) and inhibits PPAR α (Leaver et al., 2007). Moreover, recent evidence from humans suggests it induces the expression of genes involved in lipid oxidation similarly to PPAR α (Dressel et al., 2003; Wang et al., 2003). In contrast to mammals, relatively little is known about the function of PPARs in non-mammalian vertebrates such as fish.

M-CoA, as mentioned above, is formed during the first committed step of *de novo* fat synthesis and will inhibit CPT I. This unique and simple system provides a mechanism to prevent the oxidation of newly formed fatty acids in the liver. Liver CPT I is thus under strict control via this mechanism. In muscle, where lipogenesis is low, M-CoA appears to be present only to control CPT I and not as a result of lipogenesis (McGarry et al., 1983). Muscle M-CoA concentration is regulated by the activity of acetyl-CoA carboxylase (ACC) and M-CoA decarboxylase (MCD) rather than nutritional status or energetic demand (Alam and Saggerson, 1998).

In addition to the M-CoA concentration in the tissue, the binding affinity of M-CoA to CPT I can also change. The difference in IC₅₀ between muscle and liver in mammals can either be attributed to differences in the protein sequence or changes in mitochondrial membrane fluidity. There has been a great deal of research into the amino acid sequence of mammalian CPT I, and multiple amino acids have been identified as being influential to the binding on M-CoA (Jackson et al., 2000; Morillas et al., 2003; Shi et al., 2000). In most cases, these experiments examined liver CPT I, with very little research on muscle CPT I. This research has predominantly focused on the amino acids

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within the amino- and carboxy- termini, as their interaction has been implicated in M-CoA sensitivity. In most cases, the effects of artificial mutations on M-CoA sensitivity have been investigated, but there has been no systematic determination of differences in these amino acids between CPT I isoforms and how this may affect M-CoA sensitivity. Aside from conformational changes caused by amino acids, the interaction of the amino- and carboxy- termini can be affected by the mitochondrial membrane fluidity (Zammit et al., 1997). Increases in membrane fluidity have been shown to disrupt the intramolecular interaction of the CPT I termini and reduce M-CoA sensitivity *in vitro* (Faye et al., 2005). This is presumed to also occur in rat liver *in vivo*, as changes in membrane fluidity have been correlated with decreases in M-CoA sensitivity (Zammit et al., 1998). This interaction has not yet been as extensively studied in muscle CPT I, although a similar interaction has been shown *in vitro* (Faye et al., 2005).

Changes in cellular pH and the phosphorylation state of CPT I have also been implicated as regulators of CPT I, although to a lesser extent than transcriptional and allosteric regulation. A decrease in pH will reduce CPT I activity at least in humans (Bezaire et al., 2004; Starritt et al., 2000). This phenomenon has received little attention in other animals and may only be a factor during exercise when cellular pH tends to drop (Starritt et al., 2000). Similarly, the role of phosphorylation/dephosphorylation has not been extensively studied. This phenomenon was first noted by monitoring ^{32}P in association with CPT I activity (Harano et al., 1985). Subsequently, it was noted that inhibition of kinases also increased the activity of CPT I (Wang et al., 1998; Yamagishi et al., 2001), although it may only be the dephosphorylation of other cytoskeletal components and not CPT I directly (Hoppel, 2005).

Physiological parameters affecting the regulation of CPT I

The multitude of regulatory mechanisms imposed on CPT I function are affected differently depending on the physiological state and energetic demand of the tissues. Of particular importance for lipid metabolism are diet, fasting, exercise, temperature and hypoxia. Much of what we know about the regulation of CPT I has come from humans and other mammals (rats and mice) due to the relevance of CPT I in many human diseases. Research on other non-mammalian vertebrates such as fish is lacking, although recently it has become prominent due to declining fish populations and aquaculture.

Diet. As mentioned previously, the macromolecular composition of the diet (carbohydrates, lipids, proteins) as well as the type of ingested lipids will affect rates of lipid metabolism (Iossa et al., 2002). These changes are partly due to the effect of diet on CPT I regulation. Dietary lipids exert their force on CPT I regulation at a number of levels; most notably through transcriptional regulation and their effects on mitochondrial membrane fluidity. Dietary PUFAs are known inducers of PPAR α , and promote stimulation of CPT I, at least in mammals (Price et al., 2000). This interaction is part of the larger role of PPAR α to induce the expression of a multitude of genes involved in fatty acid oxidation (Desvergne and Wahli, 1999; Price et al., 2000). A high fat diet alone, regardless of the composition has also been found to induce CPT I expression in rats (Tabarin et al., 2005; Thumelin et al., 1994) most likely through the action of PPARs,

although this increase in expression is maintained when PPAR α is abolished suggesting alternate mechanisms (LeMay et al., 2005).

Membrane fluidity can also be affected by composition of the diet, in particular by the unsaturation index of the fatty acids and by the specific type of phospholipid in the membrane. Two of the major phospholipids that contribute to change in membrane fluidity are phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Increases in PE promotes a more fluid membrane, while increases in PC tend to stabilize the membrane by promoting a lamellar formation (Hazel, 1995). Therefore, a change in the ratio of PE:PC is often used as an indicator of membrane fluidity. These changes in fluidity have been noted in both the plasma and mitochondrial membranes (Guderley et al., 2008; Robin et al., 2003). There have been few investigations where the effects of diet on both CPT I activity and membrane fluidity have been studied, and in these few instances the response has been controversial (Brady et al., 1989; Power et al., 1994; Wong et al., 1984). Experimental design and the specific composition of the diet are extremely important in these instances and may be a contributing factor to the controversial results.

Fish frequently encounter changes in their diet, either due to seasonal changes in food availability or changes in habitat. More recently, with the advancement of aquaculture, the effect of diet has become increasingly important in many salmonid species. Despite this, relatively little is known about the effects of diet on lipid metabolism, rather, research has been focused on growth and feed conversion efficiency (Bureau et al., 2008). It is unknown whether changes in the fatty acid composition of the diet will affect fish in the same manner as mammals. Furthermore, the majority of research has been focused on the liver, while muscle has received little attention.

Fasting. Just as changes in the composition of the diet will affect CPT I regulation, so will the cessation of food consumption. In most animals, periods of fasting will induce greater rates of lipid oxidation in order to spare limited glucose/glycogen resources (Neumann-Haefelin et al., 2004). Fasting will induce the release of NEFAs from adipose tissue causing an increase in blood and cellular NEFA concentration similar to those found in high fat diets. As mentioned previously, NEFAs will activate PPAR α and induce expression of CPT I (Price et al., 2000). Circulating NEFAs may also reduce the rate of lipogenesis by inhibiting ACC and fatty acid synthase, at least in isolated mammalian hepatocytes (Goodridge, 1973). Subsequently, the inhibition of ACC will prevent the formation of M-CoA, allowing relief from inhibition of CPT I. Fasting is also known to increase mitochondrial membrane fluidity in mammalian livers (Zammit et al., 1998; Zammit et al., 1997) and has been associated with a decrease in sensitivity to M-CoA (Drynan et al., 1996; Zammit et al., 1998).

Some fish species naturally experience extended bouts of fasting during their lifetime. For example, during spawning migrations, salmonids cease feeding at the onset of migration which can last for weeks. Spawning salmonids are a natural model for the study of lipid metabolism during fasting. In spite of this, there remains little research on fasting in fish species. The mechanisms of regulation of CPT I such as M-CoA and membrane fluidity appear to be present in fish and may potentially play the same role during fasting.

Exercise. Exercise induces a host of cellular and mitochondrial changes in order to meet energetic demands. For most muscle tissues, lipids are the primary fuel to generate the necessary energy for muscle function. To this end, there is a large increase in mitochondrial density within the tissues to increase the oxidative capacity of the tissue (Moyes and Hood, 2003). The associated increase in oxidative metabolism will cause a local cellular acidosis. A drop in pH (from 7.1 to 6.8) has been known to reduce CPT I activity (Bezair et al., 2004). This decrease in CPT I activity with a drop in pH associated with exercise may seem counterintuitive, as it would be beneficial to increase CPT I activity during exercise to increase lipid oxidation. However, this drop in pH generally only occurs during high intensity exercise (Sahlin et al., 1976), a state which is known to cause preferential oxidation of carbohydrates over lipids (McClelland et al., 1994; McClelland, 2004).

Exercise instigates adjustments in many metabolites and metabolic intermediates. Of these, changes in M-CoA concentration appear to be the most important for the regulation of CPT I. Changes in M-CoA during exercise have remained controversial and appear to be species specific. For example, there is a decrease in M-CoA levels in rats (Winder et al., 1995) and trout (Richards et al., 2002a) but not in humans during exercise (Odland et al., 1996) or after exercise training (Starritt et al., 2000). Prolonged exercise also induces a reduction in CPT I sensitivity to M-CoA (Holloway et al., 2006). The location of CPT I in the mitochondrial membrane may also be altered during exercise. Only recently has the presence of FAT/CD36 been observed in mitochondrial membranes and been associated with fatty acid uptake at the mitochondria (Campbell et al., 2004). During exercise, FAT/CD36 and CPT I co-localize to the mitochondrial membrane. This co-localization promotes an increase in lipid transport across the mitochondrial membrane in close association with CPT I which limits intra-mitochondrial movement of the fatty acids.

Low intensity, but not high intensity aerobic exercise stimulates an increase in CPT I in both red and white muscle of mammals (Hildebrandt et al., 2003). Interestingly, this increase in CPT I mRNA occurred in red muscle after short (45 mins) and long (120 mins) bouts of exercise, but only after 120 mins of exercise in white muscle. There is also evidence that PPAR α mRNA expression increases in human skeletal muscle during exercise and is maintained during recovery (Pilegaard et al., 2000). Increases in NEFA concentrations, a consequence of exercise, will provide fatty acid ligands to activate PPAR α and stimulate CPT I expression.

Temperature. Mammalian cellular temperature is highly regulated and rarely fluctuates from its normal temperature. On the contrary, ectothermic animals maintain body temperature near their ambient temperature, and thus, as the ambient temperature changes, as does the cellular temperature. This change in temperature will ultimately affect cellular enzyme activity and membrane fluidity. A decrease in temperature will increase the order of a membrane, thereby reducing fluidity. Membrane bound enzymes tend to lose function as the membrane becomes more ordered and makes it difficult for conformational changes to take place. To counteract this problem many ectothermic animals such as fish will employ homeoviscous adaptation. This process induces changes

in cellular membranes towards higher a amount of PUFAs (low order) in order to maintain fluidity and enzyme function (Hazel, 1995).

CPT I is a membrane bound enzyme that is subject to changes in temperature. Previous data shows that decreasing temperature causes an increase in CPT I activity in red muscle (Rodnick and Sidell, 1994). This may indicate that there are changes in mitochondrial membrane composition although this was not confirmed. Concurrently, increases in fluidity of the membrane may reduce the sensitivity of CPT I to M-CoA further increasing CPT I activity. Moreover, increases in CPT I activity may be a result of the increases in the number of mitochondria or increases in CPT I per milligram of mitochondrial protein. There is little known about the consequences of temperature change on expression of genes encoding lipid oxidizing proteins such as CPT I.

Hypoxia. In contrast to the other physiological states, hypoxia induces a reduction in fatty acid oxidation. Fatty acid oxidation requires a greater amount of oxygen per mole ATP than carbohydrates, therefore, during hypoxia when oxygen is limited, it would be beneficial to maximize ATP yield per oxygen consumed. This problem becomes exacerbated when exercising in hypoxic conditions. Very little is known about the mechanisms which may support this theory, and results have been conflicting (McClelland, 2004). To date there has been very few studies on the effects of hypoxia on CPT I activity. Interestingly, what is known is that hypoxia reduces the PPAR α induced expression of CPT I in cardiac myocytes which may be one mechanism to reduce fatty acid oxidation during hypoxia (Huss et al., 2001). Much of this research has come from humans and mammals acclimated to hypoxia or high altitude. No research has investigated the effects of hypoxia on fish lipid metabolism, particularly CPT I although there is evidence in zebrafish embryos that hypoxia will causes changes in many metabolic genes (Ton et al., 2003).

The regulatory mechanism used to control CPT I are highly dependent on the physiological state of the animal and whether the stress is of environmental or energetic origins. To date, what we know about the role and regulation of CPT I stems from human and other mammalian research, and has primarily focused on the liver as this tissue is a metabolic hub for energy homeostasis. In this thesis, I will expand this knowledge to non-mammalian vertebrates (fish) with a focus on both liver and muscle tissue from an energetic perspective. Fish were chosen as a model animal for their anatomically distinct muscle groups and the fact that they frequently encounter the above listed physiological stresses in nature. The information generated will provide a comprehensive view of the control of CPT I and its role in lipid metabolism in fish, which may be quite different from mammals.

Objectives

The overall objective of this thesis is to investigate the regulatory mechanisms used to control CPT I during different physiological states. To achieve this, both genomic and non-genomic regulatory mechanisms will be examined and related to the overall capacity for lipid oxidation between tissues. As a first step, the known regulatory

mechanisms described in mammals will be investigated under routine conditions across liver and muscle tissues in rainbow trout. From here, the response of the lipid oxidation pathway to physiological disturbances can be evaluated. Therefore, the specific objectives of this thesis are to:

- 1) Examine the inter-tissue differences in the capacity for mitochondrial lipid oxidation and CPT I activity and regulation under routine conditions.
- 2) Investigate the effects of membrane composition on CPT I regulation using high polyunsaturated or saturated fatty acid diets.
- 3) Determine the presence of multiple CPT I isoforms across tissues and investigate their genomic and protein structure and tissue expression during development and routine conditions
- 4) Investigate the metabolic and genomic preparations for the increase in fatty acid oxidation associated with the combined fasting and endurance exercise during spawning migration in salmon.
- 5) Investigate the individual effects of both fasting and chronic exercise on CPT I regulation and the capacity for lipid oxidation in trout and compare this with the combined effects in migrating salmon.

Chapter Summary

Until now, little was known concerning the regulation of mitochondrial lipid oxidation in fish, despite their prominent use of lipids as fuel. In particular, the differences in this regulation between tissues such as liver and muscle under routine conditions were not known. This information is the basis for determining how these tissues respond when faced with physiological conditions requiring an increase in lipid oxidation to fuel metabolic demands. **Chapter 2** identifies the regulatory mechanisms employed by fish liver and muscle mitochondria to control CPT I and also highlights the inter-tissue differences in the capacity for lipid oxidation.

Chapter 3 further investigated the effects of mitochondrial membrane composition on CPT I function through changes in dietary fatty acid content. Fatty acids are a known inducer of transcription factors for CPT I, therefore, the effects of diet on genomic regulation of CPT I were also investigated.

Results from chapters 2 and 3 instigated the evaluation of the phylogeny and isoform expression of CPT I in **Chapter 4**. Specifically, five CPT I isoforms were identified and their mRNA expression determined in multiple tissues under routine conditions. The mRNA expression of these isoforms during development was also examined during the transition from endogenous yolk sac nutrients to free feeding juvenile and adult fish. Furthermore, the protein structure of these isoforms was explored to identify potential amino acids which may affect CPT I inhibition by M-CoA.

In **Chapters 5 and 6** a comparative approach is used to study the regulation of lipid metabolism in red and white muscles during spawning migration of pacific salmon. Specifically, the genomic and metabolic control of lipid metabolism was examined at four sites along the migration route to determine how these fish respond to the large energetic demand imposed by chronic exercise and fasting. Chronic exercise and fasting were then investigated individually in rainbow trout to determine their individual effects on the regulation of lipid metabolism.

Chapter 7 concludes this thesis with a general discussion of the significant results and a comparison of the major regulatory mechanisms of CPT I and lipid metabolism between tissues and taxa. To finish, the perspectives and future avenues for research in this area are discussed.

Fig. 1.1

The pathway for fatty acid transport from lipid storing adipose tissue to the mitochondria for oxidation. For fatty acids to be broken down to provide ATP, the triacylglycerols within the adipocyte are first broken down into glycerol and non-esterified fatty acids (NEFAs). NEFAs are transported into the circulation via CD36/fatty acid binding protein plasma membrane(FABP_{pm})/fatty acid transport protein (FATP). In the circulation, NEFAs are bound to albumin and transported to the necessary tissues. At the tissue, NEFAs are transported into the cell via CD36/FABP_{pm}/FATP. Inside the cell, NEFAs are transported to the mitochondria by FABPs. NEFAs may also come from intracellular lipid stores and be carried to the mitochondria by FABP. At the mitochondrial, NEFAs are transported across the outer and inner mitochondrial membranes by CD36/CPT. Fatty acids then enter the β -oxidation pathway to generate acetyl-CoA. Acetyl-CoA then enters the tricarboxylic acid cycle which generates the necessary reducing equivalents for the electron transport chain to produce ATP.

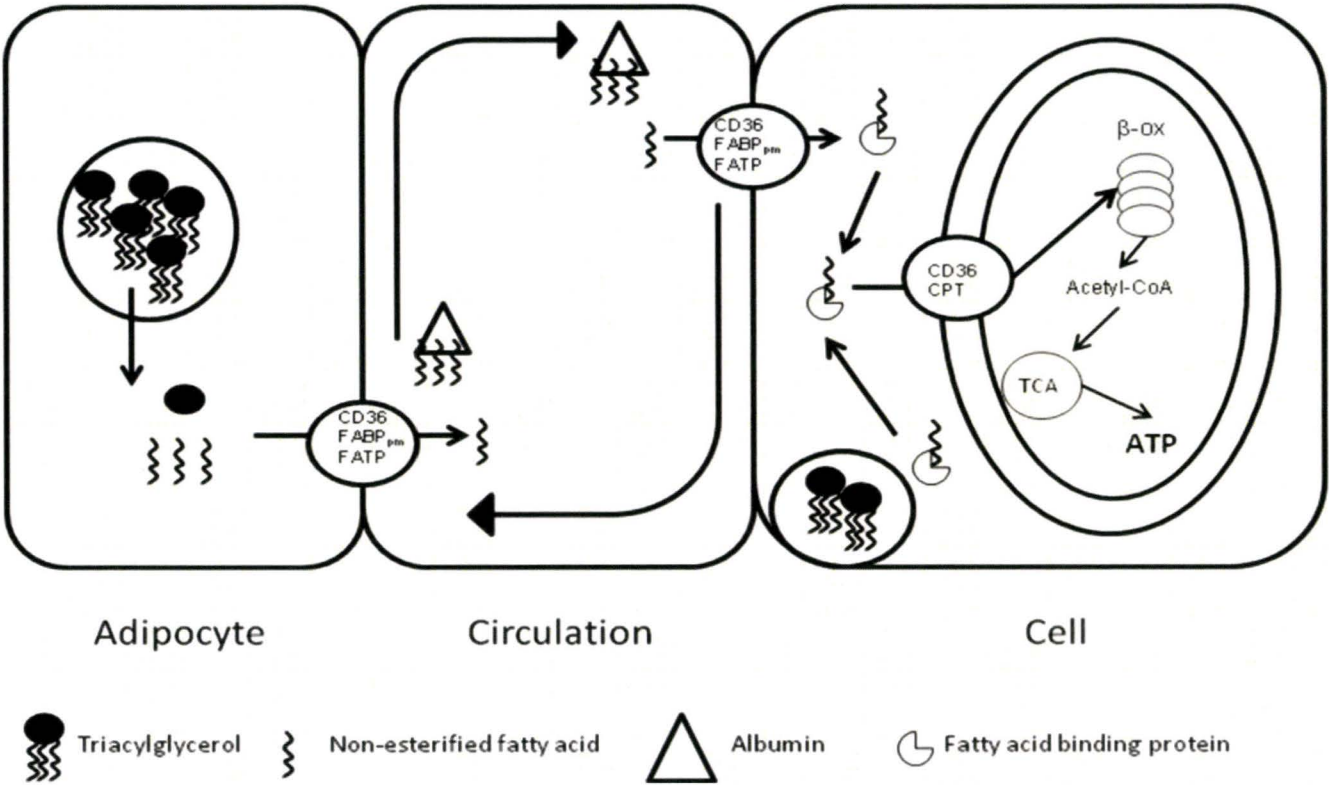
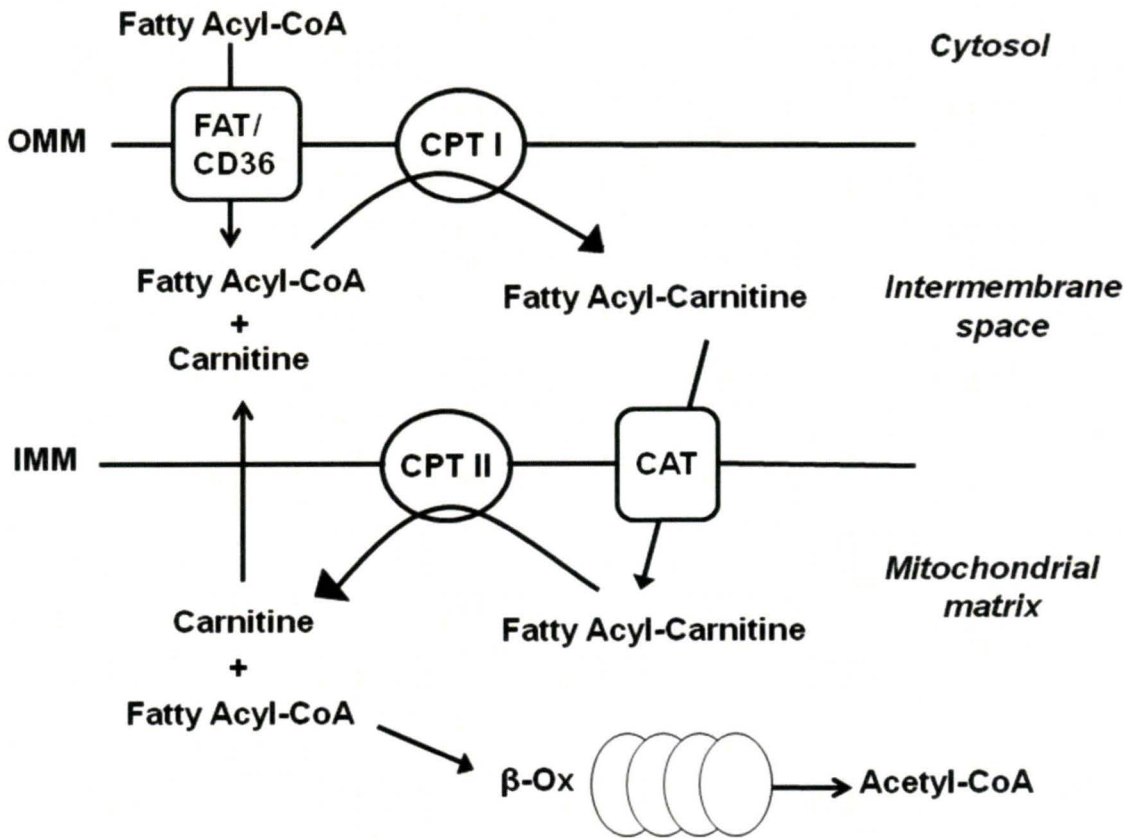


Fig. 1.2

The carnitine palmitoyltransferase (CPT) system. Fatty acyl-CoAs in the intermembrane space are converted to fatty acyl-carnitines by CPT I. Fatty acyl-carnitines are transported across the inner mitochondrial membrane (IMM) and converted back to fatty-acyl-CoAs by CPT II in the mitochondrial matrix. The fatty acyl-CoAs then enter the β -oxidation cycle and generate acetyl-CoA.



INTERTISSUE REGULATION OF CARNITINE PALMITOYLTRANSFERASE I
(CPT I): MITOCHONDRIAL MEMBRANE PROPERTIES AND GENE EXPRESSION
IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

Abstract

Carnitine palmitoyltransferase (CPT) I is regulated by several genetic and non-genetic factors including allosteric inhibition, mitochondrial membrane composition and/or fluidity and transcriptional regulation of enzyme content. To determine the intrinsic differences in these regulating factors that may result in differences between tissues in fatty acid oxidation ability, mitochondria were isolated from red, white and heart muscles and liver tissue from rainbow trout. Maximal activity (V_{max}) for β -oxidation enzymes and citrate synthase per mg tissue protein as well as CPT I in isolated mitochondria followed a pattern across tissues of red muscle>heart>white muscle>liver suggesting both quantitative and qualitative differences in mitochondria. CPT I inhibition showed a similar pattern with the highest malonyl-CoA concentration to inhibit activity by 50% (IC_{50}) found in red muscle while liver had the lowest. Tissue malonyl-CoA content was highest in white muscle with no differences between the other tissues. Interestingly, the gene expression profiles did not follow the same pattern as the tissue enzyme activity. CPT I mRNA expression was greatest in heart>red muscle>white muscle>liver. In contrast, PPAR α mRNA was greatest in the liver>red muscle>heart>white muscle. There were no significant differences in the mRNA expression of PPAR β between tissues. As well, no significant differences were found in the mitochondrial membrane composition between tissues, however, there was a tendency for red muscle to exhibit higher proportions of PUFAs as well as a decreased PC:PE ratio, both of which would indicate increased membrane fluidity. In fact, there were significant correlations between IC_{50} of CPT I for malonyl-CoA and indicators of membrane fluidity across tissues. This supports the notion that sensitivity of CPT I to its allosteric regulator could be modulated by changes in mitochondrial membrane composition and/or fluidity.

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Introduction

The regulation of lipid metabolism is complex, and is currently an extensive area of research. Although there is keen interest into pathology aspects of lipid metabolism, there are still many unanswered questions regarding the normal regulation of cellular lipid oxidation. In general terms, it has been recognized that mitochondrial fatty acid oxidation is regulated by both genetic and various other non-genetic cellular mechanisms that can affect fat entry into, and oxidation by mitochondria (for review see (McClelland, 2004)). For instance, a key enzyme in mitochondrial β -oxidation, carnitine palmitoyltransferase (CPT) I, is transcriptionally regulated in mammals, but this enzyme also experiences allosteric modulation (Murthy and Pande, 1987), is sensitive to changes in cellular pH (Bezaire et al., 2004), and possibly undergoes covalent modulation (Harano et al., 1985). Currently it is unclear how these regulatory mechanisms affect CPT I differently and if regulation is similar across tissues with varying rates of fatty acid oxidation or even if these mechanisms are evolutionarily conserved.

CPT I is located on the inner side of the outer mitochondrial membrane and catalyses the conversion of acyl-CoA to fatty acylcarnitine (Kerner and Hoppel, 2000). Fatty acyl-carnitine can then be transferred into the mitochondrial matrix by carnitine–acyl-carnitine translocase (CAT) and converted back to fatty acyl-CoA by the enzyme CPT II. Only acylcarnitines can be transferred across the inner mitochondrial membrane, thus, CPT I is thought to be a major regulating step in mitochondrial fat oxidation (Kerner and Hoppel, 2000). CPT I is allosterically modulated by malonyl-CoA (M-CoA), which is produced during the first step of de novo fatty acid synthesis by acetyl-CoA carboxylase (McGarry and Brown, 2000). This is a simple mechanism that prevents the oxidation of newly synthesized fats in the liver (McGarry and Brown, 2000). In the muscle, however, where fatty acid synthesis rates are low, M-CoA is thought to be present solely as a regulator of CPT I (McGarry and Brown, 1997).

Due to regulatory role of CPT I, changes in its abundance, activity, or substrate kinetics, as well as the concentration of M-CoA can lead to changes in overall rates of fatty acid oxidation. In humans and other mammals, there are two isoforms of the CPT I enzyme, encoded for by two different genes; L-CPT I (or CPT I α) which is dominant in the liver, and M-CPT I (or CPT I- β) which is dominant in skeletal muscle (a brain isoform has also been identified) (Britton et al., 1997). Both α and β isoforms are expressed in the heart but at different stages of development in mammals (Brown et al., 1995). McGarry and Brown have shown that in rats, these two isoforms vary greatly in their kinetics. They have also shown that the two isoforms differ significantly in their sensitivity to M-CoA with M-CPT I being approximately 100 times more sensitive than the liver isoform (McGarry and Brown, 1997).

CPT I has not been studied as extensively in non-mammalian vertebrates. For example, there is limited information on CPT I activity or gene expression in different tissues from fish. In rainbow trout CPT I has been found to be expressed in various tissues including liver and muscle, however, it has been suggested that this species expresses only one CPT I gene which corresponds to the mammalian liver-type CPT I (Gutierrez et al., 2003). To our knowledge, there has neither been a systematic

determination of the sensitivity of CPT I for M-CoA across tissues in rainbow trout (*Oncorhynchus mykiss*), nor has the relative expression levels of CPT I been determined between various tissues. The mRNA expression of CPT I is thought to be influenced by the transcription factor peroxisome proliferator-activated receptor (PPAR), as it contains a PPAR response element (PPRE) (Price et al., 2000) at least in mammals. Hence, it will be important to determine the relative expression of PPARs across tissues.

Aside from allosteric modulation and changes in gene expression, mitochondrial membrane composition and fluidity have been proposed to play a role in CPT I regulation (Kolodziej and Zammit, 1990). Since CPT I is located in the outer mitochondrial membrane changes in the properties of the surrounding membrane can affect enzyme activity and kinetics. Maintaining a particular state of fluidity in membranes is necessary for several important cellular functions including ion transport and protein function, and is the basis for homeoviscous adaptation (Hochachka and Somero, 1984). Under changing environmental conditions, such as a decrease in temperature, ectothermic animals selectively incorporate long chain polyunsaturated fatty acids into their membranes to combat the effects of temperature on changes in membrane order. As well, other modifications such as the ratio between phospholipid classes phosphatidylcholine (PC) and phosphatidylethanolamine (PE) can also affect fluidity of the membrane due to their differential effects on membrane order (Hazel, 1995).

In vitro studies using rat liver mitochondria have demonstrated that changing the fluidity of the mitochondrial membrane does affect the activity of CPT I as well as its sensitivity to M-CoA (Kolodziej and Zammit, 1990). This has also been confirmed through in vivo studies using 48-h fasted rats and streptozotocin-induced diabetic rats, both of which show decreases in M-CoA sensitivity paralleled by increases in membrane fluidity as measured by DPH fluorescence anisotropy (Zammit et al., 1997). Little is known about these mechanisms in non-mammalian vertebrates. Fish, in particular naturally experience temperature and diet induced changes in membrane composition and fluidity (Hochachka and Somero, 1984). As well, fish offer many advantages for the study of muscle fibre-specific and tissue-specific regulation of lipid metabolism since they have anatomically separated ‘red’ and ‘white’ muscle masses. Previously, clear fibre type differences have been shown in muscle mitochondrial profiles of rainbow trout, including membrane fluidity (Leary et al., 2003) but the effect on CPT I kinetics have not been determined.

The purpose of this study was to evaluate cross-tissue variation in the capacity for fatty acid oxidation and to identify potential genetic and non-genetic factors by which it is regulated in rainbow trout (*Oncorhynchus mykiss*). More specifically, we sought to determine; 1) the non-genetic regulation of CPT I across tissues by examining differences in V_{max} , M-CoA sensitivity, M-CoA concentrations, and mitochondrial membrane composition, and 2) differences in constitutive transcription for CPT I and transcription factors important for the expression of genes for fat oxidizing enzymes.

Materials and Methods

Experimental fish

Rainbow trout, *Oncorhynchus mykiss*, (~500 g) were obtained from a local hatchery (Humber Springs, Orangeville, ON) and kept in 500 l tanks with circulating water at 12 °C on a commercial fish diet, Profishent Classic Floating Trout Grower (Martin Mills, Elmira, ON).

Mitochondrial isolation

Fish were killed by a blow to the head followed by severing of the spinal cord. Mitochondria were isolated from red muscle (RM), white muscle (WM), heart (H) and liver (L) from rainbow trout according to Suarez and Hochachka (Suarez and Hochachka, 1981) and Moyes et al. (Moyes et al., 1988). Each tissue was immediately excised (WM~15 g, RM~4 g, whole H and whole L) and placed in mitochondrial isolation buffer (MIB) consisting of (in mM) 140 KCl, 10 EDTA, 5 MgCl₂, 20 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 0.5% BSA (pH 7.0) for RM, WM and H, and 250 sucrose, 1 EDTA, 20 HEPES and 0.5% BSA (pH 7.4) for L, on ice. Tissues were diced, washed twice with fresh chilled MIB, and then homogenized 3 times, first using a wide clearance Teflon pestle on a chilled glass homogenizer, then 3 times with a narrow clearance Teflon homogenizer to lyse cells. Homogenates were centrifuged at 800 ×g for 10 min at 4 °C. For RM, H and L the supernatant was spun at 9000 ×g for 10 min at 4 °C. WM supernatant was first strained through 2 layers of cheesecloth and then spun at 800 ×g for 10 min at 4 °C, strained again through 4 layers of cheesecloth and spun again at 9000 ×g for 10 min at 4 °C. Supernatants for all tissues were then discarded and pellets were resuspended in a small volume of the appropriate MIB lacking BSA. The resuspended homogenate was collected into a 15 ml centrifuge tube and spun again at 9000 ×g for 10 min at 4 °C. The supernatant was discarded and the mitochondrial pellet was resuspended in an appropriate volume of MIB lacking BSA and kept on ice.

Enzyme and protein assays

All assays (except CPT I radioisotope assay) were performed in triplicate at room temperature using a Spectramax Plus 384 and clear 96-well flat bottom assay plates and data was analysed using Softmax Pro 4.7.1 software (Molecular Devices, Sunnyvale, CA).

CPT I assay (isotope)

Radioactive CPT I assays followed McGarry et al. (McGarry et al., 1983) for mammals and modified to use the assay conditions of Rodnick and Sidell (Rodnick and Sidell, 1994) to obtain CPT I V_{max} and IC₅₀. The assay buffer (pH 7.0) contained (in mM) 20 HEPES, 40 KCl, 1 EGTA, 220 sucrose, 0.1 DTT, 0.04 palmitoyl-CoA, 1 carnitine and 1.3 mg/ml BSA. 1 µCi/sample of L-[methyl-³H] carnitine hydrochloride (specific activity 82.0 Ci/mmol) (Amersham Biosciences, Quebec) was added and 70 µl of the assay mixture was placed in 1.5 ml Eppendorf tubes and incubated with 10 µl of 0.5–500 mM malonyl-CoA or water in place of mitochondria for blanks or in place of

malonyl-CoA and maximum activity. Tubes sat for 5 min at room temperature. The reaction was started by the addition of 20 μ l of mitochondria diluted 5 \times in MIB (\sim 2 mg/ml), and incubated at room temperature for 8 min. The reaction was stopped by the addition of 60 μ l of 1M HCl. The palmitoyl-[3 H]-carnitine was collected according to Starritt et al. (Starritt et al., 2000). 20 μ l of the assay mixture with L-[methyl- 3 H]carnitine hydrochloride was also counted in duplicate for determination of individual specific activity. Background counts were determined from a blank sample containing aqueous counting scintillation. The decays per minute (DPM) were read for 5 min per sample on a Tricarb 2900 TR Liquid Scintillation Analyzer (PerkinElmer) using QuantaSmart 1.31 (Packard Instrument Company) analysis software.

CPT I assay (spec)

MIB was used as the assay buffer and contained 0.1 mM 5,5' dithiobis (2-nitrobenzoic acid) (DTNB), 0.1 mM palmitoyl-CoA and 5.0 mM L-carnitine (omitted from control) \pm 50 μ M malonyl-CoA (pH 7.0 for RM, WM, and H; pH 7.4 for L) to determine V_{\max} . The reaction was started with 10 μ l of mitochondria (\sim 10 mg/ml). Solutions were mixed and DTNB absorbance read at 412 nm for 5 min. The IC_{50} was also determined using this method; however, the sensitivity was lower than the isotope method and thus yielded much higher IC_{50} values for all tissues (data not shown). Frozen tissue samples were powdered using a mortar and pestle chilled with liquid N_2 to determine whole tissue maximum enzyme activity under saturating conditions. Powdered tissue (50–100 mg) was homogenized in 20 volumes of an enzyme extraction buffer (20 mM HEPES, 1 mM EDTA and 0.1% Triton at pH 7.4) using a glass on glass homogenizer and enzyme assays were performed on this crude homogenate.

Long-chain acyl-CoA dehydrogenase (LCAD)

LCAD was assayed according to Davidson and Schulz (Davidson and Schulz, 1982). The assay contained (in mM) 100 potassium phosphate buffer (pH 7.6) with 0.028 2,6 dichlorophenolindophenol (DCPIP), 0.65 phenazine methosulfate (PMS), 0.2 N-ethylmaleimide and 0.45 KCN. The reaction was started with the addition of 0.1 mM palmitoyl-CoA and DCPIP absorbance was monitored at 600 nm. Control wells lacking palmitoyl-CoA were assayed to correct for background hydrolase activity.

β -hydroxy-acyl-CoA dehydrogenase (HOAD)

The assay followed McClelland et al. (McClelland et al., 2005) and contained (in mM) 50 imidazole (pH 7.4), 0.1 acetoacetyl-CoA, 0.15 NADH and 0.1% Triton 100-X and NADH absorbance was monitored at 340 nm. Controls lacking substrate were used to correct for background activity.

CPT II

The assay followed McClelland et al. (McClelland et al., 2005) and contained (in mM) 20 Tris buffer (pH 8.0), 0.1 DTNB and 5 L-carnitine. The reaction was started with the addition of 0.1 mM palmitoyl-CoA and DTNB absorbance was monitored at 412 nm. Control wells lacking carnitine were assayed to correct for thiolase activity.

Citrate synthase (CS)

CS assays followed McClelland et al. (McClelland et al., 2005). Whole tissue homogenates were frozen and thawed 3× using liquid N₂ and kept on ice until further use. As well, an aliquot of the 5× diluted isolated mitochondria used for the CPT I assay was diluted a further 5× using the enzyme extraction buffer (see above) and frozen and thawed 3 times using liquid N₂ and kept on ice. Isolated intact mitochondria in MIB were also assayed for CS. These three homogenates were used to determine the amount of intact mitochondria versus ruptured mitochondria in our CPT I preparations and to extrapolate CPT I enzyme activities to the tissue level. The CS assay buffer consisted of (in mM) 20 TRIS (pH 8.0), 0.1 DTNB and 0.3 acetyl-CoA. The reaction was started by the addition of 0.5 oxaloacetate and absorbance was monitored at 412 nm. Control wells lacking acetyl-CoA were assayed to correct for hydrolase activity.

Protein content

Protein concentrations were determined by the Bradford method (Bradford, 1976) using a commercial kit (Bio-Rad).

Malonyl-CoA (M-CoA) content

M-CoA concentrations were determined using a modified method from Richards et al. (Richards et al., 2002a). Briefly frozen tissue samples were powdered using a liquid N₂ cooled mortar and pestle, lyophilized for 24 h and kept at –80 °C until analysis. 50 mg of lyophilized tissue was homogenized at 4 °C for 20 s using a Teflon pestle in 200 µl of 0.5 M perchloric acid with 50 µM DTE and 10 mg/ml propionyl-CoA used as an internal standard. Homogenized samples were centrifuged at 20,000 ×g for 10 min at 4 °C and 200 µl of the supernatant was transferred and adjusted to pH 3 using 4 M NaOH while being vortexed. The sample was transferred to an autosampler vial containing 20 µl of MOPS (pH 6.8) and the final pH was determined (always less than 5). Autosampler vials were placed in a Waters 717 Plus autosampler (Waters, Mississauga, ON) at room temperature and M-CoA was separated using reverse-phase HPLC based on a method from Demoz et al. (Demoz et al., 1995). For RM, WM and L, 200 µl of the sample was injected onto a Zorbax ODS Rx C-18 column (25 cm×0.46 mm) (Agilent Technologies, Mississauga, ON). The elution gradient was created using a Waters Model 510 pump controller. Mobile phase A was 100 mM sodium phosphate and 75 mM sodium acetate in deionized water (pH4.6). Mobile phase B was the same as A except that it contained 30% CH₃CN. The elution gradient was as follows: 0 min, 90% A; 17 min, 50% A; and 17.6 min, 90% A. Baseline conditions were established after 5 min of 90% A. The flow rate was 1.5 ml/min and absorbance measurements were made at 254 nm on a Lambda Max 481 LC spectrophotometer (Waters, Mississauga, ON). For heart, the procedure was the same except that an extra column was added to the loop and the flow adjusted to 1ml/min to separate peaks co-eluting with the M-CoA peak. Peaks were manually identified by comparisons to known M-CoA standards and quantified using the internal standard (propionyl-CoA).

mRNA quantification by real-time PCR

Total RNA from each tissue was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) based on the acid guanidiniumthiocyanate-phenol-chloroform extraction method. Total RNA concentrations were quantified by UV spectrophotometry at 260 nm and diluted to 0.5 µg/µl. cDNA was synthesized using 1 µg of DNase (Invitrogen, Carlsbad, CA) treated RNA and SuperScript RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA). SYBR green (Bio-Rad, Mississauga, ON) with ROX as a reference dye was used for quantitative real-time PCR in 25 µl reactions using a Stratagene Mx3000P realtime PCR system. Each reaction contained 12.5 µl SYBR green mix, 1 µl each of forward and reverse primer (5 µM), 5.5 µl of DNase/RNase-free water and 5 µl of 5× diluted cDNA. Primers were designed using a CPT I sequence from rainbow trout liver (Gutierrez et al., 2003) (see Table 2.1 for specific primer sequences). The thermal program included 3 min at 95, 40 cycles of 95 for 15 s, 60 for 30 s and 72 for 30 s. A no-template control and dissociation curve was performed to ensure only one PCR product and stock solutions were not contaminated. Standard curves were constructed for each gene using serial dilutions of stock cDNA to account for any differences in amplification efficiencies. All samples were normalized to the housekeeping gene, EF1- α , which did not change between tissues. Primers were designed using Primer3 software (Rozen and Skaletsky, 2000).

Analysis of mitochondrial membrane phospholipid composition

Mitochondrial total lipid was extracted and phospholipids were analysed according to Gillis and Ballantyne (Gillis and Ballantyne, 1999), based on a modified protocol from Bligh and Dyer (Bligh and Dyer, 1959). Mitochondrial lipid extracts were dried and resuspended in 25 µl chloroform:methanol (2:1) and spotted along with a standard phospholipid mix (sphingomyelin (SPH), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and cardiolipin (CL)) (Sigma, Oakville, ON) onto silica gel 60 pre-coated 250 µM thick plates (Fisher Scientific, Ottawa, ON) for thin layer chromatography. The solvent system used to separate the phospholipids was chloroform: methanol:acetic acid:water (50:37.5:3.5:2, by volume). Once the solvent had run to within 5 cm of the top of the plate, they were removed, air dried, then sprayed with a saturated solution of 2,7-dichlorofluorescein and allowed to sit in a tank containing 25% ammonium hydroxide for 5 min. Plates were viewed under UV light and individual phospholipid fractions (SPH, PC, PS, PI, PE, CL) were scraped into individual Kimex tubes for saponification and methylation. Two ml of 6% sulphuric acid in methanol and 10 µl heptadecanoic acid as an internal standard (0.6 mg/ml, C17:0) were added to each fraction and incubated for 2 h at 80 °C. The samples were allowed to cool for 10 min and 1 ml of water and 2 ml of petroleum ether were added and the tubes vortexed. Samples were spun at 2000 rpm for 6 min and the top phase containing the fatty acid methyl ester (FAME) fraction was removed into a new tube and dried under N₂. The FAMES were resuspended in petroleum ether and transferred into autosampler vials for gas chromatograph analysis. 1 µl of each sample was injected using a 7683B series automatic injection system (Agilent Technologies) onto a Hewlett–Packard 6890N series gas chromatograph (GLC) (Agilent Technologies) equipped with

either an Innowax or a DB-23 (J&W Scientific) 30-m fused silica capillary column (Supelco, Bellefonte, PA) at 250 °C and followed the following temperature profile: initial oven temperature was kept at 160 °C for 4 min, ramped up to 220 °C at 2 °C/min, held at 220 °C for 16 min, ramped up to 240 °C at 10 °C/min and held for 2 min. Post-run was 130 °C for 6 min. The flow was 1.8 ml/min and the velocity through the column was 37 cm/s. GLC retention times were verified using two standards, PUFA No. 3 from menhaden oil and fatty acid methyl esters mix C4–C24 (Supelco, Bellefonte, PA). Identified fatty acids in each phospholipid class were compared to the known internal standard concentration and then summed for each sample. The percent contribution of each fatty acid was determined by adding the concentration of that fatty acid from each phospholipid class and then dividing by the total concentration of all fatty acids in all phospholipid classes for that sample. The percent contribution of each phospholipid class was determined by adding the total fatty acid concentration in each individual phospholipid class and dividing by the total concentration of all fatty acids in all phospholipid classes for that sample.

Statistical analysis

All statistical analyses were performed using SigmaStat (Systat Software Inc., San Jose, CA). One-way ANOVA and Tukey's tests were used to test for significance between tissues. Correlational analyses were performed using a linear regression. Significance level was set at $p < 0.05$.

Results

Cross-tissue variation in enzymatic activities

The apparent V_{\max} of citrate synthase (CS), long chain acyl-CoA dehydrogenase (LCAD), carnitine palmitoyltransferase (CPT) II and β -hydroxy acyl-CoA dehydrogenase (HOAD) from whole tissue homogenates were assayed as indicators of mitochondrial content and fatty acid oxidation potential. All four enzymes demonstrate the same pattern of activity across tissues; red muscle having the highest activity, followed by heart, then liver, and then white muscle (Fig. 2.1A–B). Red muscle CS was significantly higher than all other tissues ($p < 0.001$), as well, heart CS was significantly higher than liver and white muscle ($p < 0.001$). Red muscle and heart LCAD and HOAD were both significantly higher than liver and white muscle ($p < 0.001$) but not significantly different from each other. Red muscle ($p < 0.001$) and heart CPT II were significantly higher than liver ($p = 0.013$) and white muscle ($p < 0.001$). There were no significant differences between liver and white muscle for all of the enzymes measured. Total CPT activity was assayed in isolated mitochondrial preparations by spectrophotometry. Red muscle CPT had the highest activity at $26.3 \pm 4.0 \text{ nmol} \cdot \text{min}^{-1} \text{ mg mito protein}^{-1}$, which was significantly higher than liver ($7.8 \pm 0.7 \text{ nmol} \cdot \text{min}^{-1} \text{ mg mito protein}^{-1}$) ($p < 0.001$) and white muscle ($14.9 \pm 2.7 \text{ nmol} \cdot \text{min}^{-1} \text{ mg mito} \cdot \text{protein}^{-1}$, $p = 0.028$), but not significantly different from heart CPT activity ($16.7 \pm 1.4 \text{ nmol} \cdot \text{min}^{-1} \text{ mg mito protein}^{-1}$, Fig. 2.2). In the presence of high levels of an inhibitor (50 μM M-CoA), CPT activity declined in all tissues. In liver,

CPT was inhibited by 32% whereas in red, white, and heart muscles CPT was inhibited by 51%, 70% and 65%, respectively (Fig. 2.2). This M-CoA inhibitable activity can be considered as the maximal activity of CPT I.

Malonyl-CoA inhibition of CPT I

The concentration of malonyl-CoA required to inhibit 50% of the malonyl-CoA sensitive activity (IC_{50}) of CPT I was determined for each tissue using a range of M-CoA concentrations from 0.05 μ M–50 μ M. Inhibition curves are shown in Fig. 2.3 (A–D) and the average IC_{50} for each tissue in Table 2.2. Liver had an IC_{50} of 0.079 ± 0.037 μ M which is significantly lower than red muscle (0.55 ± 0.06 μ M) and heart (0.40 ± 0.10 μ M, $p=0.008$ and $p=0.05$, respectively), but not white muscle (0.37 ± 0.09 μ M, $p>0.05$).

Malonyl-CoA content

Tissue M-CoA content varied between tissues with WM having significantly higher amounts than all other tissues ($p<0.001$) (Table 2.3). There were no significant differences between RM, H, and L. Using citrate synthase as a marker of mitochondrial density of the tissues, we show the relationship between M-CoA content and mitochondrial content for each tissue (Table 2.3). WM ($p<0.001$) and L ($p=0.049$) had significantly higher M-CoA per mitochondria than RM and H which were not significantly different from each other.

Gene expression profiles

The constitutive mRNA expression of CPT I was very low in liver compared to other muscle tissues (Fig. 2.4). Liver expression of CPT I is significantly lower than heart expression ($p=0.007$), however, not significantly different from red and white muscles ($p>0.05$). PPAR α , a major transcription factor involved in expression of CPT I in mammals, followed an unexpected pattern. Although CPT I expression was lowest in the liver, the expression of PPAR α tended to be higher than all other tissues, however only significantly different from white muscle (Fig. 2.4). In contrast, no significant differences were seen between tissues in the relative expression of PPAR β mRNA.

Mitochondrial membrane composition profiles

Membranes were dominated mainly by the saturated fatty acid C16:0, comprising 34%, 31%, 33%, and 32% in liver, red muscle, heart and white muscle, respectively and polyunsaturated fatty acid (PUFA) C22:6n3, comprising 6%, 28%, 15%, and 10% respectively. There are quite large differences between tissues in the concentration of C22:6n3, where liver has a very low amount while red muscle is quite high, however due to high variability this was not a statistically significant difference (Table 2.4). Other fatty acids that made a substantial contribution to the membrane composition include C18:0, C18:1 and C20:5n3. The percentage of each phospholipid class in each tissue is presented in Table 2.5. As expected, levels of PC and PE were dominant in all tissues with CL also contributing a large percentage of overall phospholipid content. There are no major changes in the average percentages of the various types of fatty acids or in the unsaturation index (UI) in the different phospholipid classes between tissues. However,

there are considerable differences, although not significant, in the relative proportions of each phospholipid class between tissues (Table 2.5). Most notably, the concentrations of PC and PE vary between tissues. The liver has a higher proportion of PC (53%) compared to red muscle (44%), heart (49%) and white muscle (42%). PE also varies between tissues, with the highest proportion being in red muscle (36%). The ratio of PC:PE is relatively low in red muscle (1.23 ± 0.09) but not significantly different from other tissues (liver 2.07 ± 0.32 , heart 2.06 ± 0.32 , white muscle 2.02 ± 0.31). While there are no significant differences between the average values of the classes of phospholipids between tissues, when presented in relation to CPT I inhibition, there are significant correlations (see below). Sphingomyelin was found in trace amounts indicating low contamination with other cellular membranes (data not shown).

Relationship between IC_{50} and mitochondrial membrane composition

Significant correlations can be drawn from the current CPT I inhibition data and the mitochondrial membrane composition. There is a negative correlation between IC_{50} based on isotope determination and PC:PE ratio, indicating that as the PC:PE ratio increases, IC_{50} decreases, thus, increasing the sensitivity to M-CoA ($p=0.002$) (Fig. 2.5). Moreover, a correlation exists between IC_{50} and the percent of docosahexanoic acid (DHA, C22:6n3) in the membrane ($p=0.021$, Fig. 2.5). As the %DHA increases the IC_{50} increases, thus, decreasing the sensitivity to M-CoA.

Discussion

The regulation of CPT I and fat oxidation occurs by both genetic and non-genetic mechanisms. We found intrinsic differences in sensitivity of CPT I to the allosteric inhibitor M-CoA. Across tissues differences exist in 1) M-CoA content, 2) membrane composition, and 3) in constitutive mRNA expression for genes involved in the fat oxidation pathway. In general the apparent V_{max} for enzymes involved in mitochondrial fat oxidation followed the pattern red muscle>heart>liver>white muscle on a per gram tissue basis. Maximal activity of CPT I measured in isolated mitochondria followed a similar pattern of red muscle>heart>white muscle>liver suggesting that both mitochondrial quantity and quality play a role in tissue fat oxidative capacity. This pattern can be partially explained by tissue differences in mRNA expression pattern of heart>red muscle>white muscle>liver for CPT I. In contrast, the expression pattern of transcription factors PPAR α and β did not correspond to enzyme patterns across tissues. Tissue malonyl-CoA content was highest in white muscle than other tissues and there were no differences between the other tissues. Amongst the factors that appear to be involved in non-genetic regulation of fat oxidation mitochondrial membrane fluidity has received little attention. Here we find significant correlations between key indices of membrane fluidity (%DHA and PC:PE) and sensitivity (IC_{50}) of CPT I to its allosteric regulator M-CoA.

Enzyme activity

Patterns of enzyme V_{\max} across tissues are useful in revealing differences in fatty acid oxidation capacity and also stoichiometry of various enzymes in this pathway. Red muscle and heart have distinctly higher activities for CS, LCAD, CPT II and HOAD on a per gram protein basis when compared to white muscle and liver. CS is commonly used as marker of mitochondrial density (Reichmann et al., 1985), thus, it is clear that red muscle and heart have higher mitochondrial densities which leads to increased activities of mitochondrial fatty acid oxidation enzymes and the potential for higher rates of fat oxidation. Further to this, the activity of CPT I, thought to be the regulating enzyme of fat entry into the mitochondria, also exhibits tissue differences (but on a per mg mitochondrial protein basis). This suggests that mitochondria are not equivalent and that there are tissue-specific qualitative differences in fat oxidative ability per unit mitochondria. Similar patterns have been observed by others for CPT I activity per g tissue in rainbow trout, with red muscle and heart having the highest activity followed by liver and then white muscle (Froyland et al., 1998; Gutieres et al., 2003). However, our data suggests that liver CPT I has significantly lower activity compared to red muscle and that heart and white muscle have an intermediate activity level (Fig. 2.2). This data shows that there are stoichiometric changes in enzyme content with differences in fatty acid oxidation capacity across tissues and that this occurs through quantitative differences in mitochondrial density and qualitative differences in β -oxidation ability of each mitochondrial unit.

Allosteric regulation of CPT I

M-CoA reduced CPT I activity by allosteric inhibition in all tissues, however, to varying extents (Figs. 2.2 and 2.3A–D). At high levels of M-CoA liver, red, white and heart muscles were inhibited by 32%, 51%, 70% and 65%, respectively. Residual activity in each tissue may be due to activity of CPT II which is M-CoA insensitive and can be expressed if membranes are damaged during mitochondrial preparation. The mitochondrial preparations used in these experiments were between 60–85% intact depending on the tissue (based on CS measurements). However this cannot explain all of the M-CoA insensitive activity since liver mitochondrial preparations were 85% intact but had the highest residual activity (Fig. 2.3). Alternatively, it may be due to a M-CoA insensitive splice variants of CPT I as seen in rat red muscle (Kim et al., 2002). We determined the IC_{50} for M-CoA sensitive moiety of CPT I in all tissues and have found that the liver ($IC_{50}=0.079 \mu\text{M}$) is significantly more sensitive to M-CoA than red muscle ($IC_{50}=0.55 \mu\text{M}$). In contrast, in mammals, CPT I is 100 times more sensitive to M-CoA in muscle than in liver (McGarry and Brown, 1997). In fish, it appears that the opposite is true, liver CPT I is approximately 10 times more sensitive than muscle CPT I to M-CoA. However, this is a small difference compared to that seen between tissues in mammals.

The M-CoA content varied across tissues but was only significantly different in white muscle compared to the other 3 tissues. White muscle M-CoA content (0.19 nmol/g–1 wet weight) is similar to other data published on any tissue rainbow trout (Richards et al., 2002a), however, our current values for red muscle are significantly lower than other published data (Richards et al., 2002b). When the M-CoA content is

expressed per unit citrate synthase, a mitochondrial density marker, it suggests that white muscle and liver have significantly higher M-CoA concentrations per unit mitochondria. Although these tissues also had the lowest CPT I activities per mg mitochondria this relatively high inhibitor concentration most likely contributes to low in vivo fat oxidation capacities.

Interestingly, our data indicate no changes in M-CoA between red muscle, heart and liver, which can be interpreted in a number of ways. It would be expected that liver would have higher M-CoA content than other tissues because it is the primary site of fat synthesis. On the other hand, we have shown here that liver CPT I is significantly more sensitive to M-CoA than other tissues. It is possible that in trout hepatocyte M-CoA concentrations are kept low to prevent total inhibition of CPT I and maintain fatty acid oxidation. In fact resting levels of M-CoA in all tissues were well below the IC_{50} (Table 2.2, 2.3).

Gene expression profile

CPT I has been found to be expressed in many tissues of rainbow trout (Gutierrez et al., 2003). However, the relative expression levels across tissues have not been previously determined in this species. Using real-time PCR we show that CPT I expression was very low in the liver compared to heart, but not significantly different from red and white muscles (Fig. 2.4). The low expression of CPT I in liver corresponds to its low activity (Fig. 2.2). Unlike mammals which express two different CPT I isoforms, trout are thought to express a single isoform and phylogenetic analysis has shown it to be similar to mammalian CPT I- α (Gutierrez et al., 2003). However, it is possible that our measurements reflect the expression of an uncharacterized muscle isoform which is expressed at low levels in liver. Moreover, the differences in CPT I kinetics between tissues shown here, are very suggestive of second isoform in trout. This would not be surprising since salmonids have gone through several genome duplications and possibly have retained 50–75% of the loci as duplicates (Bailey et al., 1978). However, intertissue differences in enzyme milieu could be a non-genomic explanation for the intertissue kinetic differences (see below).

In mammals many fatty acid oxidation genes have been shown to have PPAR response elements (PPRE) to which PPARs can bind and induce gene expression, including CPT I, CPT II and LCAD (Price et al., 2000). Surprisingly, the expression of PPAR α across tissues was quite different from that of CPT I, with the highest, not lowest, expression in liver. The present data may be explained if in fish: 1) the PPAR α gene expression is not translated to protein, 2) CPT I does not contain a PPRE, 3) this isoform performs a different role than in mammals, 4) there are more than one PPAR α isoform as is the case for PPAR β in zebra fish (Robinson-Rechavi et al., 2001a), or 5) CPT I gene expression is regulated independently of PPAR α as seen in rat hepatoma cells (LeMay et al., 2005). It is also possible that basal and inducible CPT expression occur by different pathways. Basal transcription of CPTI in fish might be regulated by the transcription factor SP1 as has been suggested for mammals (Steffen et al., 1999), while inducible expression is controlled by the PPARs.

There were no differences in the expression of PPAR β between any of the tissues (Fig. 2.4). Very little is known about the roles of PPAR β , however, it has recently been shown to play a variety of roles depending on developmental stage, gender and diet (Akiyama et al., 2004; Ibabe et al., 2005). In adult animals, PPAR β is expressed rather ubiquitously (Kliwer et al., 1994). Our results show a ubiquitous and uniform expression across tissues in adult rainbow trout. These results are similar to those found in brown trout (Batista-Pinto et al., 2005) and rats (Braissant and Wahli, 1998) and may suggest that in adult trout, PPAR β plays a role in homeostatic lipid metabolism but not in determining tissue differences in fat oxidation machinery.

Mitochondrial membrane composition and relation to CPT I IC₅₀

Both the fatty acid tail composition and the phospholipid head groups contribute to the overall fluidity, with increases in PUFAs (large “kinked” fatty acid tails) and PE (a membrane destabilizing phospholipid) contributing to increased fluidity (Hazel, 1995; Hulbert and Else, 1999). There has been no systematic investigation of tissue-specific differences in mitochondrial membrane composition of the same animal that may be contributing to the differences in membrane fluidity or fat oxidation capacity (Leary et al., 2003). Past interest in membrane remodeling has focused on single tissue changes during temperature acclimation that ensure proper membrane fluidity to preserve function at low temperature (for review see (Hazel, 1995)). As well, comprehensive studies across reptiles and mammals have shown that the activity of mitochondrial membrane bound enzymes is positively correlated to the unsaturation of the mitochondrial membrane fatty acids (Hulbert et al., 2006).

In the present study fatty acids contributing the most to the composition of the mitochondrial membranes were C16:0, C18:0, C18:1, C20:5n3 and C22:6n3 (Table 2.4). This composition is similar to those observed by Kraffe et al. in their investigation of trout red muscle mitochondria (Kraffe et al., 2007). Moreover, here the majority of the fatty acids were found in phospholipid classes PC and PE and to some extent in CL (Table 2.5). We found no significant differences in the fatty acid composition between tissues. However, red muscle tended to have higher concentrations of PUFAs (Table 2.4) and lower PC:PE ratios (Table 2.5), both of which may indicate an increase in fluidity. Leary et al. directly measured mitochondrial membrane fluidity in rainbow trout muscles and found that red muscle is more fluid when compared to white muscle and heart (Leary et al., 2003). Recently, it has been suggested that increases in membrane fluidity may disrupt the interaction between the N- and C-termini of CPT I and be partly responsible for the decrease in the sensitivity of CPT I to M-CoA (Faye et al., 2005; Jackson et al., 2000). We investigated if there were any correlations that might explain the variation in the data and suggest a role of membrane composition in the regulation of CPT I. Significant correlations exist when the IC₅₀ values are plotted against important determinants of membrane fluidity, PC:PE ratio and %DHA (Fig. 2.5A–B). Lower PC:PE ratio and higher %DHA may lead to increases in membrane fluidity, with changes in phospholipid headgroups (PC:PE) probably having a greater affect than changes in DHA proportions (Hazel, 1995). The differences, particularly in PC:PE across tissues

could potentially decrease the sensitivity of CPT I to M-CoA. It should be noted that the measurements presented in this study are for both inner and outer mitochondrial membranes while CPT I only resides in the outer membrane. Direct fluidity measurements using DPH anisotropy, as opposed to membrane composition studies will be valuable in assessing actual outer mitochondrial membrane fluidity and verify our current findings.

This evidence suggests that in fish, sensitivity to M-CoA may be regulated by the fluidity of the membrane as indicated by the mitochondrial membrane composition, but currently it is unclear if this occurs by a common mechanism to that proposed for mammals (Faye et al., 2005). Perhaps as part of homeoviscous adaptations, fish may be able to maintain suitable rates of mitochondrial fat oxidation at low environmental temperatures through this interaction between membrane fluidity and CPT I kinetics. This would help compensation for the decelerating effects of temperature on metabolism.

Fish vs. mammals

It is interesting to note the dramatic differences between rainbow trout and rat CPT I kinetics, especially in IC₅₀ values. The liver mitochondria of rainbow trout tend to oxidize pyruvate at a higher rate than palmitoyl-carnitine (Leary et al., 2003), whereas in mammals, acyl-carnitines are preferentially oxidized over pyruvate (Bremer, 1966). While this may explain the difference in liver CPT I sensitivity to M-CoA, it does not explain the differences in muscle sensitivity seen between rats and fish, as lipids are an important fuel source for endurance exercise in both species (Lauff and Wood, 1996; Lauff and Wood, 1997). In rats, the IC₅₀ of CPT I for M-CoA is approximately 0.03 μ M (McGarry and Brown, 1997), whereas, in trout red muscle it is approximately 18 times higher (IC₅₀=0.55 μ M). There may be differences in skeletal muscle fiber types in rats that are being masked in the mixed muscle samples that are most conveniently elucidated by separating out the anatomically distinct fiber types in fish.

Conclusions

Fatty acid oxidation is regulated by both genetic and non-genetic mechanisms. There is a growing body of evidence from mammals (Faye et al., 2005; Kolodziej and Zammit, 1990; Mynatt et al., 1994; Power et al., 1994; Zammit et al., 1997) indicating that the fluidity of the mitochondrial membrane influences the sensitivity of CPT I to M-CoA. The present study suggests that this phenomenon extends across taxa. Isolated mitochondria from white, red and heart muscles of rainbow trout have been shown to exhibit dramatic differences in a variety of components such as proton leak kinetics and fluidity (Leary et al., 2003). We have shown tissue specific differences in a variety of factors affecting fatty acid oxidation in rainbow trout. Most dramatically, red muscle and liver show significant differences in CPT I kinetics. These kinetic differences match changes in gene expression of CPT I and tissue differences in mitochondrial membrane composition across tissues. In contrast to a previous study (Gutierrez et al., 2003), these differences suggest the possibility of a second CPT I isoform in fish. It will now be important to determine if changing these factors through in vivo perturbations such as diet and temperature acclimation will affect these tissues in the same or different manner and

if it will have effects on overall mitochondrial fatty acid oxidation. Studying the tissue-specific differences in fat oxidation and gene expression which reflect developmental differences in tissue function also provides a window into species-specific differences in abilities to use fat as a metabolic substrate.

Table 2.1: Forward (F) and reverse (R) primers used for real-time PCR analysis of mRNA expression in trout.

Gene	Primer
Efl α	F - 5' CAT TGA CAA GAG AAC CAT TGA 3' R - 5' CCT TCA GCT TGT CCA GCA C 3'
CPTI	F - 5' GCC GCA AAC TAG AGA GAG GA 3' R - 5' CCC GTA GTA CAG CCA CAC CT 3'
PPAR α	F - 5' CCA AGT TCA GTT TGC CAT GA 3' R - 5' ATT GGG GAA GAG GAA GGT GT 3'
PPAR β	F - 5' CTG GAG CTG GAT GAC AGT GA 3' R - 5' GTC AGC CAT CTT GTT GAG CA 3'

Table 2.2: The concentration of malonyl-CoA (μM) to reduce the activity of malonyl-CoA sensitive carnitine palmitoyltransferase (CPT) I activity by 50% (IC_{50}) as determined by isotope assay. Values are means \pm SE. Results that share a symbol are not significantly different.

	Tissue			
	Liver	Red Muscle	Heart	White Muscle
IC_{50}	$0.079 \pm 0.037^{\dagger}$	$0.55 \pm 0.06^*$	$0.40 \pm 0.10^*$	$0.37 \pm 0.09^{*\dagger}$

Table 2.3: Malonyl-CoA (M-CoA) content across tissues. Values are means \pm S.E. for 4 animals. Results that share a symbol are not significantly different.

	Tissue			
	Liver	Red Muscle	Heart	White Muscle
[M-CoA] (nmol·g ⁻¹ wet tissue)	0.014 \pm 0.004	0.030 \pm 0.004	0.013 \pm 0.002	0.196 \pm 0.027*
[M-CoA] (nmol·g ⁻¹ wet tissue)/ U citrate synthase	0.0005 \pm 1.8 \times 10 ⁻⁴ †	0.0001 \pm 2.5 \times 10 ⁻⁵	0.00008 \pm 1.5 \times 10 ⁻⁵	0.006 \pm 8.8 \times 10 ⁻⁴ *

Table 2.4: Total average mol% percent contributions of individual fatty acids (FA) to total FA from the mitochondrial membrane phospholipids across tissues*. Values are means \pm S.E., n=4.

Fatty Acid	Liver	Red Muscle	Heart	White Muscle
C14:0	2.5 \pm 0.5	3.8 \pm 1.3	4.3 \pm 1.7	5.1 \pm 1.4
C16:0	34.2 \pm 2.0	30.6 \pm 3.0	32.9 \pm 4.4	32.1 \pm 0.7
C16:1	5.5 \pm 1.2	6.2 \pm 2.5	7.6 \pm 3.2	9.6 \pm 2.6
C18:0	8.0 \pm 2.3	7.8 \pm 1.3	8.5 \pm 0.9	7.4 \pm 0.3
C18:1n9	5.1 \pm 3.0	3.5 \pm 1.3	10.7 \pm 2.1	6.6 \pm 2.0
C18:1n7	10.8 \pm 2.1	8.0 \pm 3.7	6.5 \pm 2.6	7.1 \pm 1.6
C18:2n6	8.5 \pm 0.8	6.3 \pm 0.8	5.0 \pm 1.2	5.4 \pm 1.2
C18:3n3	0.6 \pm 0.1	0.6 \pm 0.1	n.d.	0.7 \pm 0.1
C18:4n3	0.6 \pm 0.3	n.d.	1.1 \pm 0.6	n.d.
C20:1n9	3.5 \pm 0.7	3.8 \pm 0.9	2.3 \pm 0.8	1.7 \pm 0.3
C20:4n6	2.7 \pm 0.5	1.5 \pm 0.2	2.4 \pm 0.5	1.2 \pm 0.2
C20:5n3	10.9 \pm 3.6	2.7 \pm 1.9	1.6 \pm 1.0	7.3 \pm 2.6
C22:5n3	1.9 \pm 1.4	3.4 \pm 0.8	4.8 \pm 2.0	7.5 \pm 4.0
C22:6n3	5.8 \pm 4.8	21.5 \pm 12.3	11.6 \pm 6.2	10.4 \pm 8.3
saturates	44.9 \pm 3.6	42.2 \pm 4.4	45.8 \pm 3.8	44.6 \pm 0.7
monounsaturates	25.1 \pm 1.9	21.5 \pm 7.3	27.1 \pm 5.3	25.1 \pm 3.7
polyunsaturates	29.9 \pm 1.8	36.3 \pm 11.0	27.0 \pm 3.1	30.3 \pm 4.4
η -3 polyunsaturates	18.6 \pm 1.4	28.4 \pm 10.8	19.6 \pm 3.8	23.6 \pm 4.4
η -6 polyunsaturates	11.3 \pm 0.8	7.8 \pm 0.7	7.5 \pm 1.3	6.6 \pm 1.0
η -3/ η -6	1.7 \pm 0.1	3.6 \pm 1.3	3.1 \pm 0.9	3.9 \pm 1.2
UI [†]	1.5 \pm 0.1	2.0 \pm 0.6	1.5 \pm 0.2	1.6 \pm 0.2

*Values are means \pm SE; n.d. = not detectable

[†]Unsaturation index = $\sum m_i \cdot n_i$; where m_i is the mole percentage and n_i is the number of C-C double bonds in the fatty acid “i”

Table 2.5: Mol% of mitochondrial membrane phospholipid classes across tissues. Values are means \pm S.E., n=4.

Phospholipid	Liver	Red Muscle	Heart	White Muscle
Phosphatidylcholine	52.7 \pm 2.5	43.7 \pm 3.5	49.0 \pm 7.7	41.9 \pm 2.1
Phosphatidylserine	2.5 \pm 0.6	4.6 \pm 1.7	6.5 \pm 2.8	6.2 \pm 2.3
Phosphatidylinositol	8.0 \pm 3.0	5.7 \pm 2.7	7.7 \pm 2.9	13.3 \pm 4.4
Phosphatidylethanolamine	27.2 \pm 4.3	35.7 \pm 2.5	23.9 \pm 1.2	22.4 \pm 4.3
Cardiolipin	9.5 \pm 1.1	13.9 \pm 2.8	13.0 \pm 2.2	16.0 \pm 2.0
PC:PE [†]	2.1 \pm 0.3	1.2 \pm 0.1	2.1 \pm 0.3	2.0 \pm 0.3

[†]PC = phosphatidylcholine, PE = phosphatidylethanolamine

Fig 2.1:

The apparent V_{\max} (in mU per mg total protein) for (A) citrate synthase (CS) and β -hydroxyacyl-CoA dehydrogenase (HOAD), and (B) long chain acyl-CoA dehydrogenase (LCAD) and carnitine palmitoyltransferase (CPT) II in white muscle, heart, red muscle and liver. Values are means \pm SE for 5 animals. Different symbols denote significance between tissues for each enzyme, $p < 0.05$, similar symbols indicate no significant difference between tissues for each enzyme, $p > 0.05$.

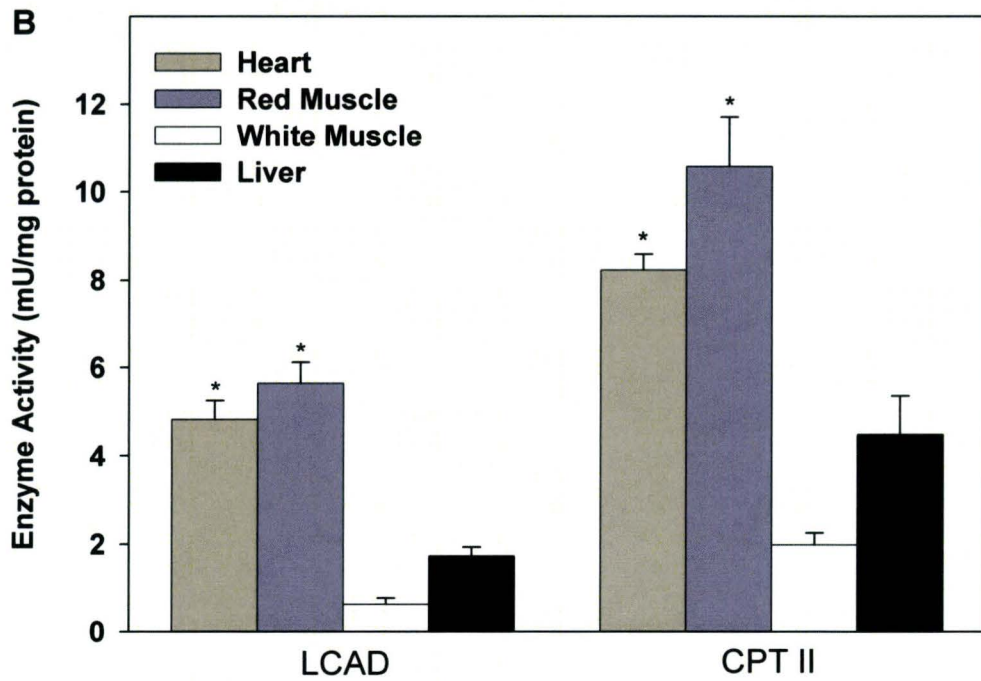
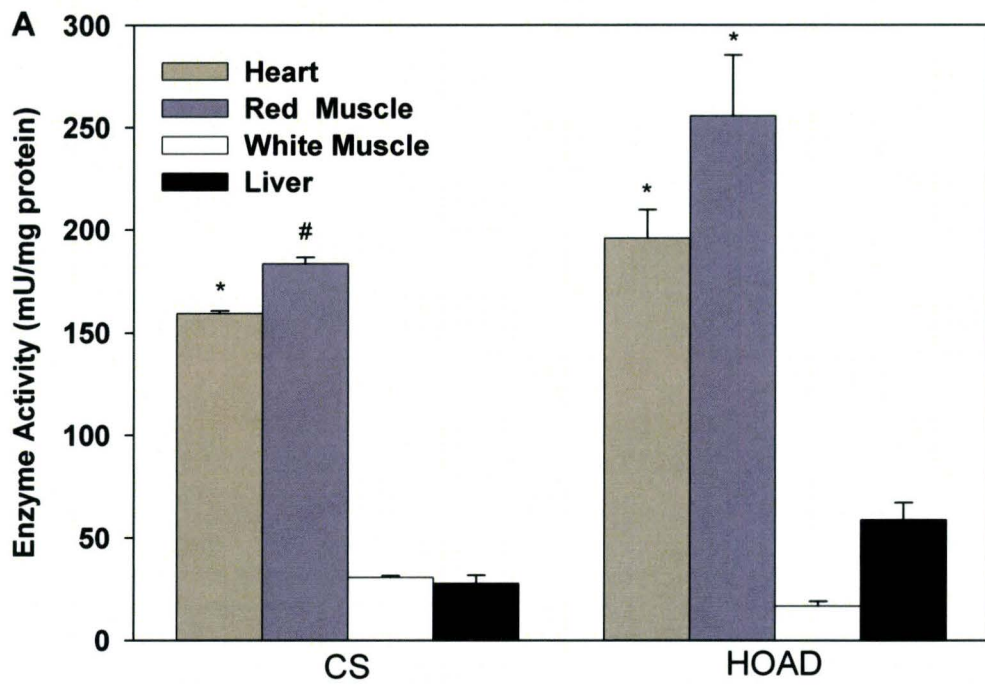


Fig. 2.2

CPT activity (mU per mg mitochondrial protein) in liver (L), red muscle (RM), heart (H) and white muscle (WM). Colored bars, 0 μ M M-CoA; black bars, 50 μ M M-CoA. Values are means \pm SE for 4 animals. Different letters denote significance, $p < 0.05$.

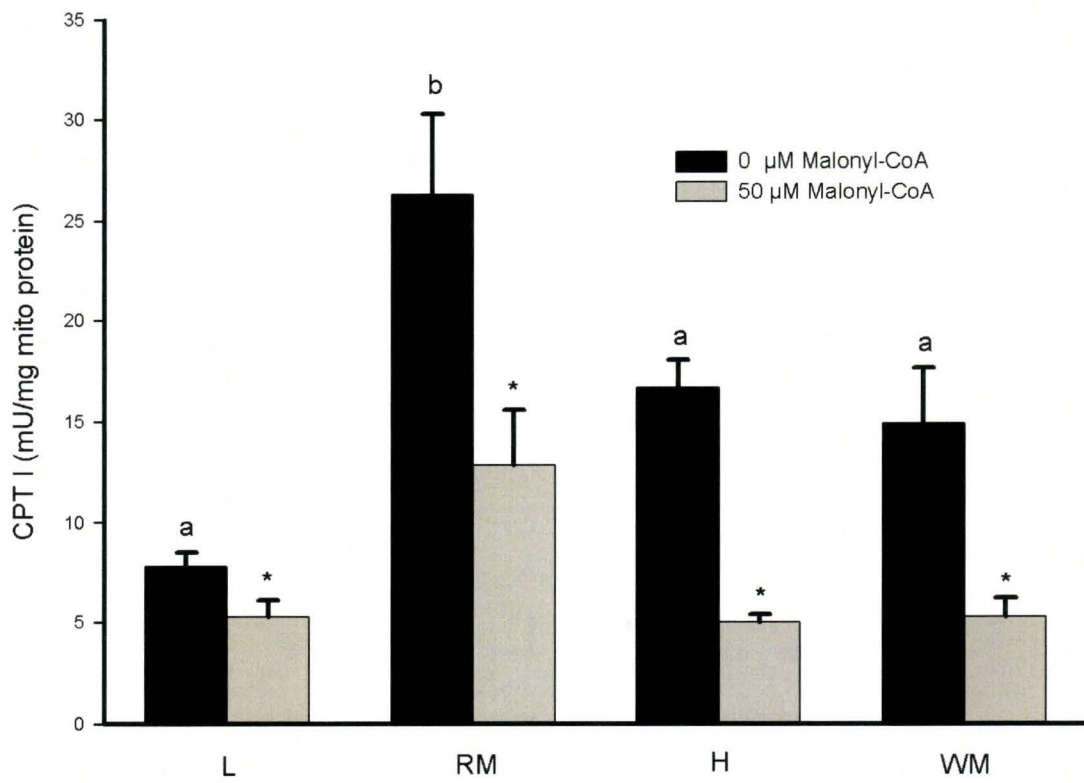


Fig 2.3

Inhibition curves determined using a radioisotope assay for carnitine palmitoyltransferase (CPT) I activity in isolated mitochondrial preparation with increasing malonyl-CoA (M-CoA) concentration in (A) red muscle, (B) heart, (C) white muscle and (D) liver. M-CoA concentrations ranged from 0.05 μM to 50 μM . Liver and white muscle, n=5; heart, n=4; red muscle, n=3.

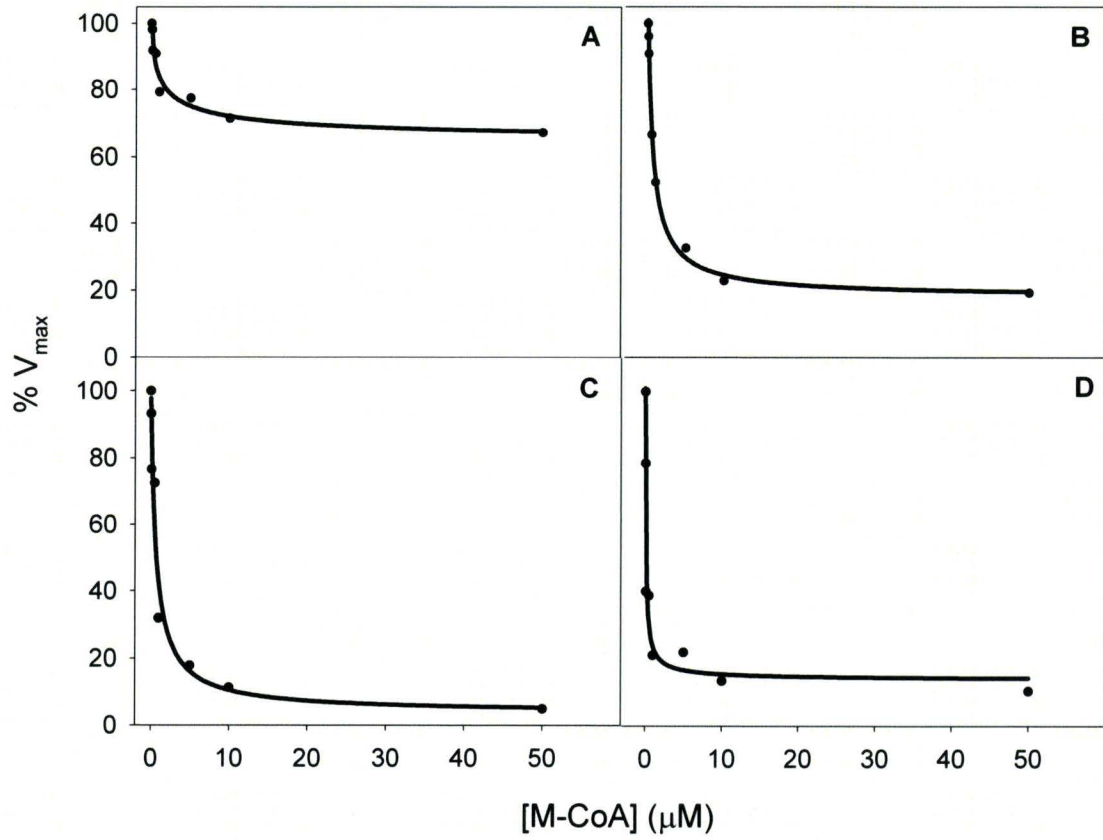


Fig 2.4

Real-time PCR gene expression profiles in liver, red muscle, heart and white muscle for carnitine palmitoyltransferase (CPT) I, peroxisome proliferator-activated receptor (PPAR) α and PPAR β . Values are expressed relative to EF1 α . Values are means \pm SE. n=4 for each tissue. Different letters denote significance within group, p<0.05.

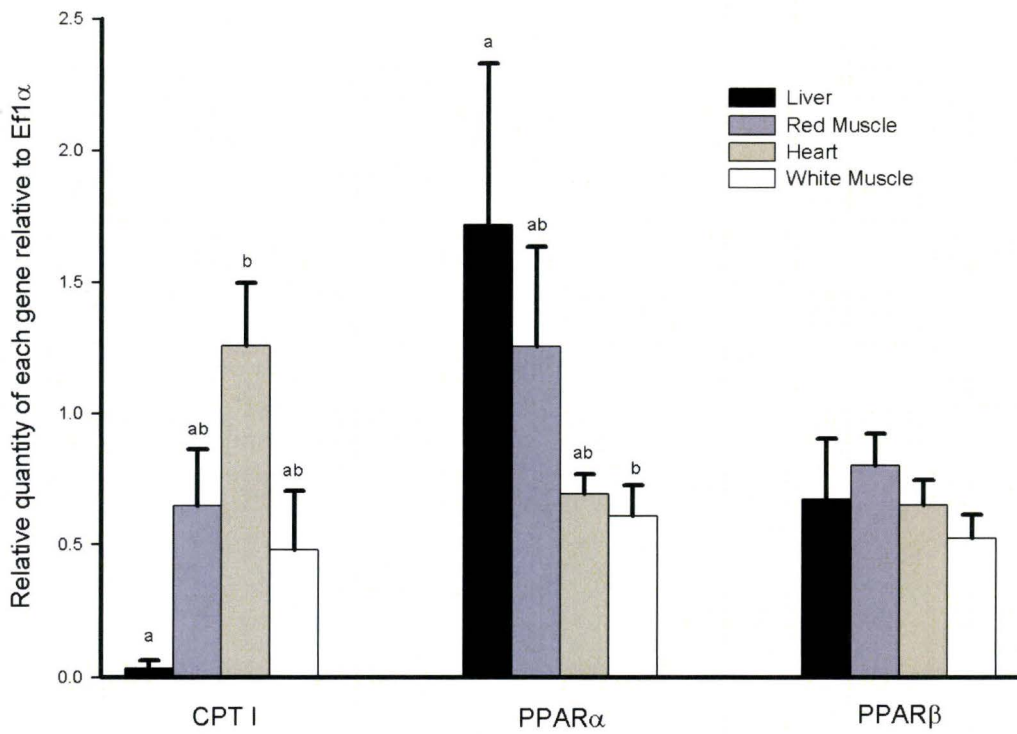
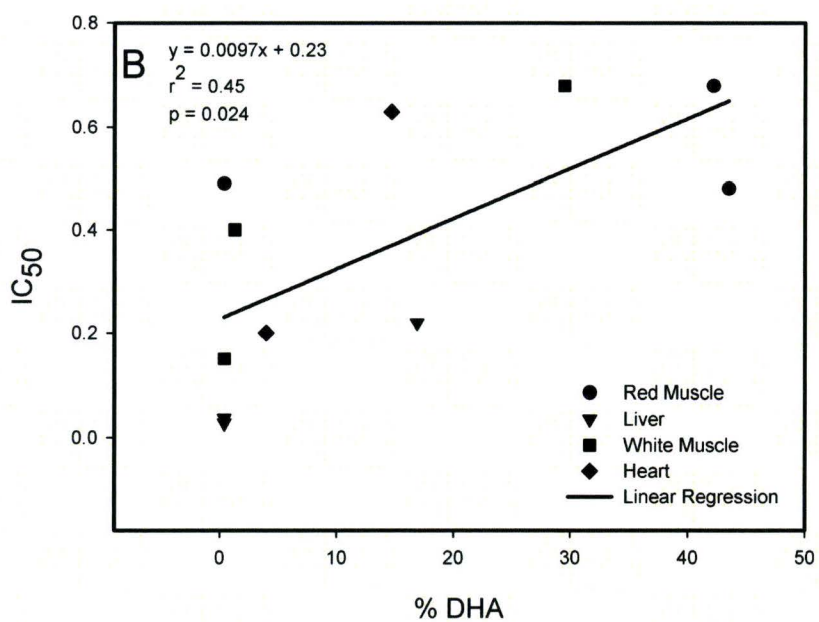
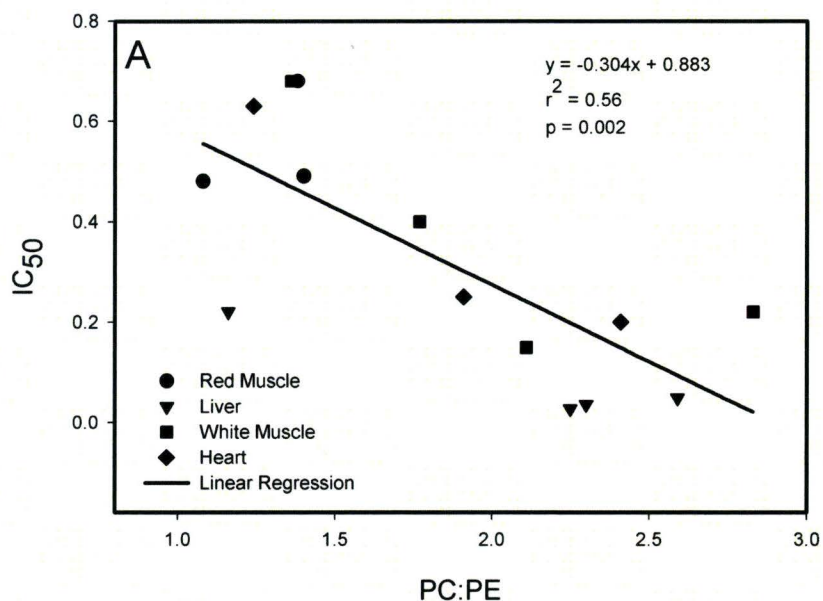


Fig. 2.5

The relationship between mitochondrial membrane composition and sensitivity of carnitine palmitoyltransferase (CPT) I to malonyl-CoA (IC_{50}) across tissues. (A) Negative correlation between the PC:PE ratio and IC_{50} . Liver, n=4; red muscle, n=3; heart, n=3; white muscle, n=4. (B) Positive correlation between the % of docosahexanoic acid (DHA) and IC_{50} . Liver, n=3; red muscle, n=3; heart, n=2; white muscle, n=3.



CHAPTER 3

EFFECTS OF DIETARY FATTY ACID COMPOSITION ON THE REGULATION OF CARNITINE PALMITOYLTRANSFERASE (CPT) I IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

Abstract

Dietary fatty acid composition, particularly polyunsaturated fatty acids, can affect both genetic and non-genetic regulatory mechanisms of carnitine palmitoyltransferase (CPT) I, the main regulatory enzyme of mitochondrial fatty acid oxidation. We aimed to determine how these regulatory mechanisms were affected by changes in the fatty acid composition of the diet in fish. Specifically, we fed rainbow trout (*Oncorhynchus mykiss*) either a high polyunsaturated fatty acid (PUFA) diet, a high saturated fatty acid (SFA) diet or a mixed fatty acid control (CTL) diet for 8 weeks to determine if modifications of the dietary fatty acids would affect 1) the genetic expression of CPT I and its transcription factor peroxisome proliferator-activated receptor (PPAR), 2) the mitochondrial membrane composition and if these modifications would affect CPT I sensitivity to malonyl-CoA, and 3) levels of malonyl-CoA in the tissues. We found that fish fed the high PUFA diet significantly increased CPT I mRNA expression in red muscle, liver and adipose tissue, while PPAR α and β expressions were variable across tissues. Few significant changes were observed in the mitochondrial membrane composition with the exception of DHA in the red muscle. There were no significant differences in CPT I sensitivity to malonyl-CoA or the malonyl-CoA content of the tissues with either experimental diet. Our present data suggest that a change in gene expression of CPT I and PPARs are the main regulatory mechanism controlling CPT I function in fish using our experimental diet.

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Introduction

Carnitine palmitoyltransferase (CPT) I is considered to be the main regulatory enzyme in mitochondrial fatty acid oxidation because it catalyses the conversion of fatty acyl-CoAs into fatty acyl-carnitines for entry into the mitochondrial matrix (Kerner and Hoppel, 2000). There are numerous mechanisms governing the regulation of CPT I including allosteric inhibition by malonyl-CoA (M-CoA) (Murthy and Pande, 1987), changes in the expression of the CPT I gene and and/or transcription factors (Price et al., 2000), as well as the composition and fluidity of the outer mitochondrial membrane (Kolodziej and Zammit, 1990; Morash et al., 2008). Previously we have shown that there is a relationship between key indices of membrane fluidity and CPT I sensitivity to M-CoA across tissues in rainbow trout (Morash et al., 2008). However it is unclear how manipulation of membrane composition and physicochemical properties will affect CPT I kinetics. There is evidence that the various mechanisms involved in CPT I regulation and, consequently, mitochondrial β -oxidation of fatty acids are modulated by various nutrients. For example, dietary polyunsaturated fatty acids (PUFA), through their ability to act as ligands for specific nuclear receptors, can modulate gene expression of CPT I, at least in many mammalian species (Power et al., 1994).

M-CoA can inhibit CPT I activity thereby reducing the oxidation of newly formed fatty acids (McGarry and Brown, 2000). M-CoA is synthesized in the liver during the first step of de novo fatty acid synthesis. Levels of M-CoA can reflect the metabolic status of the organism. For instance, increases in circulating blood glucose and insulin associated with feeding have been shown to promote hepatic lipogenesis and fat storage via increases hepatic M-CoA levels and inhibition of CPT I activity (Chien et al., 2000). In muscle, M-CoA levels are more sensitive to regulation by acetyl-CoA carboxylase (McGarry et al., 1983) and its main role appears to be modulating CPT I activity.

Increasing lipid intake generally results in significant increases in lipid oxidation and deposition in animals (Iossa et al., 2002). Changes in the composition of lipids of the diet can also influence lipid oxidation and deposition partly through modulation of gene expression of various metabolic enzymes, notably enzymes involved in mitochondrial fatty acid oxidation (Price et al., 2000). Polyunsaturated fatty acids (PUFAs) are known activators of peroxisome proliferator activated receptors (PPARs) (Price et al., 2000). PPARs are a family of nuclear receptors and transcription factors that bind to corresponding response elements to activate numerous genes in the fatty acid oxidation and other pathways. In mammals for instance, CPT I has a PPAR response element (PPRE) and therefore, increases in PUFAs induce changes in CPT I expression through their activation of PPARs (Price et al., 2000).

In addition to genomic effects, PUFAs can also act on CPT I activity indirectly via changes in the mitochondrial membrane composition. The fatty acid composition of the outer mitochondrial membrane has been shown to be of particular importance to the regulation of CPT I because it can affect membrane properties and the binding affinity of the allosteric regulator M-CoA to the enzyme (Jackson et al., 2000). The binding site for M-CoA is located on the cytosolic side of CPT I and is formed through amino acid linkages of the N- and C-termini of the CPT I protein (Jackson et al., 2000). This

interaction relies on amino acids adjacent to the mitochondrial membrane and consequently changes in membrane fluidity. The degree of movement in the membrane can affect this interaction (Faye et al., 2005). The mitochondrial membrane is very flexible and is constantly being modified through, amongst other things, temperature (Hochachka and Somero, 1984) or diet (Yamaoka et al., 1988). Dietary PUFAs can be incorporated into the mitochondrial membrane phospholipids and modify their physical properties. Increases in the incorporation of long chain highly unsaturated fatty acids may lead to increased membrane fluidity. Fluidity of the membrane can also be affected by changes in phospholipid type. For example, an increase in phosphatidylethanolamine (PE) destabilizes the membrane lipid bilayer by promoting hexagonal (HII) phase conformation versus phosphatidylcholine (PC) which stabilizes the membrane by promoting a lamellar formation (Hazel, 1995).

This interaction between membrane fluidity and M-CoA sensitivity has been demonstrated in rat liver mitochondria both in vivo and in vitro. Isolated mitochondria exposed to membrane fluidizing agents such as benzyl alcohol or increases in temperature both showed decreases in M-CoA sensitivity (Kolodziej and Zammit, 1990). Starved and diabetic rats also exhibited the same decrease in sensitivity to MCoA which was correlated with increases in membrane fluidity (Zammit et al., 1998). Using rainbow trout, we have demonstrated a similar correlation between indices of fluidity derived from mitochondrial membrane composition and CPT I IC_{50} (concentration of an inhibitor to reduce enzyme activity by 50%) in both liver and muscle tissue (Morash et al., 2008). Other studies have shown that the fatty acid composition of mitochondrial phospholipids of fish could be significantly modified by changing the fatty acid composition of the diet (Guderley et al., 2008). Thus, modifying the mitochondrial membrane composition through changes in dietary fatty acid composition may elicit the same change in CPT I sensitivity as seen in mammalian liver in both the liver and muscle tissue of rainbow trout.

Aside from the regulation of CPT I sensitivity to M-CoA by mitochondrial membrane fluidity and composition, the genetic expression of various isoforms or splice variants of CPT I can change the sensitivity within a tissue. For example, in the embryonic stage, rat heart expresses the liver type CPT I, which switches to the muscle type CPT I after birth. Furthermore, there also exists splice variants of CPT I in mammals which are M-CoA insensitive (Kim et al., 2002). While these isoforms have yet to be identified in fish, it is possible that these mechanisms may exist for altering sensitivity of CPT I to M-CoA.

We have previously shown that the capacity and regulation for fatty acid oxidation is different between liver and muscle tissue of fish, and that it is distinctly different from mammals (Morash et al., 2008). Most studies on the effects of diet on membrane properties have been focused on mammals, and principally on the liver. It is not known if diet has an effect on muscle M-CoA and more specifically, how changing dietary fatty acid composition (degree of unsaturation) affects M-CoA levels in both the liver and muscle. Therefore, we investigated the effects of diets containing varying amounts of SFAs and PUFAs on CPT I regulation in both red muscle and liver of rainbow trout (*Oncorhynchus mykiss*). Our overall objectives were to determine if fatty acid

composition of the diet (focusing on the degree of fatty acid saturation in the diets) affects: 1) mitochondrial membrane composition, 2) concentrations of malonyl-CoA, 3) gene expression of PPARs and CPT I and 4) sensitivity of CPT I to its allosteric inhibitor MC_oA (IC₅₀). Taken all together, these data will give insight into the dietary regulation of mitochondrial fatty acid oxidation in the liver and red muscle.

Materials and methods

Experimental diets

Three isoproteic, isolipidic, and isoenergetic diets (43% crude protein, 20% crude lipid, 18 MJ digestible energy/kg) were formulated to meet all known nutrient requirements of rainbow trout and contain increasing concentration of n-3 PUFA by using a combination of fish oil, beef tallow or a fish oil concentrate rich in DHA (55:05 TG, Ocean Nutrition, Halifax, NS, Canada) (Table 3.1). Fatty acid composition of the different lipid sources are provided in (Bureau et al., 2008). In the diets, n-3 PUFA (mainly DHA 22:6 n-3) increase at the expense of saturated fatty acids (mainly 16:0,18:0) and monounsaturated fatty acids (18:1 n-9) with minimal modification of n-6 PUFA concentration. The three diets were identified as follows: SFA=high saturated fatty acid diet, CTL=control (“balanced” fatty acid profile) diet, PUFA=high polyunsaturated fatty acid diet (Table 3.2). The diets were mixed using a Hobart mixer (Hobart, Don Mills, ON, Canada) and pelleted to the appropriate size using a steam pellet mill (California Pellet Mill, San Fransisco, CA, USA). The pellets were dried under forced air at room temperature for 24 h and then sieved. Feed was kept at -4 °C until used. Each week appropriate amounts were kept at room temperature for feeding. 2.2.

Experimental fish and conditions

Rainbow trout, *Oncorhynchus mykiss*, (~500 g) were obtained from a local hatchery (Humber Springs, Orangeville, ON, Canada) and fed a commercial fish feed, (Profishent Classic Floating Trout Grower, Martin Mills, Elmira, ON, Canada) until the start of the experiment. Upon the start of the experimental feeding, 15 fish were randomly distributed into each of six 500 L tanks with circulating water at 12 °C with two tank replicates per diet. Fish were hand fed twice daily to satiation for 8 weeks.

Each tank was individually aerated and kept on a 12 h light/12 h dark cycle and mortality and temperature were recorded daily. Fish were anesthetized using MS-222 (0.5 g/L, buffered with sodium bicarbonate to maintain a neutral pH), weighed and their length measured at the start and the end of the experiment. Condition factor (CF) was calculated using the following equation, $CF = 100w=l^3$ where w is the weight of the fish in grams and l is the length of the fish in centimeters.

Mitochondrial isolation

Fish were killed by a blow to the head followed by severing of the spinal cord. Mitochondria were isolated from red muscle (RM) and liver (L) from rainbow trout according to published protocols (Moyes et al., 1988; Suarez and Hochachka, 1981). Briefly, each tissue was immediately excised (RM ~4 g, whole L) and placed in

mitochondrial isolation buffer (MIB) consisting of (in mM) 140 KCl, 10 EDTA, 5 MgCl₂, 20 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 0.5% bovine serum albumin (BSA) (pH 7.0) for RM and 250 sucrose, 1 EDTA, 20 HEPES and 0.5% BSA (pH 7.4) for L, on ice. Tissues were diced, washed twice with fresh chilled MIB then homogenized three times, first using a wide clearance teflon pestle on a chilled glass homogenizer, then three times with a narrow clearance teflon homogenizer to lyse cells and expose the mitochondria. Homogenates were centrifuged at 800 × g for 10 min at 4 °C. The supernatant was centrifuged at 9000 × g for 10 min at 4 °C. Supernatants for both tissues were then discarded and pellets were resuspended in a small volume of the appropriate MIB lacking BSA. The resuspended homogenate was collected into a 15 mL centrifuge tube and centrifuged again at 9000 × g for 10 min at 4 °C. The supernatant was discarded and the mitochondrial pellet was resuspended in an appropriate volume of MIB lacking BSA and kept on ice.

Enzyme and protein assays

All assays (except the CPT I radioisotope assay) were performed in triplicate at room temperature (~22 °C) using a Spectramax Plus 384 and clear 96-well flat bottom assay plates and data was collected using Softmax Pro 4.7.1 (Molecular Devices, Sunnyvale, CA, USA).

CPT I assay

CPT I assay followed a protocol from McGarry et al. (McGarry et al., 1983) for mammals altered using the assay conditions of (Rodnick and Sidell, 1994) to obtain CPT I V_{max} and IC_{50} as previously described (Morash et al., 2008). The assay buffer (pH 7.0) contained (in mM) 20 HEPES, 40 KCl, 1 EGTA, 220 sucrose, 0.1 DTT, 0.04 palmitoyl-CoA, 1 carnitine and 1.3 mg/mL BSA. One μ Ci/sample of L-[methyl-³H]carnitine hydrochloride (specific activity 82.0 Ci/mmol: Amersham Biosciences, Baie d'Urfé, Quebec, Canada) was added and 70 μ L of the assay mixture was placed in 1.5 mL Eppendorf tubes and incubated with 10 μ L of 0–500 mM malonyl-CoA for 5 min at room temperature. Water was used instead of malonyl-CoA for blank measurements and for the determination of maximal activity. The reaction was started by the addition of 20 μ L of mitochondria diluted 5× in MIB (final concentration ~2 mg/mL), and incubated at room temperature for 8 min. The reaction was stopped by the addition of 60 μ L of 1 M HCl. The palmitoyl-[³H]-carnitine was collected according to published methods (Starritt et al., 2000). 20 μ L of the assay mixture with L-[methyl-³H] carnitine hydrochloride was also counted in duplicate to determine individual specific activity for each sample as well as one blank sample containing aqueous counting scintillation fluid to determine background counts. The decays per minute (DPM) were read for 5 min per sample on a Tricarb 2900 TR Liquid Scintillation Analyzer (PerkinElmer) using QuantaSmart 1.31 (Packard Instrument Company) analysis software.

Citrate synthase (CS)

CS assays were performed as described in McClelland et al. (McClelland et al., 2005). Whole tissue homogenates were frozen and thawed three times using liquid N₂

and kept on ice until further use. An aliquot of the 5× diluted isolated mitochondria used for the CPT I assay was diluted a further 5× using the enzyme extraction buffer (see above) and frozen and thawed three times using liquid N₂ and kept on ice. Isolated intact mitochondria in MIB were also assayed. These three homogenates were used to determine the amount of intact mitochondria versus ruptured mitochondria and to extrapolate CPT I enzyme activities to the tissue level. The CS assay buffer consisted of (in mM) 20 TRIS (pH 8.0), 0.1 DTNB and 0.3 acetyl-CoA. The reaction was started by the addition of 0.5 oxaloacetate and absorbance was monitored at 412 nm. Control wells lacking oxaloacetate were assayed to correct for acetyl-CoA hydrolase activity.

Protein content

Protein concentrations were determined by the Bradford method (Bradford, 1976) using a BioRad protein assay kit (BioRad, Mississauga, Ontario, Canada).

Malonyl-CoA (M-CoA) content

M-CoA concentrations were determined using a modified method from Richards et al. (Richards et al., 2002a). Briefly, frozen whole tissue samples were powdered using a liquid N₂-cooled mortar and pestle. Samples were lyophilized for 24 h and kept at –80 °C until analysis. Fifty mg of lyophilized tissue was homogenized at 4 °C for 20 s using a teflon pestle in 200 µL of 0.5 M perchloric acid with 50 µM dithioerythritol (DTE) and 10 µg/mL propionyl-CoA as an internal standard. Homogenized samples were centrifuged at 20,000 × g for 10 min at 4 °C and 200 µL of the supernatant was transferred and adjusted to pH 3 using 4 M NaOH while being vortexed. Twenty µL of MOPS (pH6.8) was added and the final pH determined (always less than 5). M-CoA was then separated using reverse-phase HPLC based on a method from Demoz et al. (Demoz et al., 1995) using a Waters 717 Plus autosampler (Waters, Mississauga, ON, Canada) at room temperature (~22 °C). Two hundred µL of the sample was injected onto a Zorbax ODS Rx C-18 column (25 cm×0.46 mm) (Agilent Technologies, Mississauga, ON, Canada). The elution gradient was created using a Waters Model 510 pump controller. Mobile phase A was 100 mM sodium phosphate and 75 mM sodium acetate in deionized water (pH 4.6). Mobile phase B was the same as A except that it contained 30% CH₃CN. The elution gradient was as follows: 0 min, 90% A; 17 min, 50% A; 17.6 min, 90% A. Baseline conditions were established after 5 min of 90% A. The flow rate was 1.5 mL/min and absorbance measurements were made at 245 nm using a Lambda Max 481 LC spectrophotometer (Waters, Mississauga, ON). Peaks were manually identified by comparisons to known M-CoA standards and quantified using the internal standard.

mRNA quantification by real time PCR

Total RNA from red muscle, liver, heart, white muscle and adipose tissue was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA concentrations were quantified by UV spectrophotometry at 260 nm and diluted to 0.5 µg/µL. cDNA was synthesized using 1 µg of DNase (Invitrogen) treated mRNA with SuperScript RNase H- reverse transcriptase (Invitrogen). SYBR green (Bio-Rad, Mississauga, ON, Canada) with ROX as a reference dye was used for quantitative real

time PCR in 25 μL reactions using a Stratagene Mx3000P real-time PCR system. Each reaction contained 12.5 μL SYBR green mix, 1 μL each of forward and reverse primer (5 μM), 5.5 μL of DNase/RNase free water and 5 μL of 5 \times diluted cDNA. Primers were designed using a CPT I sequence from rainbow trout liver (Gutierrez et al., 2003) (see Table 3.3 for specific primer sequences). The thermal program included 3 min at 95 $^{\circ}\text{C}$, 40 cycles of 95 $^{\circ}\text{C}$ for 15 s, 60 $^{\circ}\text{C}$ for 30 s and 72 $^{\circ}\text{C}$ for 30 s. A no-template control and dissociation curve were performed to ensure only one PCR product was being amplified per reaction. Standard curves were constructed for each gene using serial dilutions of stock cDNA to account for any differences in amplification efficiencies. All samples were normalized to the housekeeping gene, EF1- α , which did not change between our experimental treatments. Primers were designed using Primer3 software (Rozen and Skaletsky, 2000).

Analysis of mitochondrial membrane phospholipid composition

Mitochondrial total lipid was extracted and phospholipids were analyzed according to Gillis and Ballantyne (Gillis and Ballantyne, 1999), based on a modification of the protocol of Bligh and Dyer (Bligh and Dyer, 1959). Mitochondrial lipids were extracted in chloroform:methanol (1:2) and after drying were resuspended in 25 μL chloroform:methanol (2:1) and spotted onto silica gel 60 precoated 250 μM thick plates (Fisher Scientific, Ottawa, ON, Canada) for thin layer chromatography along with a standard phospholipid mix (sphingomyelin, phosphotidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine and cardiolipin) (Sigma, Oakville, ON, Canada). The solvent system used to separate the phospholipids was chloroform:methanol:acetic acid:water in a 50:37.5:3.5:2 (by volume) mixture. Once the solvent had run to within 5 cm of the top of the plate, the plate was removed and allowed to air dry then sprayed with a saturated solution of 2,7- dichlorofluorescein and allowed to incubate in a 25% ammonium hydroxide solution for 5 min. Plates were viewed under UV light and individual phospholipid fractions were scraped off into individual Kimex tubes for saponification and methylation. Two ml of 6% sulphuric acid in methanol and 10 μL heptadecanoic acid as an internal standard (0.6 mg/mL C17:0) were added to each fraction and incubated for 2 h at 80 $^{\circ}\text{C}$. The samples were allowed to cool for 10 min and 1 mL of water and 2 mL of petroleum ether were added and the mixture was vortexed. Samples were centrifuged at 600 g for 6 min and the top phase containing the methylated phospholipid fractions was removed into a new tube and dried under N_2 . The lipids were resuspended in petroleum ether and transferred into autosampler vials for gas chromatograph analysis. 1 μL of each sample was injected using a 7683B series automatic injection system (Agilent Technologies) onto a Hewlett-Packard 6890N series gas chromatograph (GLC) (Agilent) equipped with an Innowax or a DB-23 (Agilent) 30-m fused silica capillary column (Supelco, Bellefonte, PA, USA) at 250 $^{\circ}\text{C}$ and proceeded using the following temperature profile: 160 $^{\circ}\text{C}$ for 4 min, increased 2 $^{\circ}\text{C}/\text{min}$ for 30 min, 220 $^{\circ}\text{C}$ for 16 min, increased 10 $^{\circ}\text{C}/\text{min}$ for 2 min, 240 $^{\circ}\text{C}$ for 2 min. Post-run was 130 $^{\circ}\text{C}$ for 6 min. The flow was 1.8 mL/min and the velocity through the column was 37 cm/s. GLC retention times were verified using two standards, PUFA No. 3 from menhaden oil and fatty acid methyl esters mix C4–C24 (Supelco).

Statistical analysis

All statistical analyses were performed using SigmaStat (Systat Software Inc., San Jose, CA, USA). One-way ANOVA and Tukey's post hoc tests were used to test for significance between diets and between the condition factors of the three groups of fish. Where data did not pass a normality test, a log transformation was used to normalize the data. Significance level was set at $p < 0.05$.

Results

Growth Performance

No significant differences were observed in the growth of the fish fed the different diets ($p > 0.05$) (Table 3.4). On average, fish weight increased 220% and fork length 110%. Mortality was low ($< 1\%$) and unaffected by treatment.

Mitochondrial membrane composition

Mitochondrial phospholipid fatty acid composition was determined in red muscle and liver of fish fed the three different diets (Table 3.5). In general, fish fed a high PUFA diet tended to have higher proportions of PUFAs in their phospholipids, especially compared to the SFA diet, although this was not significant except for 22:6n3. The fish fed the PUFA diet had significantly more 22:6n3 in the red muscle mitochondrial membranes when compared to the fish fed the SFA diet ($p < 0.05$; Table 3.5). There appeared to be a similar pattern when looking at the individual phospholipid classes within the mitochondrial membrane (Table 3.6). The ratio of PC:PE tended to be lower in both tissues of fish fed the PUFA diet, however this did not reach the level of statistical significance (Table 3.6).

CPT I inhibition by M-CoA and V_{max}

The IC_{50} was determined in red muscle and liver across the three different diets. In the red muscle there was a trend towards differences between the diets (e.g. PUFA = 0.35 ± 0.11 and SFA = 1.83 ± 0.75) however, there was considerable variation in samples and thus these differences were not statistically significant (Table 3.7). In the liver, all three diets produced similar IC_{50} values (SFA = 0.11 ± 0.03 , CTL = 0.12 ± 0.05 , PUFA = 0.07 ± 0.03) with no statistically significant differences (Table 3.7).

M-CoA content

M-CoA content of the liver and red muscle tissues of fish fed the three experimental diets were not significantly different ($p > 0.05$, Fig. 3.1). In red muscle the average concentration of M-CoA was approximately 0.028 nmol/g while in the liver it was 0.0046 nmol/g.

Gene expression profiles

The changing saturation of the fats in diet had significant effects on the gene expression across all tissues.

CPT I mRNA expression was significantly increased in both liver and red muscle of fish fed the PUFA diet versus the control and SFA diet ($p < 0.05$; Fig. 3.2A). As well, the PUFA diet significantly increased CPT I mRNA expression in adipose tissue when compared to the SFA diet ($p = 0.033$; Fig. 3.2A). No changes were seen in the expression of CPT I in the white muscle or heart between diets (Fig. 3.2A).

The expression of PPAR α mRNA did not follow the same pattern as CPT I. In heart tissue, PPAR α expression was significantly higher in fish fed the PUFA diet compared to the control and SFA diets ($p = 0.032$, 0.005 respectively; Fig. 3.2B). Adipose tissue also had significantly higher PPAR α expression in fish on the PUFA diet when compared to the SFA diet ($p < 0.05$; Fig. 3.2B). In contrast, the expression of PPAR α in white muscle was significantly lower in the PUFA and SFA diets when compared to the control diet ($p < 0.05$; Fig. 3.2B). No changes were seen in the liver and red muscle expression of PPAR α (Fig. 3.2B).

The pattern of PPAR β expression was different across tissues and diets. In heart tissue the expression of PPAR β was significantly higher in fish fed the PUFA diet than those fed the SFA diet ($p = 0.029$) while in the liver the opposite occurred ($p = 0.020$; Fig. 3.2C). In white muscle, the same pattern was observed for PPAR β expression as for PPAR α expression. In both the PUFA and SFA diets PPAR β expression was significantly lower ($p < 0.05$). No significant differences were found in red muscle and adipose tissue ($p < 0.05$; Fig. 3.2C).

Discussion

Changes in dietary fatty acid composition can have profound effects on the regulation of fatty acid oxidation through a variety of genomic and non-genomic mechanisms. This is the first study to examine the effects of dietary fatty acid composition on several regulatory factors mediating mitochondrial fat oxidation in both red muscle and liver of fish. We show here that manipulating the fatty acid composition of the diet can result in significant changes in gene expression for CPT I and transcription factors PPAR α and PPAR β . Small changes in red muscle mitochondrial fatty acid composition of rainbow trout were also observed in response to changes in dietary fatty acid composition. However, no other indices of fluidity such as PC:PE ratio determined through mitochondrial membrane composition changed significantly between diets. Although DHA increases in the PUFA fed fish compared to the SFA fed fish, there were no significant differences in the sensitivity of CPT I to its allosteric inhibitor M-CoA. The major finding of this experiment is that feeding of a diet rich in n-3 PUFA (mainly DHA) resulted in significant effects on the expression of various fat oxidation genes. In most cases, feeding the high PUFA diet resulted in an increase in either CPT I, PPAR α and PPAR β , with some exceptions. PUFAs appear to act differentially on CPT I and PPAR expression as there is no clear connection between PPAR expression and induction of CPT I expression.

Gene expression profiles

The induced expression of CPT I is influenced, in part, by a group of transcription factors, peroxisome proliferator-activated receptors (PPAR). In mammals, CPT I contains a PPAR response element (PPRE) (Price et al., 2000). PPARs have been found to be activated by fatty acids, particularly n-3 PUFAs (Desvergne and Wahli, 1999). Here we have shown that modifying the proportions of saturated and unsaturated fatty acids in the diet can have profound effects on the expression of PPARs and CPT I, but differentially across tissues (Fig. 3.2A-C). There is evidence in mammals that feeding high fat diets will increase CPT I expression compared to low fat diets (Tabarin et al., 2005; Thumelin et al., 1994) presumably through activation of PPAR α . However, other PPAR α -independent mechanisms exist for the induction of CPT I as shown in rat hepatocytes (LeMay et al., 2005). In fish however, there is limited knowledge on how lipids affect PPAR expression, or indeed how this might impact CPT I expression. PPAR isoforms found in mammals (α , β , γ) have been identified in numerous fish species (Leaver et al., 2005; Leaver et al., 2007; Robinson-Rechavi et al., 2001a) but studies on their functional roles are very limited. In mammals, PPAR α and β are more highly involved in regulating fatty acid oxidation in most tissues, whereas PPAR γ is generally restricted to adipocyte differentiation (Desvergne and Wahli, 1999). The situation may be even more complicated in fish since whole genome duplication events have led to multiple isoforms of PPAR β and PPAR α previously shown in zebrafish and Atlantic Salmon (Leaver et al., 2007; Robinson-Rechavi et al., 2001a). These multiple isoforms may or may not share the same function as they do in mammals or in other fish, may be silenced, or may have new roles not existent in other species. As well, their expression may vary across tissues making their genomic and functional studies much more difficult.

We predicted that feeding a high PUFA diet would result in significantly higher expression of CPT I across tissues as PUFAs are known activators of PPAR α (Brandt et al., 1998). CPT I expression was increased in the red muscle, adipose tissue and liver between three and seven fold in fish fed the high PUFA diet compared to the control but there were no changes in heart or white muscle expression (Fig. 3.2A). Interestingly, this increased CPT I expression in red muscle and liver was not reflected in the maximal activity of the enzyme as there was no increase in CPT I V_{max} in fish fed the PUFA diet (data not shown). Only in the adipose tissue did we see the corresponding increase in PPAR α expression (Fig. 3.2B). Conversely, in the heart, we saw a significant increase in PPAR α expression but no corresponding increase in CPT I expression. In fact, feeding the high SFA diet increased PPAR α expression in the liver while feeding the PUFA diet did not. In mammals, PPAR α is generally expressed at substantial levels only in highly oxidative tissues such as heart, red muscle liver and intestinal mucosa (Escher et al., 2001) while we have previously showed it to be highest in the liver of trout (Morash et al., 2008). From the present results it seems that there is no clear association between PPAR α and CPT I expression with increased dietary n-3 PUFA intake except for in the adipose tissue. However, the present results can be explained in a few other ways: 1) changes in PPAR mRNA expression does not necessarily result in changes in PPAR protein content, therefore, there may be changes in protein content that we have not tested for that could be inducing CPT I expression, 2) that there is a specific temporal pattern of expression and that PPAR α may have been expressed earlier during the treatment, 3)

there is an isoform specific difference in PPAR response elements across tissues, or 4) other signaling mechanisms exist up- or down-stream of PPAR affect CPT I expression in trout. In fact, in rat hepatoma cells inhibition of PPAR α expression has no effect on the long chain fatty acid induced CPT I expression (LeMay et al., 2005). A PPAR α -independent pathway of induction may also be present in fish but requires further investigation.

PPAR β expression was quite variable across tissues in trout. We found that feeding the high PUFA diet increased expression of PPAR β in the heart but not in any other tissue (Fig. 3.2C). Interestingly, like PPAR α , the feeding of the SFA diet resulted in an increase of PPAR β expression in the liver. In mammals, PPAR β has been shown to be ubiquitously expressed and plays a role in whole body lipid homeostasis and is only moderately induced by PUFAs (Forman et al., 1997). Our results show few changes across tissues in PPAR β expression with an increase in PUFAs in the diet suggesting that in fish, similar to mammals, that PPAR β may possibly be more involved in whole body lipid homeostasis and is not inducible by changes in the unsaturation of fatty acids in the diet.

Mitochondrial membrane composition and CPT I inhibition by M-CoA

Recently we have shown a correlation between membrane DHA(22:6n3) and PC/PE, implicated in fluidity, and CPT I sensitivity to M-CoA in rainbow trout (Morash et al., 2008). In the current study, changing the composition of the fats in the diet did not have a statistically significant effect on the composition of the mitochondrial membranes from liver and red muscle, although there was a relatively small statistically significant change in the percent of DHA in the red muscle. Most likely, variations in experimental diets, including types of fatty acids, total fat content, etc. and achieved nutrient deposition all have significant implications on this effect. In our study the lack of response in the mitochondrial membrane may be that there was not a sufficient deposition of dietary fatty acids into the mitochondrial membrane after 8 weeks on this particular diet or that the differences in the concentration of unsaturated fat in the diets were not adequate enough to elicit the expected changes in membrane composition. These two factors combined may have resulted in there being a minimal change in the existing lipid stores already present in the fish at the beginning of the experiment. Furthermore, we may have failed to see a significant remodeling of the mitochondrial membranes due to high inter-individual variability, or potentially due to differences in taste, although the amount of food consumed by each group was similar (data not shown).

There is considerable information on how manipulating fatty acid composition in the diet will result in changes in the plasma membrane composition of fish (Robin et al., 2003). However, there is very little known about how mitochondrial membranes in fish respond to changes in dietary lipid quality, especially at the level of muscle. In mammals and fish, membranes can be modified through the action of desaturases, which add double bonds to long chain fatty acids. In the liver, desaturases are controlled in part by dietary PUFAs, via the activation of PPAR α and sterol regulatory element binding protein-1c (SREBP-1c) (Nakamura and Nara, 2004). Increases in exogenous PUFAs will reduce the formation of endogenous PUFAs via inhibition of a variety of desaturases (Nakamura and Nara, 2004). Furthermore, large increases in dietary PUFAs such as 18:3n3 can result

in storage in adipose tissue as they are often used in the liver for the formation of triacylglycerol (TAG) (Garland et al., 1998).

Our previous work with rainbow trout has shown that tissue specific differences in mitochondrial membrane composition result in changes in CPT I IC_{50} (Morash et al., 2008). There is a lack of data regarding tissue-specific dietary effects on mitochondrial membrane composition and CPT I kinetics. Other studies in rainbow trout have found that diet can significantly change the concentration of DHA in the mitochondrial membrane (Guderley et al., 2008) while studies in brown trout have shown that an increase in n-3 PUFAs in the diet will produce increases in CPT I activity, although membrane composition and expected fluidity were not assessed in this case (Turchini et al., 2003). There were no changes in CPT I IC_{50} in either the red muscle or the liver of the fish fed the three different diets (Table 3.7). It may be that changing the concentration of DHA alone in the mitochondrial membrane is not enough to elicit a response in CPT I IC_{50} . Although we were unable to detect any other significant changes in membrane composition, it is still possible that the fluidity may have changed as the composition is not always directly related to fluidity. We did not evaluate other aspects of the mitochondrial membrane which may affect the composition/fluidity such as cholesterol content (Hazel, 1995). There may be differences in the concentration of cholesterol in the mitochondrial membranes which may change the fluidity.

There is considerable variation in individual measures of membrane composition and IC_{50} in both tissues when comparing the effects of the different diets of this study. This phenomenon is also apparent in mammalian studies as well and resulted in contradictory results. There have been studies in rat livers that indicate that dietary manipulations will alter membrane composition and effect CPT I activity (Power et al., 1994), as well as studies to the contrary where there is no effect (Brady et al., 1989; Wong et al., 1984). The correlation between CPT I IC_{50} for M-CoA and membrane fluidity has been shown in both mammals (Zammit et al., 1998) and indirectly in fish (Morash et al., 2008) and our current data neither negate or strengthen this correlation.

M-CoA content

In mammals M-CoA content in the liver is tightly connected to fat metabolism and the levels of fat in the diet. An increase in lipids in the diet will decrease hepatic lipogenesis as the fatty acid requirements are being met through dietary intake. In this case, M-CoA levels may fall and the capacity for fat oxidation should increase. Very little is known about the regulation of M-CoA in the muscle in either fish or mammals. To date, there have been no studies investigating the effects of fatty acid composition of the diet on M-CoA content of the liver or muscle of mammals or fish. In the present study the dietary lipid concentration was kept constant and only the concentration of saturated and unsaturated fatty acids were varied to assess whether or not the type of fat in the diet would affect the levels of M-CoA in the liver and red muscle. We found similar concentrations of M-CoA in the control samples as a previous study (Morash et al., 2008), but did not find any significant differences in the amount of M-CoA in either of the tissues with either high PUFA or high SFA diets (Table 3.7). This may indicate that

the total fat content of the diet, and not the unsaturation of the fatty acids, plays a larger role in regulating M-CoA levels.

Conclusions

There are inherent differences in the capacity for fat oxidation across tissues as well as between fish and mammals. The regulation of fatty acid oxidation is regulated by a number of genetic and non-genetic factors that ensure energetic demands are being met within the cells. As well, fish frequently encounter changing diets in the wild. In an aquaculture context, lipid content and fatty acid composition of the diet can frequently be modified (Bureau et al., 2008). Therefore, the regulation of lipid oxidation in muscle requires further investigation in the context of changing diet. We expected to find an increase of PUFAs in the mitochondrial membranes of fish fed the high PUFA diet which may ultimately increase the fluidity of the mitochondrial membrane. This increase in PUFAs was expected to increase the IC_{50} for M-CoA of CPT I. However, we found few significant changes in the mitochondrial membrane composition, and no significant differences in the PC:PE ratio between diets. Consequently membrane fluidity is not expected to have been modified significantly in this experiment. As well, there were no significant changes in IC_{50} for M-CoA of CPT I. The absence of changes in both types of parameters in this study, does not allow us to confidently state that membrane fluidity affects the sensitivity of CPT I to M-CoA. Furthermore, we found no significant differences in the concentration of M-CoA between diets indicating that the unsaturation of the fatty acids in the diet likely does not play a large role in regulating M-CoA. The present study implies that during changes in dietary fatty acid composition the genetic control of CPT I plays a larger role in regulating fatty acid oxidation capacity than other non-genetic mechanisms studied here, as there were significant changes in gene expression of CPT I and its transcription factors, but not in CPT I sensitivity to M-CoA, mitochondrial membrane composition, or M-CoA content between the diets.

Table 3.1: Composition of experimental diets.

Ingredients (g/Kg as is basis)	Diet		
	SFA	CTL	PUFA
Fish meal	400	400	400
Corn gluten meal	200	200	200
Wheat middlings	226	226	226
Fish oil	-	25	50
Ethoxyquin	0.5	0.5	0.5
Fish oil concentrate	-	20	40
Olive oil	60	60	60
Beef Tallow	90	45	-
Vitamin Premix	6	6	6
Lysine	5	5	5
Methionine	2	2	2
Choline chloride	3	3	3
Mineral premix	3	3	3
NaCl	3	3	3
Rovimixsaty-C (25%)	1.5	1.5	1.5
Total	1000	1000	1000
<i>Determined diet composition, as is basis</i>			
Dry matter (%)	92.9	92.9	92.9
Crude protein (%)	43.1	43.1	43.1
Crude fat (%)	20.7	20.7	20.7
Ash (%)	6.2	6.2	6.2
Gross energy (MJ/kg)	22.0	22.0	22.0

SFA=high saturated fat, CTL=control, PUFA=high polyunsaturated fat.

Table 3.2: Mol% of individual fatty acids in experimental diets.

Fatty Acid	Diet		
	SFA	CTL	PUFA
C14:0	1.67	1.06	0.34
C14:1	0.24	0.15	0.02
C15:0	0.29	0.21	0.07
C16:0	18.78	13.00	6.46
C16:1	1.86	1.55	1.07
C18:0	11.74	7.03	2.01
C18:1	54.34	46.87	36.97
C18:2n6	7.77	6.37	4.73
C18:3n6	0.03	0.05	0.06
C18:3n3	0.69	0.61	0.48
C18:4n3	0.01	0.14	0.25
C20:0	0.52	0.44	0.45
C20:1	0.48	0.55	0.68
C20:2n6	0.11	0.11	0.15
C20:3n6	0.05	0.05	0.09
C20:4n6 AA	0.08	0.13	0.20
C20:3n3	0.02	0.06	0.10
C20:4n3	0.01	0.02	0.03
C20:5n3 EPA	0.07	1.83	3.90
C22:0	0.18	0.22	0.33
C22:1	0.46	1.18	1.89
C22:2n6	0.02	0.49	1.08
C22:4n6	0.03	0.22	0.39
C22:5n6	0.00	0.47	0.97
C22:5n3	0.11	2.67	5.59
C22:6n3 DHA	0.38	13.92	29.71
C24:0	0.04	0.10	0.33
C24:1	0.01	0.52	1.65
Total	100.00	100.00	100.00
Saturated	33.23	22.07	9.98
Monounsaturated	57.39	50.81	42.29
Polyunsaturated	9.38	27.12	47.74
η-3	1.29	19.23	40.06
η-6	8.09	7.89	7.67
η-3/η-6	0.16	2.44	5.22

SFA=high saturated fat, CTL=control, PUFA=high polyunsaturated fat.

Table 3.3: Forward (F) and reverse (R) primers used for real-time PCR analysis of mRNA expression in trout.

Gene	Primer
Efl α	F - 5' CAT TGA CAA GAG AAC CAT TGA 3' R - 5' CCT TCA GCT TGT CCA GCA C 3'
CPTI	F - 5' GCC GCA AAC TAG AGA GAG GA 3' R - 5' CCC GTA GTA CAG CCA CAC CT 3'
PPAR α	F - 5' CCA AGT TCA GTT TGC CAT GA 3' R - 5' ATT GGG GAA GAG GAA GGT GT 3'
PPAR β	F - 5' CTG GAG CTG GAT GAC AGT GA 3' R - 5' GTC AGC CAT CTT GTT GAG CA 3'

Table 3.4: Growth of experimental fish. Initial condition measurements were taken prior to the start of experimental feeding. Final condition measurements were taken after 8 weeks of being fed either a high saturated fat (SFA) diet, control diet (CTL) or a high polyunsaturated fat (PUFA) diet.

Diet	Initial Condition			Final Condition			
	BW (g)	BL (cm)	CF	BW (g)	BL (cm)	CF	% weight gain
SFA	123.1 ± 1.2	23.6 ± 0.2	0.93 ± 0.02	262.5 ± 6.5	27.8 ± 0.2	1.21 ± 0.02	213
CTL	125.7 ± 1.2	23.7 ± 0.3	0.94 ± 0.02	290.7 ± 24.4	27.7 ± 0.8	1.35 ± 0.05	231
PUFA	122.7 ± 1.2	23.8 ± 0.2	0.91 ± 0.01	298.1 ± 26.5	28.1 ± 0.5	1.34 ± 0.09	243

BW=body weight.
 BL=body length.
 CF=condition factor

Table 3.5: Total average mol percent contributions of individual fatty acids (FA) to total FA from the mitochondrial membrane phospholipids in red muscle and liver after 8 weeks of an experimental diet. Values are means \pm S.E., n=4. Different symbols denote significant difference (p<0.05).

Fatty Acid	Red Muscle			Liver		
	SFA	CTL	PUFA	SFA	CTL	PUFA
C14:0	1.1 \pm 0.1	1.0 \pm 0.1	0.8 \pm 0.4	0.5 \pm 0.3	1.2 \pm 0.3	1.4 \pm 0.1
C16:0	20.2 \pm 4.8	21.3 \pm 2.3	18.8 \pm 0.8	23.2 \pm 3.4	21.4 \pm 1.8	20.3 \pm 1.3
C16:1N7	0.8 \pm 0.3	0.8 \pm 0.2	0.8 \pm 0.3	0.6 \pm 0.2	1.0 \pm 0.1	0.8 \pm 0.4
C18:0	7.7 \pm 1.1	7.9 \pm 1.4	5.2 \pm 1.3	6.3 \pm 1.0	5.9 \pm 1.3	5.6 \pm 0.5
C18:1N7	9.3 \pm 2.9	9.9 \pm 1.2	8.7 \pm 0.8	11.4 \pm 2.0	9.8 \pm 1.5	8.9 \pm 1.2
C18:2N6	4.4 \pm 1.0	3.7 \pm 0.2	8.9 \pm 5.1	4.4 \pm 0.7	4.2 \pm 0.8	3.8 \pm 0.8
C18:3N3	4.9 \pm 3.0	2.8 \pm 1.4	2.4 \pm 1.9	2.9 \pm 1.7	2.5 \pm 2.5	1.7 \pm 1.7
C20:1N9	6.2 \pm 3.8	3.3 \pm 1.9	3.5 \pm 3.0	4.0 \pm 1.8	3.1 \pm 3.1	2.5 \pm 2.2
C20:4n6	7.1 \pm 3.7	3.3 \pm 1.7	3.7 \pm 3.0	5.6 \pm 1.8	4.1 \pm 3.0	3.6 \pm 2.2
C20:5N3	2.9 \pm 1.8	2.4 \pm 1.4	2.7 \pm 1.2	1.4 \pm 1.4	3.9 \pm 2.1	3.7 \pm 1.8
C22:5N3	6.5 \pm 2.1	2.8 \pm 0.7	3.3 \pm 1.7	3.8 \pm 1.1	3.0 \pm 2.1	1.8 \pm 1.2
C22:6N3	21.2 \pm 9.7 ^a	35.6 \pm 7.4 ^{ab}	40.4 \pm 6.6 ^b	31.5 \pm 4.5	36.9 \pm 8.5	42.9 \pm 5.1
saturates	29.0 \pm 4.9	30.4 \pm 3.4	24.8 \pm 0.9	29.9 \pm 2.2	28.5 \pm 0.4	27.3 \pm 0.7
monounsaturates	16.3 \pm 2.1	14.0 \pm 1.8	13.0 \pm 2.9	16.0 \pm 0.5	14.0 \pm 1.4	12.3 \pm 1.3
polyunsaturates	47.16 \pm 5.4	50.7 \pm 5.5	61.6 \pm 3.8	49.7 \pm 1.3	54.8 \pm 3.2	57.6 \pm 2.8
η -3 polyunsaturates	35.6 \pm 6.6	43.6 \pm 6.9	48.9 \pm 6.3	39.6 \pm 2.6	46.5 \pm 5.0	50.2 \pm 4.0
η -6 polyunsaturates	11.5 \pm 2.7	7.1 \pm 1.9	12.6 \pm 5.0	10.0 \pm 2.1	8.3 \pm 1.8	7.4 \pm 1.9
η -3/ η -6	3.6 \pm 1.7	8.5 \pm 3.2	7.4 \pm 5.8	5.0 \pm 1.7	6.6 \pm 1.7	7.8 \pm 2.1
UI	2.5 \pm 0.4	2.8 \pm 0.4	3.3 \pm 0.3	2.7 \pm 0.1	3.0 \pm 0.2	3.2 \pm 0.2

SFA=high saturated fat, CTL=control, PUFA=high polyunsaturated fat.

†Unsaturation index= $\sum mi \cdot ni$; where mi is the mole percentage and ni is the number of C–C double bonds in the fatty acid “i”.

Table 3.6: Mol% of mitochondrial membrane phospholipid classes in red muscle and liver after 8 weeks of an experimental diet. Values are means \pm SE, n=4.

Phospholipid Class	Red Muscle			Liver		
	SFA	CTL	PUFA	SFA	CTL	PUFA
Phosphatidylcholine	38.6 \pm 6.7	41.9 \pm 6.4	45.3 \pm 6.2	53.4 \pm 5.0	49.2 \pm 4.5	50.1 \pm 4.4
Phosphatidylserine	8.2 \pm 2.9	1.7 \pm 0.1	2.7 \pm 1.2	3.0 \pm 1.7	1.8 \pm 0.4	1.7 \pm 0.7
Phosphatidylinositol	9.3 \pm 4.9	7.8 \pm 3.1	9.1 \pm 4.3	6.6 \pm 3.0	16.2 \pm 4.8	6.4 \pm 2.5
Phosphatidylethanolamine	21.8 \pm 3.0	34.1 \pm 6.6	29.8 \pm 2.0	26.5 \pm 2.5	23.0 \pm 2.5	32.5 \pm 6.0
Cardiolipin	14.5 \pm 3.5	9.5 \pm 3.9	11.1 \pm 1.2	6.1 \pm 1.9	7.0 \pm 2.5	6.4 \pm 1.7
PC:PE†	1.6 \pm 0.2	1.4 \pm 0.3	1.5 \pm 0.2	2.0 \pm 0.03	2.1 \pm 0.06	1.3 \pm 0.3

SFA=high saturated fat, CTL=control, PUFA=high polyunsaturated fat.

†PC=phosphatidylcholine, PE=phosphatidylethanolamine.

Table 3.7: The concentration of malonyl-CoA (μM) to reduce the activity of malonyl-CoA sensitive carnitine palmitoyltransferase (CPT) I activity by 50% (IC₅₀) under high saturated fat (SFA) diet, control (CTL) diet or high polyunsaturated fat (PUFA) diet. Values are means \pm SE. Liver and white muscle, n=5; heart, n=4; red muscle, n=3.

Diet	Tissue	
	Liver	Red Muscle
SFA	0.11 \pm 0.03	1.83 \pm 0.75
CTL	0.12 \pm 0.05	1.19 \pm 0.89
PUFA	0.07 \pm 0.02	0.35 \pm 0.11

Fig. 3.1

Malonyl-CoA (M-CoA) content in red muscle and liver under high saturated fat (SFA) diet, control (CTL) diet or high polyunsaturated fat (PUFA) diet. Values are means \pm S.E. for four animals.

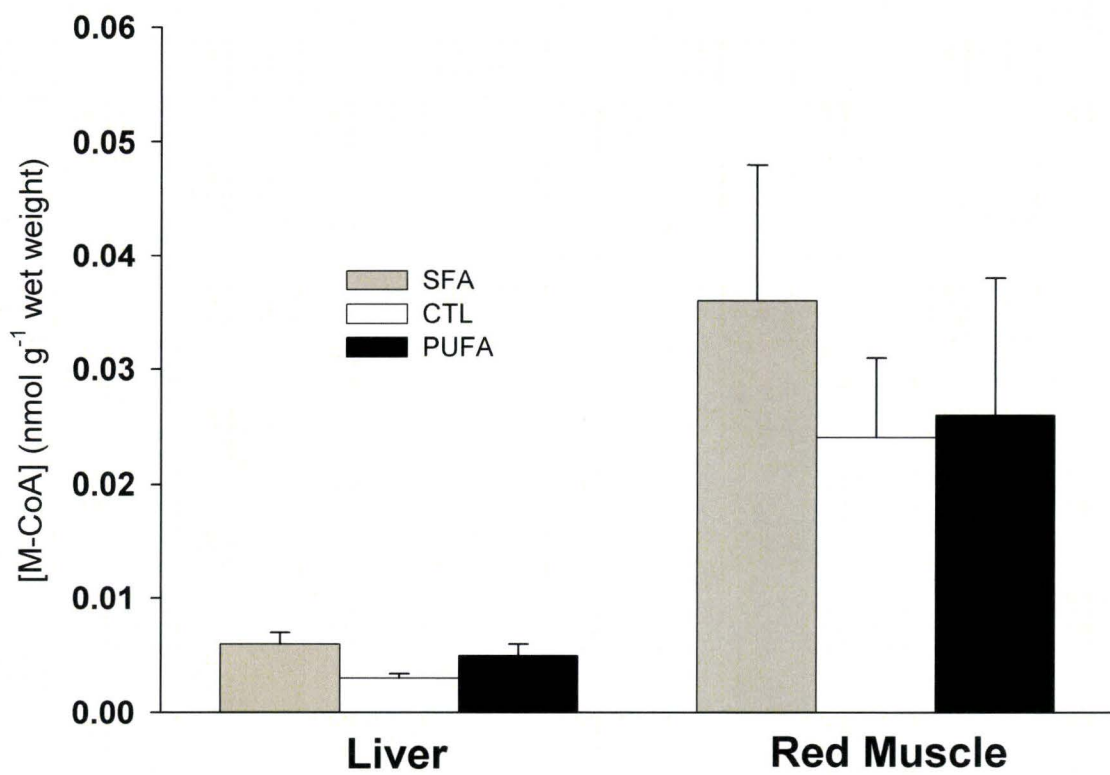
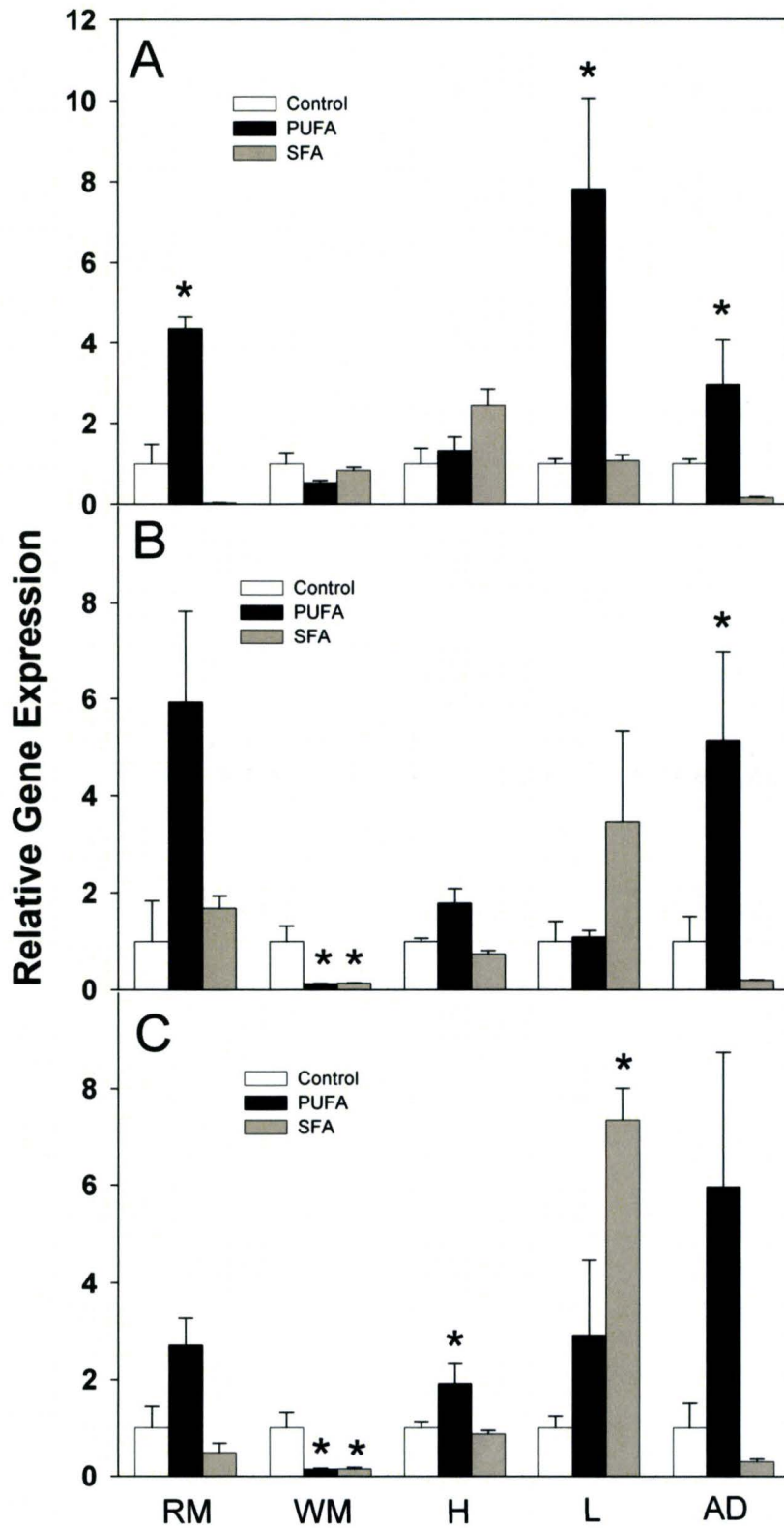


Fig. 3.2

Real time PCR gene expression profiles of A) carnitine palmitoyltransferase I (CPT I), B) peroxisome proliferator activated receptor α (PPAR α) and C) peroxisome proliferator activated receptor β (PPAR β) in red muscle (RM), white muscle (WM), heart (H), liver (L), and adipose tissue (AD). Values are expressed relative to EF1 α and are means \pm S.E. n=4 for each tissue. Asterisks denotes significance between diets, $p < 0.05$.



GENOME DUPLICATION EVENTS HAVE LEAD TO A DIVERSIFICATION IN
THE CPT I GENE FAMILY IN FISH

Abstract

The enzyme, carnitine palmitoyltransferase (CPT) I, is a major regulator of mitochondrial fatty acid oxidation in vertebrates. Numerous genome duplication events throughout evolution have given rise to three or multiple genetically and functionally different isoforms of this enzyme in mammals and fish, respectively. In particular, these isoforms represent a diversification of kinetic and regulatory properties stemming from mutations at the genomic and proteomic levels. Using phylogenetic reconstructions a comprehensive view of the CPT I family in vertebrates emerges, and reveals genomic modifications leading to structural changes in proteins and functional differences between tissues and taxa. In a model fish species (rainbow trout), the presence of five CPT I isoforms suggest repeated duplication events in bony fishes and salmonids. Subsequently, an array of nucleotide and amino acid substitutions in the isoforms exist and may contribute to a tissue- and previously observed species-specific difference in the IC_{50} for malonyl-CoA. Moreover, all five isoforms are expressed in trout at the mRNA level in skeletal muscle, heart, liver, kidney and intestine. In general, muscle tissues had higher transcript levels of the β isoforms while other tissues had higher levels of the α isoforms. Rainbow trout also exhibit developmental plasticity in relative mRNA expression of CPT I isoforms from fry to juvenile to adult. Thus, the evolution of CPT I has resulted in a very diverse family of isoforms. These differences represent a degree of specificity in the ability of species to regulate function at the protein and tissue level which in turn, may allow for precise control of lipid oxidation in individual tissues during physiological perturbations.

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Introduction

As a result of ancient whole genome duplication events vertebrates species have multiple copies of certain genes. These duplicated genes are considered a major source of biological diversity (Ohno, 1970). After duplication, genes may have several fates including, 1) pseudogenization, in which extra copies become functionless or unexpressed, 2) sub-functionalization, where the duplicated copies each adopt part of the functions of the ancestral gene, or 3) neo-functionalization, in which duplicated gene evolves rapidly and develops a novel function (Zhang, 2003). In mammals, for example, the enzyme carnitine palmitoyltransferase (CPT) I exists in multiple isoforms with specific tissue expression and distinct functional characteristics (McGarry et al., 1983). After the divergence of fish and mammals there were several lineage-specific genome duplication events in fish which gave rise to multiple isoforms of proteins for which mammals have few or even a single copy. Moreover, for many fish species, genes with neo- or sub-functionalization have not been fully sequenced or characterized (Robinson-Rechavi and Laudet, 2001). It is possible that proteins coded for by these duplicated genes in fish may have different tissue expression patterns or physiological functions when compared to the corresponding genes in mammals. Thus, lineage- and species-specific genome duplication events can lead to increased diversity in protein regulation and function. CPT I is an important regulator of mitochondrial fatty acid oxidation, essential for the entry of long chain fatty acids into mitochondria (McGarry and Brown, 1997). In mammals, there are three CPT I isoforms expressed in a well defined tissue-specific pattern; hepatic CPT I α , muscle CPT I β and CPT I γ , a brain-specific isoform. As these proteins all share very similar functional domains, and similar physiological functions, it is likely that these isoforms arose from an ancient genome duplication early in vertebrate history. In mammals, they are encoded by three separate genes and are strictly expressed in their distinct tissues, except for the heart which expresses both α and β isoforms but to different degrees depending on developmental stage (Brown et al., 1995). The kinetics of mammalian muscle and liver CPT I isoforms have been extensively studied. CPT I β is approximately 80 times more sensitive to its allosteric inhibitor, malonyl-CoA (M-CoA, $IC_{50} = 0.034\mu\text{M}$ vs $2.7\mu\text{M}$), and has a much higher K_m for its substrate, carnitine ($K_m = 500\mu\text{M}$ vs. $30\mu\text{M}$), than CPT I α (McGarry and Brown, 1997). However, it is not clear if these isoforms are either conserved across vertebrates or how many additional isoforms arose in non mammalian species, particularly in fish where multiple large duplication events could have created opportunities for the diversification of this family. By using phylogenetic analysis we can evaluate when these duplications may have taken place in vertebrate evolution and determine which fish-specific isoforms may have a distinct functional role compared to their mammalian paralogs.

Multiple putative copies of the α and β isoforms appear in several species of fish whose genomes have now been fully sequenced. Although known to exist, there is relatively little information on their relative tissue expression, protein structure or kinetics. In fact, to date, only a single CPT I isoform has been fully sequenced from rainbow trout and its tissue distribution and putative kinetics presented. This isoform has been proposed to be similar in kinetics to the mammalian CPT I liver isoform (Gutierrez et

al., 2003). Although other CPT I gene sequences for rainbow trout can be found in databases, there has been little research on the tissue distribution, kinetics or relationship of these paralogues to other isoforms (Kolditz et al., 2008). Interestingly, we determined that activity varies across tissues in rainbow trout and that the IC_{50} for M-CoA was higher in red muscle ($0.55\mu\text{M}$) than in liver ($0.079\mu\text{M}$) (Morash et al., 2008), opposite to the situation in mammals (Morash et al., 2008). This suggests species-specific differences in CPT I kinetics that could likely be a result from diversity in protein structure in the substrate and modulator binding sites. It also highlights trout as a good model to examine isoform diversity arising from duplication events.

The difference in IC_{50} between different isoforms in mammals has been attributed to structural changes in the M-CoA binding site and its interaction with the transmembrane domains of the mature protein (Jackson et al., 2000; Morillas et al., 2003; Shi et al., 2000). CPT I has two transmembrane domains with the terminal N- and C-termini protruding into the cytosol. When this interaction is interrupted, either through changes in mitochondrial membrane fluidity (Zammit et al., 1998) or conformational changes due to key amino acid substitutions, the ability of M-CoA to bind and inhibit CPT I is modified. There has been a great deal of research into the amino acid sequence of mammalian CPT I, and multiple amino acids have been identified as being influential to the binding on M-CoA (Jackson et al., 2000; Morillas et al., 2003; Shi et al., 2000). In most cases, these experiments examined CPT I α , with very little research on CPT I β . Furthermore, these differences have yet to be compared to the sequences of other isoforms and between species.

The variety of CPT I isoforms in fish species represents a putative diversity of primary amino acid sequences. Moreover, changes in key amino acids may lead to diversity in the regulation of mitochondrial fatty acid oxidation. Neither this diversity nor its putative functional significance have been extensively studied. Consequently, in this study we present a comprehensive phylogenetic reconstruction of the CPT I family in vertebrates, specifically in fish lineages using rainbow trout as our reference species. We assessed the primary protein structure for changes in amino acids in areas which may influence the changes in M-CoA sensitivity seen between tissues and species. We also examined the expression of all isoforms across tissues at different stages of development in trout. We show that repeated duplication events have led to at least five CPT I isoforms in rainbow trout. These isoforms exhibit sequence and structural differences when compared to each other as well as to their mammalian counterparts. Furthermore, their tissue distribution during development and throughout adulthood may help to explain known differences in CPT I characteristics across tissues in rainbow trout. In addition, our phylogenetic reconstruction of known CPT I in vertebrates reveals lineage-specific isoform diversification.

Materials and Methods

Experimental Fish

Adult rainbow trout (*Oncorhynchus mykiss*) were obtained from a local commercial trout hatchery (Humber Springs, Orangeville, ON), kept in 500 L tanks with

circulating dechlorinated Hamilton tap water at 12°C and fed a commercial diet (Profishent Classic Floating Trout Grower, Martin Mills, Elmira, ON). Rainbow trout embryos were obtained from Rainbow Springs Trout Farm in Thamesford, Ontario and maintained at the University of Guelph in the Hagen Aqualab. Embryos were kept in mesh bottom Heath trays at 10°C with circulating local well water. After hatching and yolk sac absorption juvenile trout were moved to McMaster University and kept in small 20L tanks with circulating 12°C dechlorinated Hamilton tap water and fed a commercial diet (Profishent Classic Floating Trout Grower) for 2 weeks. All experiments were approved by the McMaster University Animal Research Ethics Board according to the Canadian Council on Animal Care guidelines.

Trout were sampled at three different developmental stages; 1) 21 days after hatching while still using their yolk sac as their food source, 2) 14 days after total yolk sac absorption and feeding on external food, and 3) during adulthood (approx. 200g). For each time point trout were sacrificed by a lethal blow to the head and their spinal cord severed; muscle (mixed muscle samples were used for fry and juvenile trout, but separate red and white muscle samples were used in adults), heart and liver were extracted and frozen immediately in liquid nitrogen. Due to their size we were able to sample Intestine, kidney and brain from adult trout for a more comprehensive tissue distribution.

RNA extraction and cDNA synthesis

Frozen tissues were powdered in a liquid nitrogen-chilled mortar and pestle. Total RNA was extracted from each tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. RNA was quantified by UV spectroscopy at 260nm and then diluted to 0.5µg/µl. cDNA was synthesized using 1 µg of DNase (Invitrogen, Carlsbad, CA) treated mRNA with SuperScript RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA) as described previously (Morash et al., 2008).

Polymerase chain reaction (PCR), cloning and sequencing

CPT I sequences from multiple fish and mammals species were aligned and specific PCR primers for CPT Iα1b isoform (below) were designed using Primer3 software (Rozen and Skaletsky, 2000) from conserved regions.

CPT Iα1b was sequenced repeatedly using three separate overlapping (~20b.p.) sections of the gene from multiple samples of cDNA. Each section was amplified using 0.2mM dNTP, 1.5mM MgCl₂, 0.5 µM of each forward and reverse primer (Table 4.1; CPT IαS1,S2,S3), 1 unit of Taq DNA polymerase (Fermentas, Burlington, Ontario, Canada) and 1× Taq amplification buffer in a PTC-200 thermal cycler (MJ Research) with the following profile; 3 minutes at 95°C, 30 cycles 95°C for 15 seconds, 58 or 60°C (see table 1 for each section) for 30 seconds, 72°C for 60 seconds. PCR products were purified using QiaQuick gel extraction kit (Qiagen, Mississauga, Ontario, Canada), cloned using the pGEM-T easy vector system (Promega, Nepean, Ontario, Canada) and sequenced at the Mobix Lab (McMaster University). The 5' and 3' end of the sequence were obtained using a kit for rapid amplification of cDNA ends (RACE) (Roche, Mississauga, Ontario, Canada) using the protocol provided and gene specific primers

(Table 4.1). The final PCR product was purified, cloned and sequenced as described above.

mRNA quantification by real time PCR

The expression of each CPT I isoform mRNA was quantified using real time PCR with SYBR green and ROX as a reference dye using a Stratagene Mx3000P (Stratagene, Texas, USA) real-time PCR system. Each 25 μ L reaction contained 12.5 μ L SYBR green mix, 1 μ L each of forward and reverse primer (5 μ M), 5.5 μ L of DNase/RNase free water and 5 μ L of 5 \times diluted cDNA. Primers for CPT I α 1b were designed using Primer3 software (Rozen and Skaletsky, 2000) while other primers were obtained from previously published work (Table 4.2). The thermal program included 3 minutes at 95°C, 40 cycles of 95°C for 15 sec, 57°C for 30 sec and 72°C for 30 sec. A no-template control and dissociation curve was performed to ensure only one PCR product was being amplified and stock solutions were not contaminated. Standard curves were constructed for each gene using serial dilutions of stock cDNA to account for any differences in amplification efficiencies. All samples were normalized to the housekeeping gene, EF1- α whose mRNA expression did not change between tissues and treatments ($p > 0.05$).

Sequence alignments and analysis

Phylogenetic Analysis. CPT I nucleotide and protein sequences were obtained from public databases (Ensembl and GenBank; Table 4.2) or sequenced from rainbow trout tissues (see above). In all subsequent analyses, *Ciona intestinalis* was used as an out group of the modern vertebrate CPT I because it is phylogenetically outside of the lineages we focused upon. The predicted protein sequences were aligned with ClustalX v.2.0.5 software (Thompson et al., 1997). The phylogeny of the CPT I proteins was generated by Bayesian analysis using a mixed model approach (MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001)) with 10^6 generations, a sampling frequency of 100 and a burnin of 10000. The nucleotide sequences were aligned using ClustalX and Mega v.4.1 (Tamura et al., 2007) to reduce the impact of any non-homologous regions with either gaps or alignment discrepancies on the nucleotide phylogenetic analysis. Aligned sequences were subjected to ModelTest v.2.2 (Posada and Crandall, 1998) implemented in PAUP* v.4.0 (Swofford, 2000) to select the best evolutionary model to explain the data according to the Akaike Information Criterion (AIC). Based on this analysis the model chosen was GTR+I+G in all instances. Finally, we used the AIC as a prior in the Bayesian analysis (MrBayes, 5×10^6 generations and a sampling frequency of 100 and a burnin of 10000), and generated the nucleotide phylogenies of CPT I α and β in vertebrates according to the 50% majority rule. Stationarity and adequacy of settings of the phylogenetic analyses was confirmed in Tracer v1.5 (Lemey et al., 2009).

Protein structure analysis.

The transmembrane domains of each isoform were determined using TMHMM v.2.0 (Moller et al., 2001), except for CPT I α 2 where the full length sequence was not available.

Statistical analysis

All statistical analyses were performed using SigmaStat v3.5 (Systat Software Inc., San Jose, CA). One-way ANOVA and Holm-Sidak post tests were used to test for significance between tissues and treatments. Significance level was set at $\alpha=0.05$.

Results

CPT I phylogeny

We used Bayesian analyses to infer the phylogenetic relationship of the CPT I isoforms (both DNA and protein sequences) to investigate the evolution of CPT I in major vertebrate lineages (Fig. 4.1A-B). Overall, both nucleotide and protein analysis show individual clustering of mammalian and fish isoforms and present similar relationships of CPT I evolution. There are three major CPT I isoforms in mammalian vertebrates, two of which are also present in non-mammalian vertebrates; CPT I α and β . CPT I γ is only found in mammalian brain and does not appear in the *Xenopus* or any of the fish species (Fig. 4.1A) and therefore was excluded from the nucleotide analysis (Fig. 4.1B). Based on the tree topologies there appears to be several fish-specific gene duplications resulting in multiple expressed CPT I isoforms. We chose to name the isoforms based on these duplications, in that duplications of α become $\alpha 1$ and $\alpha 2$, and second duplications became $\alpha 1a$ and $\alpha 1b$ etc. This nomenclature allows us to retain information regarding the putative ancestral gene as well as the number of duplications. There appears to have been at least two CPT I α duplications; 1 resulting in CPT I $\alpha 1$ and CPT I $\alpha 2$, and a second round creating CPT I $\alpha 2a$ and b . Both zebrafish and rainbow trout also exhibit two CPT I $\alpha 1$ genes; a and b , while other fish species do not. Interestingly, while all other fish species investigated show two CPT I $\alpha 2$ isoforms (a and b), we detected only one isoform in trout which clusters with CPT I $\alpha 2b$ (Fig. 4.1B). CPT I β , on the other hand, has only a single isoform in mammals and most fish species, except for rainbow trout which exhibit two, $\beta 1a$ and $\beta 1b$.

CPT I sequence and structure

Since numerous amino acids confer M-CoA sensitivity in mammals, we aligned all five full length rainbow trout isoforms with rat CPT I α and β to investigate any changes in protein sequence which may affect their ability to effectively bind M-CoA (Fig. 4.2). There are many conserved regions between rat and trout throughout the protein, however, there are several major differences in both the N- and C-termini of the protein (boxes in Fig. 4.2). In the N-terminus isoleucine¹⁹ is substituted with valine¹⁹ in rat CPT I β . Furthermore, rat CPT I β and trout CPT I $\beta 1b$ are missing 11 and 20 residues between amino acids 27 and 47, respectively, when compared to the other isoforms. In the C-terminus, the putative M-CoA catalytic core identified in mammals between alanine⁴⁷⁸ and histidine⁴⁸³ is also present in all of the trout isoforms, however, there are major amino acid substitutions throughout (Fig. 4.2).

Aside from the primary protein structure, the regions adjacent to the transmembrane domains of CPT I have been also implicated in M-CoA sensitivity. We used TMHMM analysis to determine the transmembrane domains of the five trout CPT I

proteins and compared their location to that in rats (Fig. 4.3). CPT I α 2 was not included in the analysis because the 5' amino acid sequence was not complete. However, the transmembrane domains of the two trout α isoforms began at the same amino acid position as rat CPT I α . Conversely, the transmembrane domains in the trout CPT I β 1a and β 1b were different from each other as well as from rat CPT I β . Both trout β 1b and rat CPT I β had a shorter N-terminus than trout β 1a.

CPT I tissue and developmental expression

We investigated via real time PCR the tissue specific expression of the five known CPT I isoforms in adult rainbow trout (Fig. 4.4). While all five isoforms are present in all tissues examined, there are significantly different patterns of mRNA expression between tissues. β 1a and β 1b and α 1a mRNA expression levels were significantly higher in heart and red muscle and significantly lower in the liver ($p < 0.05$). Furthermore there were no significant differences between liver, white muscle and kidney for these isoforms ($p > 0.05$). α 1b and α 2 expression was significantly higher in kidney and intestine ($p < 0.05$). Interestingly, the expression of α 1b and α 2 was the same in liver and red muscle.

We found that during the development of rainbow trout from fry to adults the mRNA expression changed for the five CPT I isoforms in the heart, red muscle and liver (Fig. 4.5). During the cardiac development there appears to be a very significant switch in the gene expression of the CPT I isoforms. In the fry heart, CPTI β 1a and α 1b are expressed significantly higher than in juvenile fish, while conversely, β 1b and α 1a have significantly lower levels of mRNA expression in the fry than in the juvenile ($p < 0.05$; Fig. 4.5A). However, during the progression from fry to juvenile fish there is an exact reversal of the expression of these four isoforms ($p < 0.05$). This juvenile pattern of expression appears to remain unchanged in the adult trout (Fig. 4.5A).

The expression pattern of the CPT I isoforms in red muscle during development is distinct from heart and liver (Fig. 4.5B). Both β 1a and b as well as α 1a show a transient non-significant decrease in expression from fry to juvenile followed by a significant increase in expression from juvenile to the adult stage ($p < 0.05$), although this increase is not significantly higher than the original expression level in the fry. Furthermore, there are no significant changes in the expression of α 1b, while the expression of α 2 significantly decreases during adulthood. Comparatively, there were few changes in mRNA expression in the liver during development (Fig. 4.5C). Indeed, the mRNA levels of most isoforms (β 1b, α 1b and α 2) remained unchanged throughout the different developmental stages examined. Although, adult trout exhibited a marked increase in the expression of β 1a expression levels and a significant reduction in α 1a mRNA compared to fry and juvenile animals ($p < 0.05$).

Discussion

Gene duplication is a major foundation for biological diversity and can result in the neo-functionalization or sub-functionalization of one or both duplicate genes, creating temporal or spatial differences in the resulting paralogues (Zhang, 2003). This is the first

study to present the evolution and duplication of the CPT I gene family in the context of the possible functional divergence of these proteins. Duplication events have created multiple genetically and functionally divergent CPT I isoforms in all vertebrates. Moreover, we present five apparent CPT I isoforms in rainbow trout with distinct tissue and developmental expression patterns. We reveal discrete amino acid substitutions and potential structural differences between the isoforms which may be driving the species- and tissue-specific functional differences in CPT I (McGarry et al., 1983; Morash et al., 2008). Thus, genome duplication events have diversified the CPT I gene family, especially in fish.

Evolution of the CPT I family

Our amino acid and nucleotide phylogenetic reconstruction of the vertebrate CPT I isoforms suggests that there was a duplication of an ancestral CPT I gene which gave rise to the α and β paralogues in both mammals and fish (Fig. 4.1). A second duplication event may have occurred after the divergence of the teleost fish lineage and resulted in the $\alpha 1$ and $\alpha 2$ isoforms. The likelihood of this duplication event is tentative, however, given the relatively low probability at that node (0.87). Alternatively, there may have been a pseudogenization of the second alpha gene in mammals. However, previous attempts to identify CPT I α or β pseudogenes in mammals have been unsuccessful (Cox et al., 1998; van der Leij et al., 2000). Beyond these first two duplications, the number of isoforms present becomes very lineage-specific and likely arose through independent duplication events (Robinson-Rechavi et al., 2001b). Firstly, a duplication of the $\alpha 1$ gene resulted in $\alpha 1a$ and $\alpha 1b$ in both zebrafish and rainbow trout, but not in the other fish species investigated and likely occurred after the divergence from these other species. Conversely, the duplication may have occurred early in the teleost lineage, but $\alpha 1b$ was lost in the more modern fish species (Fig. 4.1). Secondly, a duplication of $\alpha 2$ resulted in $\alpha 2a$ and $\alpha 2b$ in all fish species examined except for rainbow trout. In this instance, $\alpha 2a$ may not have been retained in rainbow trout. However, because both isoforms are present in zebrafish and the other more recently evolved fishes the more probable cause for a lack $\alpha 2a$ in trout is that it has not been identified yet despite our extensive efforts. Thirdly, there appears to have been a salmonid specific duplication of $\beta 1$ giving rise to $\beta 1a$ and $\beta 1b$ only in rainbow trout. Alternatively, these two isoforms may be splice variants, as they show 92% and 94% similarity at the nucleotide and protein level, respectively. However, this is unlikely as there are differences in amino acids throughout the length of the protein, and not localized in any specific region.

CPT I sequence and structure

It has been proposed that after gene duplication, each paralog retains the same activity level as the ancestral gene but becomes more specifically regulated at the mRNA level (Hughes, 1994). Alternatively, it has been suggested that highly constrained amino acids may diverge after duplication leading to protein with new function (Robinson-Rechavi and Laudet, 2001). Given the divergence in CPT I isoform function between tissues and species, we wanted to investigate the roles of particular amino acids involved in CPT I M-CoA sensitivity. Amino acids which have an important role in M-CoA

sensitivity have been determined in mammals by mutagenesis experiments (compiled in Table 4.4). Using these data we probed the sequences of the rainbow trout CPT I isoforms and compared regions important in M-CoA inhibition to those of a representative mammal (rat; Fig. 4.2). The majority of amino acids were conserved between the CPT I isoforms in trout and rat. However, a few key substitutions are present and extend throughout the other species included in the phylogenetic analysis (data not shown). The first important substitution occurred at the location corresponding to amino acid 19 of the rat CPT I β protein. This site in all other isoforms from trout and rat contains an isoleucine, whereas in rat CPT I β a valine is present. Interestingly, mutagenesis experiments show a decrease in M-CoA sensitivity when valine is replaced by alanine in this position (Zhu et al., 2003). This substitution may partially explain why mammalian CPT I β is approximately 80 times more sensitive than CPT I α , and 3 and 18 times more sensitive than trout liver and muscle CPT I, respectively (McGarry et al., 1983; Morash et al., 2008). Secondly, specific characteristics of residues 19-30 impart a low M-CoA sensitivity in mammalian CPT I α distinct from the more sensitive CPT I β (Jackson et al., 2000; Morillas et al., 2002). Trout CPT I β 1b is missing four amino acids within this region, which may increase its sensitivity to M-CoA (Fig. 4.2). Potentially an increased sensitivity of trout muscle CPT I compared to rat liver may have to do with the significant expression of the CPT I β 1a and b isoforms. Lastly, three of these five amino acids are not conserved between isoforms or species for the putative functional binding domain of M-CoA located between alanine⁴⁷⁸ and histidine⁴⁸³ in mammalian CPT I α (Morillas et al., 2002). Although the effects of mutations at these particular residues have not been investigated to date, they may be affecting the binding, and therefore, sensitivity to M-CoA, and should be included in future research.

The interaction between N- and C-termini adjacent to the transmembrane domains (TMDs) have been implicated in M-CoA sensitivity (Faye et al., 2005; Jackson et al., 2000). Therefore, we analysed the location and length of the TMDs (Fig. 4.3). The amino terminus of both rat β and trout β 1b is 10 and 20 amino acids shorter than trout CPT I β 1a, respectively. Furthermore, there are approximately 10 additional amino acids between the first and second TMD in CPT I α compared to CPT I β . These changes may impact the interaction of the two TMDs thereby affecting the sensitivity to M-CoA. Taken together, there are numerous amino acid substitutions and structural changes between the isoforms in trout, as well in comparison to mammals. These differences are likely to be the root of the functional divergence of CPT I between tissues and taxa.

CPT I expression

In addition to potential structural modifications at the protein level of CPT I, the transcriptional regulation of the duplicated genes may also be altered. In fact, it has been suggested that at the tissue level it is the transcriptional regulation of duplicated genes which plays a greater role in determining the overall functional role of the gene rather than any changes in protein kinetics. There is evidence in fact, that in fish, the transcriptional expression pattern and transcriptional regulation of duplicated genes may play a larger role than protein activity in the overall function of a gene at the whole tissue

level (Hughes, 1994; Robinson-Rechavi and Laudet, 2001; Robinson-Rechavi et al., 2001a).

In evaluating the mRNA expression of the five CPT I isoforms we found that all were expressed in the tissues tested, but at varying levels (Fig. 4.4). In general, the β isoforms and $\alpha 1a$ were predominantly expressed in highly oxidative muscle tissues (red, heart) $\alpha 1b$ and $\alpha 2$ may play a larger role in the liver as their expression was similar to muscle whereas the beta isoforms and $\alpha 1a$ were expressed much lower in the liver. Previous research concluded that CPT I $\alpha 2$ (previously called CPT Id; see Kolditz et al., 2008) was only present in liver and was not detectable in skeletal muscle (Kolditz et al., 2008). However, detectable levels in all tissues, albeit low were observed in muscle and liver tissue (Fig. 4.4). Conversely, this isoform is expressed significantly higher in kidney and intestine, potentially with a tissue-specific role. This global expression pattern is somewhat surprising given the tissue-specificity of CPT I in mammals. However, even in mammals some tissues do express both isoforms but in varying amounts (Brown et al., 1995).

Transcriptional differences aside, protein expression and functional differences between isoforms may not directly relate to mRNA expression patterns. For instance, the total rate of fatty acid entry into, and oxidation by, the mitochondria depends on several factors including the expressed protein isoforms, the IC_{50} for M-CoA and K_m for carnitine for each isoform plus substrate and cofactor levels. Additionally, future research into the promoter region will help to determine the diversity of the transcriptional regulation of CPT I. Indeed, adding to the complexity of metabolic regulation in fish are the multiple potential isoforms of the transcription factors involved in modulating expression of genes in the fatty acid oxidation pathways (e.g Leaver et al., 2007). In particular, peroxisome proliferator-activated receptor α (PPAR α) is a known transcriptional regulator of CPT I in mammals (Price et al., 2000), however, in fish, this relationship is much less clear since correlations in expression of transcription factors and target genes have not always been observed (Morash et al., 2009; Morash et al., 2008). This suggests that tissue-specific differences in regulation and rate of fatty acid oxidation is far more complex in fish than in mammals.

To investigate the developmental plasticity in isoform expression we observed the expression of CPT I at different life stages of rainbow trout in the heart, muscle and liver. In rodents, during the neo-natal and newborn stages the heart expresses primarily CPT I α . However, there is a progressive switch to expressing primarily CPT I β once the animal is weaned from its mother and begins to feed on its own (Brown et al., 1995). Similarly, rainbow trout express significantly more $\beta 1a$ and $\alpha 1b$ during the fry stage in which they are dependent on nutrients in the yolk sac, and significantly more $\beta 1b$ and $\alpha 1a$ during juvenile and adult stages which are free feeding (Fig. 4.5A). In mammals, the change in isoform expression is thought to occur as a result of changes in carnitine and M-CoA concentrations. The expression of CPT I α in rodent neonates may well be necessary since the concentration of M-CoA is above the IC_{50} for CPT I β (Brown et al., 1995). Moreover, in rabbits and most likely other mammals, there is a dramatic reduction in M-CoA in the heart with age which would allow for the expression of the more sensitive CPT I β (Lopaschuk et al., 1994). Whether this shift in isoform expression produces the same

shift in fuel use in trout hearts as seen in mammals is not clear. However, development as well as other cardiovascular changes that induced metabolic shifts (i.e. hypertrophy) should be examined in context of multiple CPT I isoform expression.

In contrast to the heart, red muscle shows a transient decrease in both β isoforms and $\alpha 1a$ during the juvenile stage, while the expression resembles that of the fry in the adult stage (Fig. 4.5B). Furthermore, there was a significant reduction in expression of $\alpha 2$ during both juvenile and adult life stages, indicating that it may have a more important role during development but not in free feeding juvenile and adult trout. Comparatively, we see very few changes in CPT I isoform expression in the liver with age (Fig. 4.5C). The only major changes during development are a significant increase in $\beta 1a$ and a significant decrease in $\alpha 1a$ during the transition from juvenile to adult. These changes in mRNA expression from fry to adult would most certainly be impacted by the change in diet from endogenous to exogenous resources (Morash et al., 2009; Price et al., 2000) and potentially corresponding changes in hormonal status or the change in rearing environment as the fry were moved to a higher volume tank in a new location during their free swimming/feeding juvenile life stage. Furthermore, they may also be reflective of the metabolic state of muscle as in the mammalian heart.

Perspectives and Significance

The evolution of CPT I has resulted in a diverse family of isoforms with significant structural differences which may influence the functional differences observed between tissues. Although some isoforms appear to be conserved across taxa, genome duplication events have resulted in lineage-specific diversification of these genes important to fatty acid oxidation. Moreover, these differences represent a degree of specificity in the ability of species to regulate fatty acid entry into, and oxidation by, mitochondria. This fine-tuned control of lipid oxidation may help better preserve tissue metabolism during physiological and environmental perturbations. Future studies should determine the functional characteristics of individual isoforms to discern their kinetics and response to inhibition.

Table 4.1: Primers used for the sequencing of CPT I α 1a from rainbow trout.

Primer	5' to 3' Forward Primer	5' to 3' Reverse Primer	T_m °C	Size (bp)
oligo-dT	gaccacgcgtatcgatgctgatttttttttttttv	-	59	-
Anchor	gaccacgcgtatcgatgctga	-	57	-
CPT I α S1	gttcattcgcctcaagaatgg	ctgggctctgtccagtt	58	750
CPT I α S2	aactggacagagcccagatca	ctggtccggcacttctttatc	60	837
CPT I α S3	gataaagaagtgccggacca	agcgtttgctggagatgtg	60	545
SP1 α	-	accaggacttgagcttgagg	60	-
SP2 α	-	atcaacggacgagctgactc	60	-
SP3 α	-	gtctcctgccagagaaca	60	-
SP4 α	catgcaccgtcacctcct	-	60	-

Table 4.2: Rainbow trout primers used for real-time PCR analysis.

Gene	5' to 3' Forward Primer	5' to 3' Reverse Primer	T_m °C	Size (bp)
CPT I β 1	gatgttcctgagggttagga	ttgtcttgcattggctctgac	58	80
CPT I β 2	gccgcaaactagagagagga	cccgtagtacagccacacct	58	199
CPT I α 1a	atgaggaatgccctcaagtg	gcttctgccagagaacaac	58	120
CPT I α 1b	cgcttcaagaatgggggtgat	caaccacctgctgtttctca	58	187
CPT I α 2	ccgttcttaacagaggtgct	acactccgtagccatcgtct	58	154
EF1 α	cattgacaagagaaccattga	ccttcagctgtccagcac	58	94

Table 4.3: Gene and Protein ID numbers of target sequences.

Species_CPTI Isoform	Protein ID	Gene ID	Database
O.mykiss_a1a	CAF03604.1	AJ619768	GenBank
O.mykiss_a1b	Bankit1316105	Bankit1316105	GenBank
O.mykiss_a2	CAF04490.1	AJ620356	GenBank
T.rubripes_α1	ENSTRUT00000024420	ENSTRUG00000009681	Ensembl
G.aculeatus_α1	ENSGACT00000014796	ENSGACG00000011141	Ensembl
D.erio_α1a	ENSDARP00000083632	ENSDARG00000062054	Ensembl
T.nigroviridis_α1	ENSTNIT00000013563	ENSTNIG00000010461	Ensembl
D.erio_α1b	ENSDARP00000077866	ENSDARG00000059770	Ensembl
G.aculeatus_α2a	ENSGACT00000010606	ENSGACG00000007987	Ensembl
T.rubripes_α2a	ENSTRUT00000031872	ENSTRUG00000012539	Ensembl
T.nigroviridis_α2a	ENSTNIT00000018470	ENSTNIG00000015188	Ensembl
O.latipes_α2a	ENSORLT00000008557	ENSORLG00000006807	Ensembl
D.erio_α2a	ENSDARP00000082601	ENSDART000000088168	Ensembl
T.nigroviridis_α2b	ENSTNIT00000007428	ENSTNIG00000004619	Ensembl
D.erio_α2b	ENSDARP00000055293	ENSDARG00000037943	Ensembl
T.rubripes_α2b	ENSTRUT00000035476	ENSTRUG00000013830	Ensembl
G.aculeatus_α2B	ENSGACP00000008742	ENSGACG00000006606	Ensembl
O.latipes_α2b	ENSORLT00000021139	ENSORLG00000016883	Ensembl
O.mykiss_B1a	CAE54358.1	AJ606076	GenBank
O.mykiss_B1b	AAN37385.2	AF327058	GenBank
D.erio_β	ENSDART00000083421	ENSDARG00000058285	Ensembl
T.nigroviridis_β	ENSTNIT00000021339	ENSTNIG00000017945	Ensembl
T.rubripes_β	ENSTRUT00000029413	ENSTRUG00000011589	Ensembl
G.aculeatus_β	ENSGACT00000016348	ENSGACG00000012306	Ensembl
O.latipes_β	ENSORLT00000012821	ENSORLG00000010223	Ensembl
A.carolensis_a	ENSACAP00000013904	ENSACAG00000013801	Ensembl
G.gallus_a	ENSGALP00000011452	ENSGALG00000007077	Ensembl
H.sapiens_a	ENSP00000265641	ENSG00000110090	Ensembl
P.troglodytes_a	ENSPTRP00000006862	ENSPTRG00000003987	Ensembl
M.mulatta_a	ENSMMP00000002371	ENSMMPG00000001780	Ensembl
M.musculus_a	ENSMUSP00000025835	ENSMUSG00000024900	Ensembl
R.norvegicus_a	ENSRNOP00000019652	ENSRNOG00000014254	Ensembl
C.porcillus_a	ENSCPOP00000003917	ENSCPOG00000004344	Ensembl
E.caballus_a	ENSECAP00000015118	ENSECAG00000016852	Ensembl
C.familiaris_a	ENSCAFP00000015834	ENSCAFG00000010757	Ensembl
X.tropicalis_a	ENSXETP00000044307	ENSXETG00000020491	Ensembl
A.carolensis_B	ENSACAP00000001055	ENSACAG00000000623	Ensembl
X.tropicalis_B	ENSXETP00000033674	ENSXETG00000015426	Ensembl
H.sapiens_B	ENSP00000353945	ENSG00000205560	Ensembl
P.troglodytes_B	ENSPTRP000000045669	ENSPTRG00000014548	Ensembl
M.musculus_B	ENSMUSP00000104936	ENSMUSG00000078937	Ensembl
R.norvegicus_B	ENSRNOP00000013985	ENSRNOG00000010438	Ensembl
E.caballus_B	ENSECAP00000010542	ENSECAG00000012793	Ensembl
C.familiaris_B	ENSCAFP00000000832	ENSCAFG00000000586	Ensembl
P.pygmaeus_B	ENSPPYP00000013379	ENSPPYG00000011994	Ensembl
F.catus_B	ENSFCAP00000009834	ENSFCAG00000010593	Ensembl
B.taurus_B	ENSBTAP00000021357	ENSBTAG00000016048	Ensembl
H.sapiens_C	ENSP00000319343	ENSG00000169169	Ensembl
M.musculus_C	ENSMUSP00000069539	ENSMUSG00000007783	Ensembl
R.norvegicus_C	ENSRNOP00000032802	ENSRNOG00000026163	Ensembl
E.caballus_C	ENSECAP00000013707	ENSECAG00000015556	Ensembl
C.familiaris_C	ENSCAFP00000005319	ENSCAFG00000003568	Ensembl
P.pygmaeus_C	ENSPPYP00000011791	ENSPPYG00000010527	Ensembl
F.catus_C	ENSFCAP00000005655	ENSFCAG00000006084	Ensembl
B.taurus_C	ENSBTAP00000016188	ENSBTAG00000012205	Ensembl
C.intestinalis	ENSCINP00000007072	ENSCING00000003450	Ensembl

Table 4.4: Amino acid mutations and their effect on CPT I maximal activity and sensitivity to malonyl-CoA.

Mutation	Tissue	Δ IC₅₀ M-CoA	V_{max}	Reference
Glu ³	Liver	100x ↑	no change	Shi et al., 1999
His ⁵	Liver	12.5x ↑	no change	Shi et al., 1999
AA1-18	Liver	190x ↑	0.67x ↓	Shi et al., 1998
AA1-18	Muscle	4.2x ↑	no change	Shi et al., 2000
AA3-18	Liver	4.63x ↑	2.17x ↑	Jackson et al., 2000
AA3-18	Muscle	1.36x ↑	no change	Jackson et al., 2000
Val ¹⁹ /Leu ²³ /Ser ²⁴	Muscle	142x ↑	no change	Zhu et al., 2003
His ¹⁴⁰	Liver	50x ↑	6.73x ↑	Swanson et al., 1998
His ²⁷⁷	Liver	18.6x ↑	no change	Morillas et al., 2002
Ala ⁴⁷⁸	Liver	3.25x ↑	no change	Morillas et al., 2002
Pro ⁴⁷⁹	Liver	33x ↑	0.5x ↓	Brown et al., 2001
His ⁴⁸³	Liver	2.3x ↑	no change	Morillas et al., 2002
His ²⁷⁷ /His ⁴⁸³	Liver	80x ↑	no change	Morillas et al., 2002

Fig. 4.1

(A) Rooted tree resulting from Bayesian phylogenetic analysis of CPT I proteins in vertebrates. (B) Rooted tree resulting from Bayesian phylogenetic analysis according to Akaike information criterion of vertebrate CPT I nucleotide sequences. The tree topologies are supported by $\geq 99\%$ posterior probabilities, except where indicated. # indicates $< 50\%$, * indicates $= 50\%$. All other nodes are indicated by their numerical value. The scale represents estimated mean number of substitutions (amino acid or nucleotide) per site. “A” and “B” represent “ α ” and “ β ”, respectively.

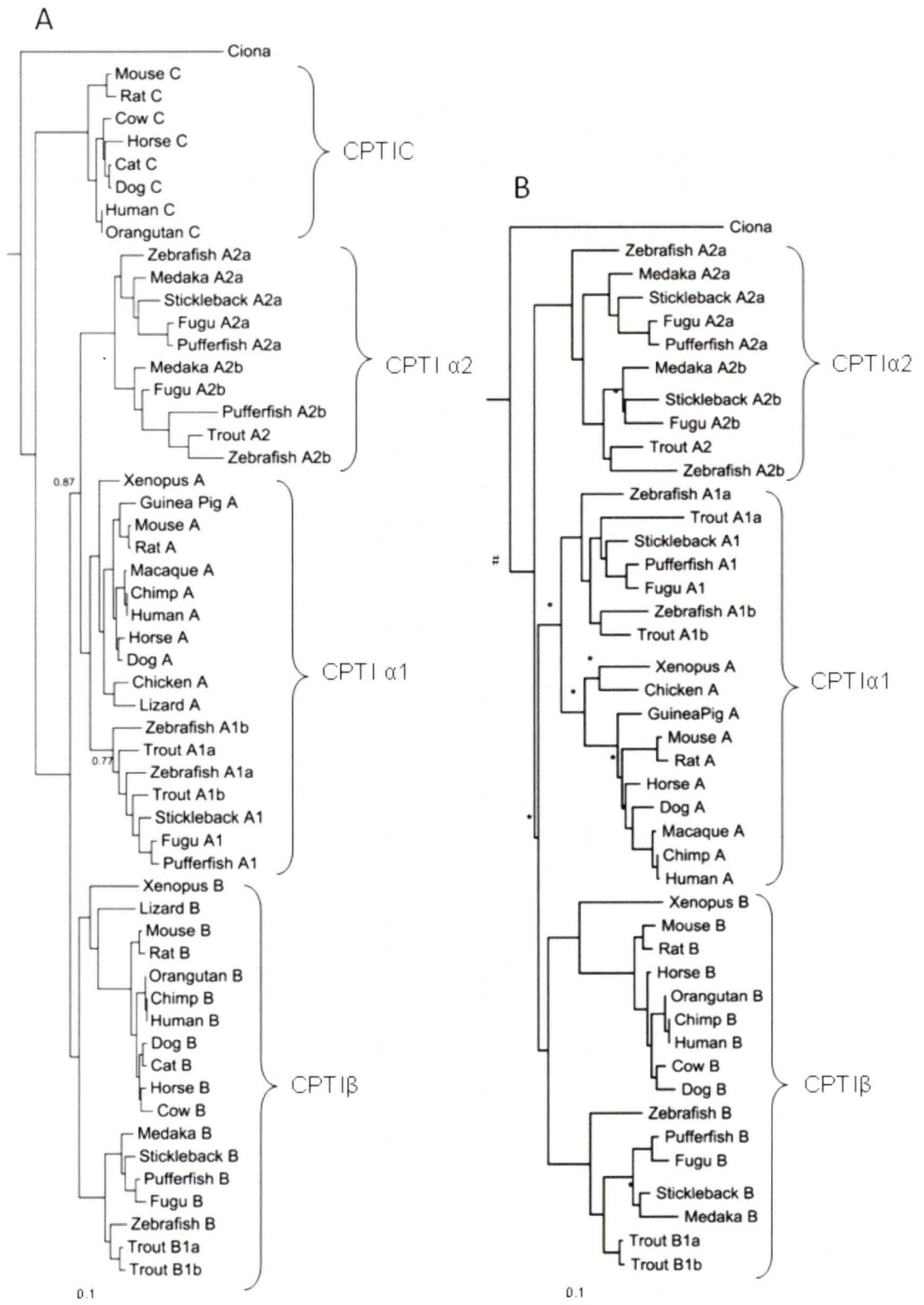


Fig. 4.2

Protein alignment of selected sections from trout and rat CPT I isoforms. Boxes indicate area of interest where amino acid substitutions or deletions occurred. “A” and “B” represent “ α ” and “ β ”, respectively.

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Trout_A1a MAEAHQAVAFQFTVGPDCIDMQLCHEALRQIYLSGKHSWKKRFIRFKNGVMTGVYEGSPS 60
Trout_A1b MAEAHQAVAFQFTVSPDCIDLQLSHEALRQIYLSGLHSWKKKRFIRFKNGVMTGVYEGSPT 60
Rat_A MAEAHQAVAFQFTVT PDGIDLRLSHEALKQICLSGLHSWKKKRFIRFKNGIITGVF PANPS 60
Trout_A2 -----
Trout_B1a MAEAHQAVGFQFTVT PDGIDLHLSREYLKHIYLSGVT SWIKRAIRFKNGILTGVYPASPS 60
Trout_B1b MAEAHQAVGFQFTVT PDGIDLHLSRE-----NGILTGVYPASPS 39
Rat_B MAEAHQAVAFQFTVT PDGVDFRLSREALRHIYLSGINSWKKRLIRIK-----PT 49

Trout_A1a TLDDSEQKYEIDNPVKS LSYAKSLLHGKCYDRWF DKSFNLIVFKNGTMGLNAEHWADA 478
Trout_A1b TLDDTEQRYKAEDPVRS LSYAKSLLHGKCYDRWF DKSFNLIVFRNGTMGLNAEHSWADA 479
Rat_A TLDESEQGYREEDPEASIDSYAKSLLHGRCFDRWF DKSFITFVVFKNKSGINAEHSWADA 477
Trout_A2 TLDDEEQGMMGEDPVGNDRYAKSLLHGKCYDRWF DKSF SVVYKNGKGLNAEHSWADA 126
Trout_B1a TLDDEAHGYD PDK-LRSLDLYAKSLLHGKCYDRWF DKSF TLIAYKNGKGLVNAEHSWADA 475
Trout_B1b SLDDEAHGYD PDK-LRSLDLYAKSLLHGKCYDRWF DKSF TLIAYKNGKGLVNAEHSWADA 453
Rat_B ALDEDSHCYNPDD-EASLSLYGKSLHGNCYNRWF DKSF TLI SCKNGQLGLNTEHSWADA 463
:*. : :. :. *.*****. *:*****:.. :*. *:*:*:*:*

Trout_A1a PIIGHLWEQVLCMDPLELGYTEEGHCNGE PHPNLPGPQRLQNDI PAECQSTIQSSLKFAQ 538
Trout_A1b PIIGHLWEHVLSMDPVKLGYTEEGHCKGNPHPSLPGPQRLQWNI PAECQTAIASSIMVAK 539
Rat_A PVVGHLEWEYMATDVFQLGYS EDGHCKGDTNPNIPKPTRLQWDI PGECQEVIDASLSAS 537
Trout_A2 PTVAHLEWEFTLATDAFQLGYTEEGHCKGEVEHSLPPPQRLTWDI PVEVQEQVSI SLSVAQ 186
Trout_B1a PIVGHLEWEYVLATDCEFLGYTEEGHCKGDINKGLPPTAKLQWDI PLECEVIEDSYMVAK 535
Trout_B1b PIIGHLEWEYVLATDCEFLGYTEEGHCKGDINKGLPLPTKLQWDI PLECEVIEESYMVAK 513
Rat_B PIIGHLWEFVLATDTFHLGYTETGHCVGE PNTKLPPPORMQWDI PEQCQTAIENSYQVAK 523
* . * ** . . * . ***: * ** * : . : * . : : * : * : * .
    
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Fig. 4.3

Diagram indicating the transmembrane domains (TMD) of CPT I isoforms in trout and rat. Values indicate relative position of the amino acids delimiting the TMDs.

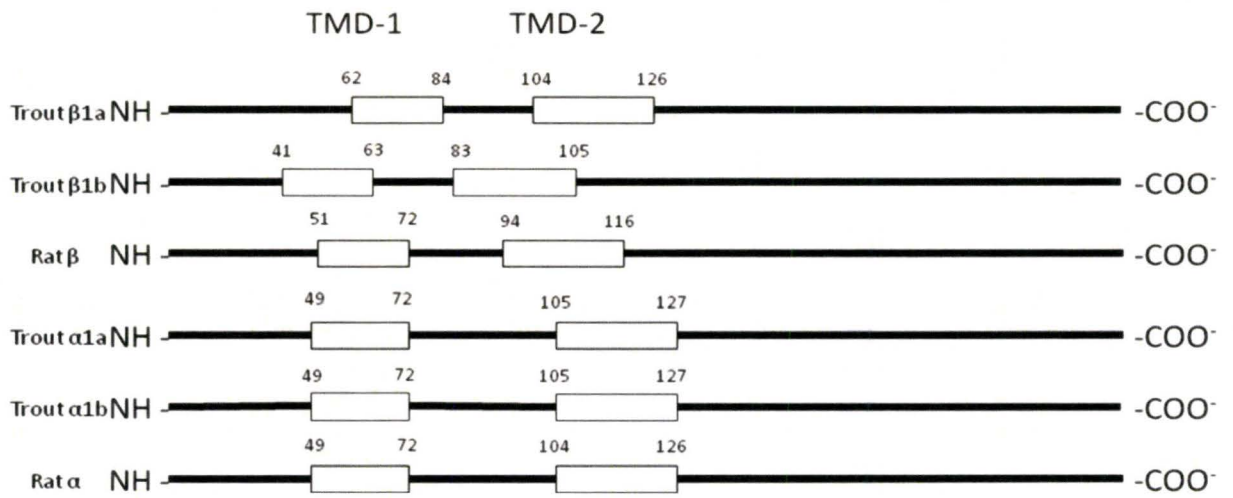


Fig. 4.4

mRNA expression of each of the five CPT I isoforms across liver, red muscle (RM), heart, white muscle (WM), kidney and intestine of rainbow trout. Values are the log of the expression of each gene relative to the expression of housekeeping gene EF1 α . N=4 \pm S.E. Bars that do not share a letter are significantly different ($p < 0.05$). “A” and “B” represent “ α ” and “ β ”, respectively.

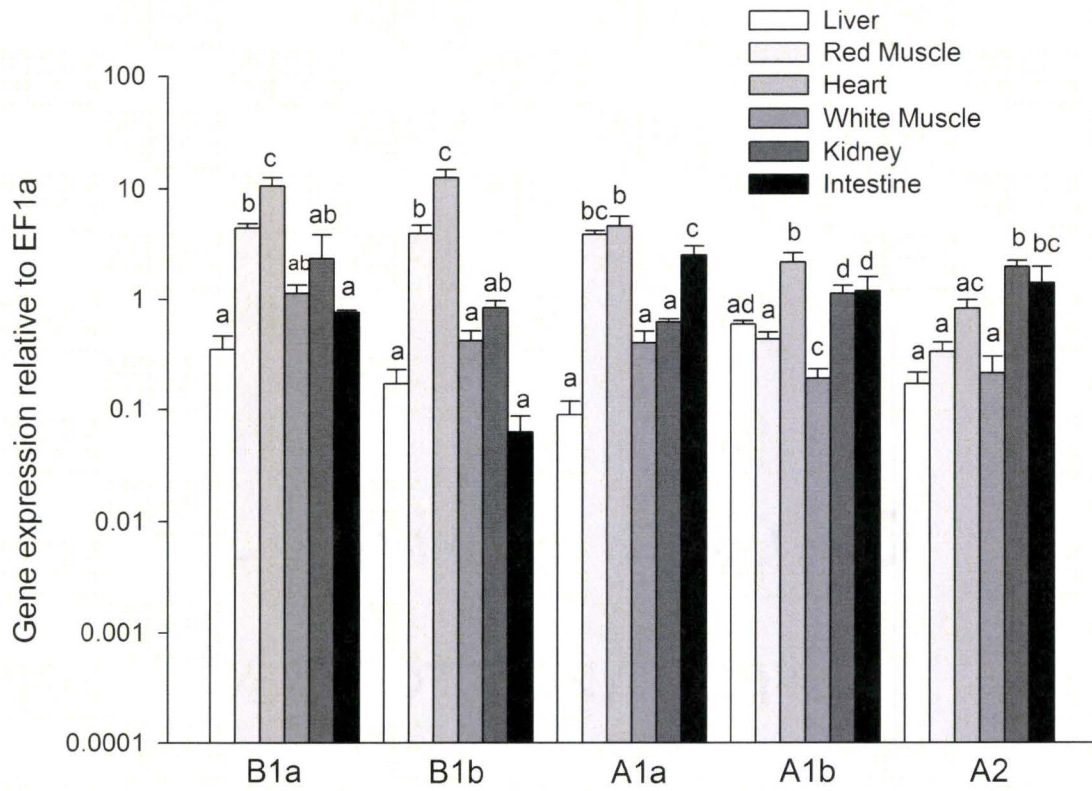
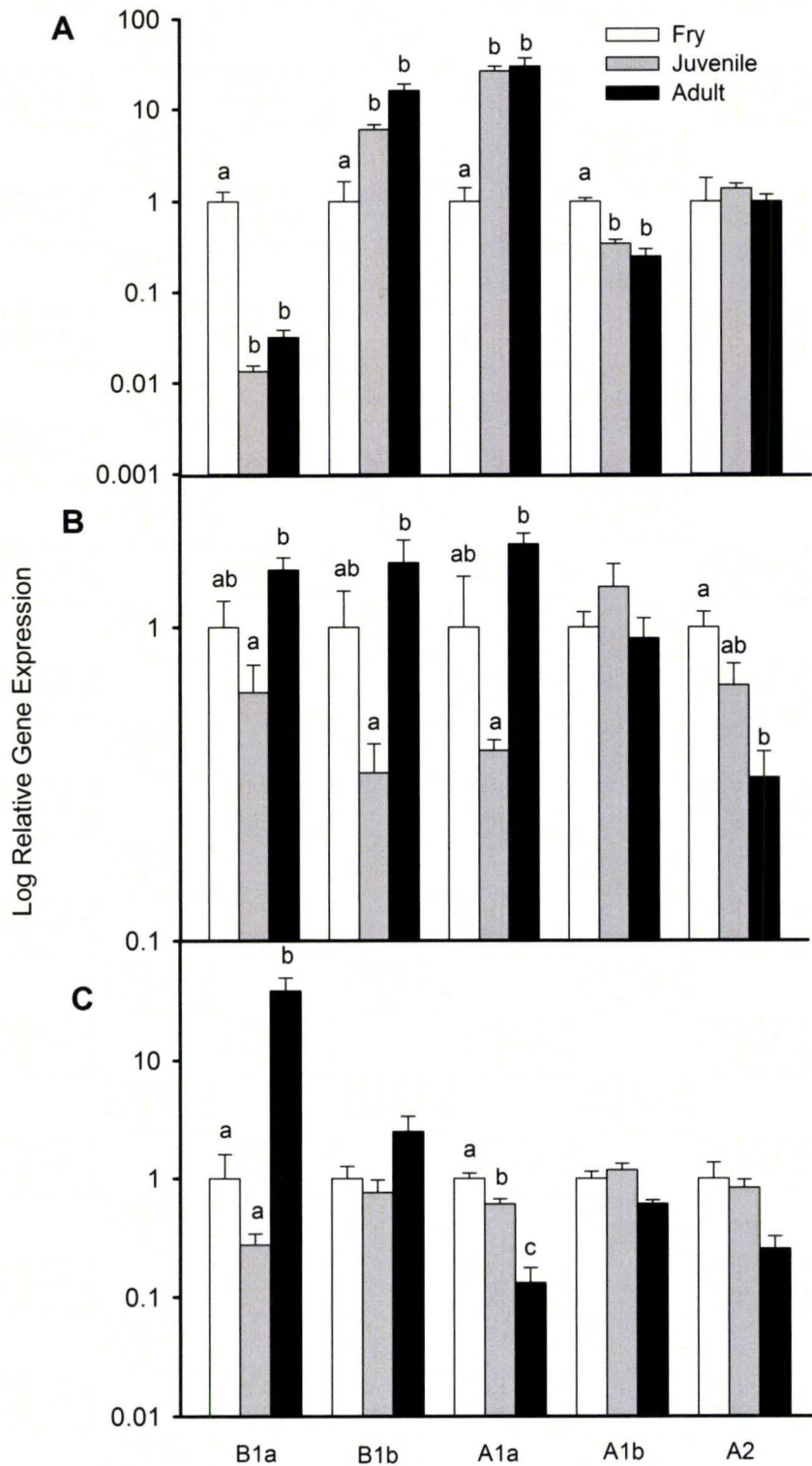


Fig. 4.5

mRNA expression of the five CPT I isoforms in rainbow trout heart (A), red muscle (B) and liver (C) during development. Values are the log of the expression of each gene relative to the expression of EF1 α . N=4 \pm S.E. Bars that do not share a letter are significantly different (p<0.05). “A” and “B” represent “ α ” and “ β ”, respectively.



THE INFLUENCE OF EXERCISE ON GENE EXPRESSION AND ENZYME
CHANGES IN MIGRATING PACIFIC SALMON

Abstract

During a migration lasting hundreds to thousands of kilometers, Pacific salmon experience a range of metabolic challenges, including but not limited to, prolonged endurance exercise and fasting. Both conditions have a stimulatory effect on lipid oxidation, particularly in the heavily recruited muscle tissues. We sought to investigate the regulatory mechanisms that initiate and maintain this increase in lipid oxidation in natural migrating populations of Pacific salmon (*Oncorhynchus nerka*) and to compare this with the effects of chronic exercise alone using rainbow trout (*Oncorhynchus mykiss*) as a closely related salmonid model. In particular, we examined the expression of multiple genes encoding for important regulators of lipid, carbohydrate and protein oxidation as well as the activity of several important mitochondrial enzymes involved in lipid and carbohydrate oxidation in red and white muscle of both species. We found that the expression of CPT I, PPAR α 1 and β 1, and AMPK β 1 were all significantly elevated at the onset of migration as well as the activity of HOAD and CS. The expression and HK I and AST increased later in migration when lipid stores were almost depleted. In exercised trout, we found similar results in the activity of HOAD, CS and HK, although the difference was not as great as in the migrating salmon suggesting that exercise alone does not account for the changes in lipid metabolizing enzymes occurring during migration. In general, the expression of CPT I, PPARs and AMPK β 1 did not follow the same pattern in exercising salmon, and most were decreased after 4 weeks of exercise. Overall, this data suggests that salmon may transcriptionally and metabolically remodel their muscle tissues to increase the capacity for lipid oxidation in preparation for migration, and that there are factors in addition to exercise which may help to promote this increase in lipid metabolizing machinery.

Morash, A.J., Yu, W., Le Moine, C.M.R., Hills, J., Farrell, A.P., Patterson, D.A., McClelland, G.B. 2010. In preparation for submission to the Journal of Experimental Biology.

Introduction

Pacific salmon (*Oncorhynchus nerka*) swim distances between 100-2000 km during their annual spawning migration (Kiessling, 2004). During this monumental trek, they are faced with numerous physiological challenges including, but not limited to, salinity transfer, changes in temperature, sexual maturation, endurance exercise and prolonged fasting. The transfer from sea water to fresh water has received much of the attention to date (Cooperman et al., 2010; Shrimpton et al., 2005), while the endurance exercise and fasting aspects of migration have received relatively little. How salmon can travel such great lengths, especially in a fasted state is certainly one of the most remarkable energetic feats in the animal world.

Past research has shown that spawning migrations are primarily fuelled by lipid stores obtained from the diet during the oceanic phase of the salmon life cycle (French et al., 1983; Gilhousen, 1980; Idler et al., 1959). Protein and carbohydrates supply relatively little energy for the majority of the migration, but are crucial for the latter period when lipid stores have been almost fully depleted (Gilhousen, 1980; Mommsen, 1980). The regulation of lipid metabolism is therefore a vital factor in ensuring a successful migration. Despite their ecological importance, there has been relatively little research on the physiology and metabolism of migrating salmon, particularly sockeye. Recently, environmental cues affecting transcription of a host of metabolic processes have been investigated in sockeye salmon throughout their migration indicating fasting and changes in salinity as the main signals for genomic remodelling of white muscle tissue (Miller et al., 2008).

Along with cell membrane transport and the cytosolic delivery of fatty acids, mitochondrial lipid oxidation is regulated, in part, by carnitine palmitoyltransferase (CPT) I which determines fatty acid entry into the mitochondria (McGarry et al., 1983). CPT I is inhibited allosterically by the metabolic intermediate malonyl-CoA (M-CoA), generated in the first step of fatty acid synthesis in liver (McGarry and Brown, 1997). Liver concentrations of M-CoA can fluctuate based on the nutritional status of the animal and the energetic demand of the tissue (Jansen et al., 1966).

Recently we have shown that in rainbow trout, there are five distinct CPT I isoforms encoded for by separate genes (Morash, 2010). These isoforms potentially have different functional characteristics which contribute to the tissue differences in CPT I IC₅₀ observed in trout (Morash et al., 2008). To date, no CPT I genes have either been sequenced or had their kinetic properties determined in sockeye salmon. Given the genetic relatedness of these two species we predict a similar isoform compliment and similar expression pattern in salmon as seen in the trout. Moreover, since the expression of CPT I is controlled, in part, by peroxisome proliferator-activated receptor transcription factor family, (PPARs; (Price et al., 2000), they may be involved in migration induced tissue remodelling. In mammals, CPT I contains a PPAR response element (PPRE) to which PPARs can bind and induce transcription (Price et al., 2000). Thus far, three PPAR isoforms have been identified in mammals; α , β and γ . PPAR α is highly expressed in tissues exhibiting high rates of lipid oxidation such as liver, heart and skeletal muscle, while PPAR β is expressed ubiquitously in all tissues (Desvergne and Wahli, 1999).

PPAR α can be activated by a wide array of fatty acid ligands to induce the expression of several key metabolic genes involved in lipid metabolism, including CPT I (Desvergne and Wahli, 1999; Price et al., 2000). In contrast, PPAR β appears to promote lipid storage (Peters et al., 2000) and inhibit PPAR α (Leaver et al., 2007). In contrast to mammals, relatively little is known about the function of PPARs in non-mammalian vertebrates such as fish. Their expression under routine conditions is relatively similar to mammals (Batista-Pinto et al., 2005; Morash et al., 2008) but it is not as clear what changes occur during physiological perturbations. For example, when trout are fasted for weeks and lipid oxidation increases, there was no change in PPAR α while PPAR β expression decreased in red muscle but increased in the liver (Morash et al., unpublished. Chapter 6). Furthermore, trout fed a diet high in polyunsaturated fatty acids showed an increase in PPAR α and β expression only in the heart but not in red muscle or liver (Morash et al., 2009). In addition, multiple paralogues of each PPAR isoform have been found in a variety of fish species and their tissue expression and functional characteristics may be distinct from the corresponding genes in mammals (Leaver et al., 2005; Leaver et al., 2007).

PPAR α appears to be regulated by adenosine monophosphate-activated protein kinase (AMPK), a major regulator of cellular energy production (Lee et al., 2006). AMPK works concurrently to stimulate fatty acid oxidation and glucose uptake in liver and muscle while inhibiting lipogenesis (Winder and Hardie, 1999). Aside from stimulating PPAR α , AMPK also works to inhibit acetyl-CoA carboxylase which is the enzyme that converts acetyl-CoA to malonyl-CoA during fatty acid synthesis (Ouchi et al., 2005). In muscle, M-CoA appears to be present only to control CPT I, as lipogenesis is quite low in this tissue (McGarry et al., 1983). Therefore, inhibition of ACC by AMPK will result in a reduction of the concentration of M-CoA relieving the inhibition of CPT I and allowing greater uptake of fatty acids into the mitochondria for oxidation. Moreover, in mammals, AMPK upregulates muscle hexokinase activity (Holmes et al., 1999). Taken together, AMPK acts to stimulate ATP generating processes, particularly during exercise and fasting when an upregulation of fat and carbohydrate oxidation is warranted.

Individually, both fasting and endurance exercise have been known to induce a greater rate of fatty acid oxidation to fuel the energetic demand of the muscle tissues of mammals. However, their effects on lipid metabolism in non-mammalian vertebrates have been relatively unexplored, particularly fasting. However, there have been several studies in teleost fish indicating that fatty acid oxidation enzymes such as HOAD and CS increase in muscle tissues after exercise training (Farrell, 1990; Johnston and Moon, 1980). Furthermore, any potential interaction between fasting and exercise within individuals remains unknown. To address this issue, along with my previous study on fasting alone (Chapter 6), I investigated the effects of chronic exercise alone on lipid metabolism and determined whether these same individual changes take place in migrating salmon who experience both fasting and endurance exercise during the same time. Here I examined major transcriptional (PPARs) and catalytic (CPT Is, HK I, HOAD, CS, AMPK β 1 subunit) regulators of lipid metabolism in sockeye salmon at four different points along the migration route from the ocean to the spawning grounds as well as in control and chronically exercised rainbow trout.

I hypothesized that there would be a transcriptional upregulation and an increase in enzyme activity of key factors in lipid metabolism prior to the start of the fresh water migration phase that would prepare the muscle cells for the large increase in the demand for lipid oxidation and a subsequent decline in these factors during the later stages of migration. Furthermore, we expect that the exercised trout will exhibit an increase in these factors when compared to control fish which will mimic changes in the migrating salmon.

Materials and Methods

Experimental species, sampling locations and exercise regime

Pacific Salmon (*Oncorhynchus nerka*) were sampled along their migration route in southern British Columbia, Canada between July and September of 2006. Fish were caught at 4 locations; 1) Port Hardy in the Johnson Strait (ocean) by a purse seine boat, 2) Whonnock (river entry) by a gill net boat, 3) Savona (mid-river) by a beach seine, 4) Adam's River (spawning site) by a beach seine (Fig. 5.1). The fish were killed and the length and weight were recorded. Condition factor (CF) was calculated using the following equation, $CF = 100w/l^3$ where w is the weight of the fish in grams and l is the length of the fish in centimeters. Red and white muscle samples were excised and immediately placed on dry ice for further analysis. The % lipid and % protein values were calculated from homogenized muscle samples according to (Higgs, 1979).

Rainbow trout were obtained from a local trout hatchery (Humber Springs, Orangeville, ON), kept in 500 L tanks with circulating dechlorinated Hamilton tap water at 12°C and fed a commercial diet (Profishent Classic Floating Trout Grower, Martin Mills, Elmira, ON) prior to exercise training. The exercised group was introduced to the swim tunnel 1 week prior to the beginning of exercise. They were exercised for four weeks at 1.5 BL sec⁻¹ for 23.5 hours per day. Exercised trout were fed the same commercial trout feed as controls twice a day to satiation for the other 30 minutes. After 4 weeks trout were killed by a lethal blow to the head and their spinal cord severed. Red and white muscle were excised and immediately frozen in liquid nitrogen.

RNA extraction and cDNA synthesis

Frozen tissues were powdered in a liquid N₂-chilled mortar and pestle. Total RNA was extracted from each tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. RNA was quantified by UV spectroscopy at 260nm and then diluted to 0.5µg/µl. cDNA was synthesized using 1 µg of DNase (Invitrogen, Carlsbad, CA) treated mRNA with SuperScript RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA) as described previously (Morash et al., 2008).

Polymerase chain reaction (PCR) and sequencing

For each gene, sequences were aligned from mammals and other fish species and specific PCR primers were designed from conserved regions using Primer3 software (Rozen and Skaletsky, 2000). A small section of each gene was amplified by PCR using

0.2mM dNTP, 1.5mM MgCl₂, 0.5μM of each forward and reverse primer (Tables 5.1 and 5.2), 1 unit of Taq polymerase (Fermentas, Burlington, Ontario, Canada) and 1X Taq amplification buffer. For all of the CPT I isoforms, EF1α, PPARβ1, AMPK β1 subunit, HK I and AST, the PCR products were purified using QiaQuick gel extraction kit (Qiagen, Mississauga, Ontario, Canada) and directly sequenced at the Mobix Lab (McMaster University). For PPARα1, the full length sequence was obtained by 3 overlapping sections (Table 5.1). PCR products were purified as mentioned above, then cloned using the pGEM-T easy vector system (Promega, Nepean, Ontario, Canada) and sequenced at the Mobix Lab (McMaster University).

mRNA quantification by real-time PCR

The expression of each mRNA was quantified using real time PCR with SYBR green and ROX as a reference dye using a Stratagene Mx3000P (Stratagene, Texas, USA) real-time PCR system. Each 25 μl reaction contained 12.5 μL SYBR green mix, 1 μL each of forward and reverse primer (5μM), 5.5 μL of DNase/RNase free water and 5 μL of 5× diluted cDNA. Primers were designed using Primer3 software (Rozen and Skaletsky, 2000); Table 5.2). The thermal program included 3 minutes at 95°C, 40 cycles of 95°C for 15 sec, 58°C for 30 sec and 72°C for 30 sec. A no-template control and dissociation curve was performed to ensure only one PCR product was being amplified and stock solutions were not contaminated. Standard curves were constructed for each gene using serial dilutions of stock cDNA to account for any differences in amplification efficiencies. All samples were normalized to the housekeeping gene, EF1-α whose expression did not significantly change between sites.

Enzyme analysis

All assays were performed in triplicate at room temperature in 96-well format using a Spectramax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA), while the data was collected using Softmax Pro 4.7.1 software (Molecular Devices, Sunnyvale, CA). Frozen tissues were powdered using a liquid N₂ chilled mortar and pestle and homogenized in 20 volumes of homogenization buffer (100mM potassium phosphate, 5mM EDTA and 0.1% Triton at pH 7.2) using a glass on glass homogenizer.

Citrate Synthase (CS). CS was measured according to previously published protocols (McClelland et al., 2005). Briefly, the CS assay buffer contained (in mM) 20 TRIS (pH 8.0), 0.1 DTNB and 0.3 acetyl-CoA. The reaction was initiated by the addition of 0.5mM oxaloacetate and absorbance was measured for 5 minutes at 412nm. Control samples were assayed without oxaloacetate to control for background hydrolase activity.

β-hydroxyacyl-CoA Dehydrogenase (HOAD). HOAD was measured according to previously published methods (McClelland et al., 2005) and consisted of (in mM) 50 imidazole (pH7.4), 0.1 acetoacetyl-CoA, 0.15 NADH and 0.1% Triton X-100 at 340nm.

Hexokinase (HK). The HK assay was modified from Houle-Leroy et al. (Houle-Leroy et al., 2000) for use in fish. The assay buffer contained (in mM) 4 ATP, 10 MgCl₂, 0.5 NADP, 1 U glucose-6-phosphate dehydrogenase, in 50 HEPES (pH 7.0). The reaction was initiated by the addition of 5 mM D-glucose (omitted in control reactions).

DNA Content. The concentration of DNA was measured using Picogreen quantification method (Invitrogen, Carlsbad, CA). Briefly, the previously homogenized samples were digested in an equal volume of 2X digestion buffer (200mM NaCl, 20mM Tris-HCl (pH 8.0), 50mM EDTA (pH 8.0) 1% SDS and 0.2mg/ml proteinase K) for 13 hours at 55°C. A DNA standard curve was constructed using a mixture of the digested samples. Purified DNA was isolated using phenol:chloroform:isoamyl extraction method. The digested DNA sample was mixed with equal parts phenol:chloroform:isoamyl solution (Invitrogen, Carlsbad, CA), vortexed and centrifuged at 12000 × g for 2 minutes. The top (aqueous) phase was removed and mixed with 1/10 the volume of 3M sodium acetate (pH 5.2) and 2× the volume of ice cold 95% ethanol. This solution was vortexed and precipitated at -80°C for 15 minutes. The sample was then microcentrifuged at 12000 × g for 5 minutes and the supernatant removed. The DNA pellet was washed with 1ml of room temperature 70% ethanol, microcentrifuged at 12000 × g, the supernatant removed and allowed to dry on ice for 15 minutes. The DNA was then resuspended in a small volume of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5) and quantified by UV spectroscopy. A standard curve was constructed from the purified quantified DNA and used to determine the DNA content of each sample. The concentration of DNA in the standard curve and samples was determined by mixing 2ul of digested samples with 200ul of picogreen diluted 400X in TE buffer for minutes in the dark and read in a fluourometer with an excitation of 480nm and emission of 535nm. Samples were then compared to known amounts of DNA in the standard curve.

Statistical analysis

All statistical analyses were performed using SigmaStat v3.5 (Systat Software Inc., San Jose, CA). One-way ANOVA and Holm-Sidak post tests were used to test for significance between tissues and treatments. Significance level was set at $p < 0.05$.

Results

Condition of salmon and trout during experimental trials

The weight, length, %lipid and %protein content were measured in salmon along migration (Table 5.3). Salmon sampled at WH and SA were significantly longer than PH and AR salmon ($p < 0.05$), but there was no change in weight between sites. As a result, the condition factor of the salmon at WH and SA was significantly lower than PH and AR salmon ($p < 0.05$). The % lipid significantly decreased at each site ($p < 0.05$), while there were no changes in % protein of the fish.

Exercised trout grew significantly more (length and weight) during the 4 weeks of chronic exercise than did control trout (Table 5.4; $p < 0.05$). There was no significant difference in the growth of control fish, or in the condition factor of either control or exercised fish.

Salmon gene expression

We examined the expression of eight genes involved in the fatty acid oxidation pathway as well two representative genes involved in carbohydrate and protein oxidation

pathways. CPT I β 1a, β 1b, α 1a and α 2 mRNA expression was highest at the onset of migration (PH) with significant declines throughout migration in both red and white muscle (Fig. 5.2A and B; $p < 0.05$). AMPK β 1 subunit mRNA expression was high at the start and maintained throughout migration until it significantly declined at AR in both tissues (Fig. 5.3A and B; $p < 0.05$). The mRNA expression of both PPAR α 1 and β 1 and HK I were similar in red and white muscle having significant declines at the river entry (WH), peaks at the mid-river site (SA), followed by declines at the spawning site (AR; Fig. 5.3A and B; $p < 0.05$). The expression of AST was significantly higher at both the river entry (WH) and spawning sites (AR) when compared to the ocean (PH) and mid river (SA) sites in both tissues (Fig. 5.3A and B; $p < 0.05$).

Trout gene expression

We measured the same eight genes as above in trout exercised for four weeks. In contrast to migrating salmon, exercised trout showed no significant differences from controls in the mRNA expression of the CPT I isoforms, with the exception of decreases in β 1a and α 1a in red and white muscle, respectively (Fig. 5.4A and B; $p < 0.05$). In red muscle, AMPK β 1 subunit, plus both PPARs and AST were expressed at a significantly lower level after exercise while HK I mRNA levels did not change at 4 weeks of exercise (Fig. 5.5A). In white muscle, PPAR β expression was significantly lower after exercise, while PPAR α and HK I expression was significantly increased (Fig. 5.5B; $p < 0.05$). There were no changes in the expression of AMPK β 1 or AST in white muscle after four weeks of exercise (Fig. 5.5B; $p > 0.05$).

Salmon enzyme activity

The activities of HOAD, CS and HK in red and white muscle are presented per gram tissue and per ng DNA in figures 6 and 7, respectively, to account for potential changes that may be taking place as a result of tissue degradation during the latter parts of the migration. HOAD activity, an indicator of β -oxidation, was significantly higher at the onset of migration (PH) and declined by ~60% at the river entry site (WH) in red muscle (Fig. 5.6A; $p < 0.05$). There was a further significant decline at SA which was maintained at that level until the end of the migration (AR) (Fig. 5.6A; $p < 0.05$). There were no significant changes in white muscle HOAD activity during migration (Fig. 5.6D; $p > 0.05$). A similar pattern exists when activity is expressed per ng DNA (Fig. 5.7A and D). CS activity, and indicator of mitochondrial content, was significantly reduced at WH and AR in red muscle when expressed per gram tissue (Fig. 5.6B; $p < 0.05$). However, this change was abolished when expressed per ng DNA (Fig. 5.7B; $p > 0.05$). No changes occurred in white muscle CS activity during migration (Fig. 5.6 E, 7E; $p > 0.05$). HK activity, and indicator of glycolytic flux, was significantly higher in red muscle at WH and SA sites, and only significantly higher at SA in white muscle (Fig. 5.6C and F; $p < 0.05$). In both tissues, HK activity significantly declined at AR (Fig. 5.6C and F; $p < 0.05$). A similar pattern is observed when expressed per ng DNA, however, HK activity is maintained higher at AR (Fig. 5.7C and F; $p < 0.05$).

Trout enzyme activity

HOAD activity in rainbow trout red muscle was significantly increased after 4 weeks of chronic exercise (Fig. 5.8A; $p < 0.05$). However, this increase is only about one third of the change in activity observed in migrating salmon (Fig. 5.6A; Table 5). HOAD activity did not change across migration or after endurance exercise in white muscle (Fig. 5.8D; $p > 0.05$). There was a significant increase in CS activity in exercised trout when compared to controls (Fig. 5.8B; $p < 0.05$). Again, however, it was not as large as that seen in salmon (Fig. 5.6B; Table 5.5). Similarly to HOAD, there were no significant changes in CS activity in white muscle after chronic exercise or during migration (Fig. 5.8D; $p > 0.05$). HK activity significantly increased in both red and white muscle of trout after 4 weeks of exercise (Fig. 5.8C and E; $p < 0.05$). The increase in red muscle HK was the comparable (82%) as the increase in migrating salmon (Table 5.5). In trout white muscle, however, the change in HK activity after exercise only accounts for about 66% of the change seen in migrating salmon (Table 5.5).

Discussion

The ability of pacific salmon to successfully complete their spawning migration relies partially on their ability to meet the energetic demands of the muscle tissue during chronic exercise in the absence of energy intake. We show here for the first time that migrating pacific salmon potentially transcriptionally and metabolically prime their muscle tissue for an increase in lipid metabolism to help fuel their muscles during migration. In both red and white muscle, genes encoding lipid metabolizing proteins (CPT I isoforms), transcription factors (PPARs) and metabolic regulators (AMPK) are all significantly higher at the onset of migration. Furthermore, the activities of an important lipid oxidizing protein (HOAD) and mitochondrial marker protein (CS) are also higher at the start of the migration in red, but not white, muscle, while the activity of a glycolytic enzyme (HK) was low at the start of migration and increased throughout. Moreover, we show that these changes in enzyme activity may only be partially accounted for by the effects of exercise and that there are likely other factors influencing these large scale metabolic changes seen in migrating salmon.

Metabolic gene expression during migration vs. exercise alone

Evidence suggests that during migration, salmon primarily use lipids as a fuel source for muscles (Gilhousen, 1980). There is a significant and continual reduction of stored lipids of the migrating salmon shown here (Table 5.3). To facilitate this increased use of lipids as fuel, the mitochondria of the muscle tissues must have the proper oxidative machinery in place to oxidize the lipids and meet the energetic demands associated with endurance exercise. To initiate this process, genes encoding for lipid oxidizing proteins may be highly expressed when compared to the final stages of migration and during spawning which primarily involves high intensity burst swimming (Rand, 1998). Indeed, we found this to be true in migrating salmon. The mRNA expression of all but one ($\alpha 1b$) of the CPT I isoforms was $\sim 70\%$ higher at the onset of migration compared to the end in both red and white muscle (Fig. 5.2A and B). If these

transcripts become translated into active proteins, this would allow the salmon to be able to take up fatty acids into the mitochondria for oxidation at a much greater rate.

Concurrently, we see an almost identical pattern in the expression of the transcription factor, PPAR, in both tissues. Both PPAR α 1 and β 1 are significantly higher at the onset of migration and this may partially explain the increased transcription of CPT I, a target gene of PPAR. It should be noted that some salmonids have multiple PPAR α and β isoforms (Leaver et al., 2007), therefore, there may be other changes taking place in those isoforms which we are unaware of.

The mRNA expression of AMPK β 1 subunit, a major regulator of energetic homeostasis, is also significantly higher at the start of migration when compared to the end (Fig. 5.3). AMPK has been shown to activate both PPAR α and peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1 α) to increase the expression of PPAR α target genes such as CPT I and stimulate mitochondrial biogenesis (Lee et al., 2006; Winder et al., 2000). Therefore, in migrating salmon, a significant increase in transcription of this gene at the onset of migration may help prepare the muscle tissue for large increases in mitochondrial content and lipid oxidizing enzymes for use throughout migration.

The use of glycolytic metabolism is thought to be reserved for the very end of migration to maintain valuable glycogen stores for the brain and to fuel the burst activity of spawning (Gilhousen, 1980; Mommsen, 1980). Unexpectedly, we found significantly higher HK I mRNA expression in both red and white muscle at the beginning of migration than at the end (Fig. 5.3). However, this was not mirrored by HK enzyme activity (see below). Lastly, we investigated the expression of AST which can be indicative of protein oxidation capacity which tends to occur towards the end of migration. The mRNA expression of AST was low at the beginning of migration, but immediately increased by 4.5- and 11-fold in red and white muscle, respectively, upon river entry. This data may suggest that the oxidation of proteins may occur much earlier than we expected as we anticipated the use of proteins as fuel to commence later in migration once lipid stores had been depleted. Little research has been conducted on AST, but previous research has suggested that the transamination of amino acids provides intermediates for the TCA cycle and that this process occurs more rapidly during large increases in lipid metabolism (Owen and Hochachka, 1974). We expect the oxidation of lipids in the salmon to be high at this point during migration, therefore, this increase in AST mRNA expression may suggest that amino acids are being used to supplement the TCA cycle to keep up with the flux of acetyl-CoA to the TCA cycle from the oxidation of lipids. At the end of the migration, the mRNA expression of the AST in the salmon is increased again, this time by 2- and 14-fold in red and white muscle, respectively. At the end of the migration we expect there to be little oxidation of lipids, as the stores have been depleted, therefore, the expression of AST may be higher to promote oxidation of proteins to continue to fuel the muscles. The massive increase in AST in the white muscle may indicate that the white muscle is the most severely affected by protein catabolism, likely because of its overall percentage of body mass.

To assess the relative contribution of exercise in mediating these changes in gene expression, we investigated the mRNA expression of the same genes in trout which were

exercised for 4 weeks. We expected that the expression of CPT I, AMPK β 1 and PPARs would be significantly higher post exercise to facilitate the use of lipids. However, in most cases, in both red and white muscles we found no change or significant declines in expression. CPT I expression was largely maintained to the same extent as resting fish with the exception of CPT I β 1a in red muscle and α 1a in white muscle, both of which decreased after exercise (Fig. 5.4). AMPK β 1 and PPAR α and β expression also decreased in red muscle after exercise. In trout white muscle, there was no change in AMPK β 1 expression while there was a significant increase in PPAR α . PPAR β decreased with exercise training similar to results obtained in zebrafish in a mixed muscle sample after 4 weeks of exercise (McClelland et al., 2006). It is most likely that the transcriptional changes we expected occurred earlier during the exercise regime, perhaps during the first week. Therefore, it would be beneficial to examine temporal changes in mRNA expression during exercise. Furthermore, as mentioned above, we have only investigated one isoform each of PPAR α and β , and there may be changes that occur in the expression of the other isoforms during exercise.

Metabolic enzyme activity during migration vs. exercise alone

Similarly to the salmon gene expression data, the enzyme activity of both HOAD and CS were higher at the onset of migration in red muscle (Fig. 5.6) which may indicate an increase in the capacity for fatty acid oxidation. There were no changes in the activity of these enzymes in white muscle as expected, because white muscle generally does not play a large role in the oxidation of lipid for sustained aerobic exercise. When the activity is expressed per nanogram DNA, we see the same pattern for HOAD activity, however, changes in CS are abolished. The elevated HOAD and CS activity at the start of migration may be due to the increased expression of AMPK and PPAR α which have a stimulatory effect on these enzymes (Lee et al., 2003; Lee et al., 2006; Winder et al., 2000).

The activity of HK increased during the later stages of migration in both red and white muscle (Fig. 5.6) most likely to facilitate a greater use of stored glycogen. We expected HK activity to remain high even at the end of the migration, however, there was a significant decline in activity at the spawning grounds. At this point, it is possible that the glycogen stores have been depleted, or that the expression and activity of all metabolic enzymes begins to decline as the tissue damage becomes too great. In fact, when we express HK activity per ng DNA we obtain the similar results, although HK activity seems to be maintained at the AR site. This increase in activity occurs despite the decrease in HK I expression that occurs in both tissues during migration (Fig. 5.3). It may be possible that the transcripts expressed in the early stages of the migration are stable throughout, and become translated when the need arises. Alternatively, the activity of HK is most likely a combination of different isoforms with differing kinetics, while our expression data solely measures the expression of one isoform, HK I. In mammals, this isoform is thought to be the “housekeeping” isoform of HK as it is ubiquitously expressed and relatively unaffected by most physiological and metabolic changes (Sebastian et al., 1999). However, based on our data, this isoform may have a different function in salmonids during migration as there are significant changes in expression and activity.

After four weeks of exercise, we found an increase in HOAD, CS and HK activity in trout red muscle (Fig. 5.8). An increase in HOAD and CS activity would increase the capacity for lipid oxidation. This increase, however, is only a fraction of the increase we observed in migrating salmon (Table 5.5). Most notably, HOAD and CS were 4.6 and 6.8 times higher, respectively, during migration, but only 1.6 and 2.4 times higher respectively, during exercise in trout. The changes resulting from exercise alone appear to only account for approximately one third of the change that occurs during migration. This suggests that either salmon respond more exercise than trout, or that there are other factors other than exercise which are acting on the activity of these enzymes. In this case, it may be the effect of fasting which is known to stimulate lipid oxidizing enzymes. However, we cannot rule out the possibility that our exercise regime may not have been as rigorous, although we attempted to keep the speed and duration similar to that of a migrating salmon, with the exception of the burst type swimming which can occur frequently during migration.

In contrast, the change in red muscle HK in both migrating salmon and exercising trout is quite similar. White muscle, on the other hand, shows a greater increase in the salmon than it does in the trout. White muscle HK activity is known to increase in response to exercise training (Hochachka and Somero, 1984) and this is clearly demonstrated in both species. However, the increase during exercise only accounts for approximately 66% that of the change in salmon (Table 5.5). These differences may due to the availability of different substrates. In the exercised trout, both lipids and glucose are being obtained through the diet, whereas, in the salmon, lipids are primarily the only available substrate while glucose is reserved. Therefore, the relative need to oxidize glucose in the presence of lipids is less in the trout. Furthermore, the migrating salmon, unlike the trout, have to fuel burst type exercise which is most likely accomplished by the white muscle, therefore, the salmon may have an increase in metabolic capacity in preparation for this type of activity.

Conclusion

The ability of migrating salmon to use lipid as a fuel source for their exercising muscles appears to come about via genomic and metabolic remodelling. Increases in the expression and activity of important enzymes involved in fatty acid oxidation occur at the onset of migration potentially to increase the capacity for lipid oxidation, and decline at the end of migration as the use of lipids as fuel declines. These changes, to some extent, may be a result of exercise, but there are most likely numerous factors contributing to the change in fatty acid oxidizing machinery. In the case of the salmon, fasting, temperature and hormonal changes are distinct possibilities, although their effects on the processes presently investigated are unknown.

Alternatively, the data could be interpreted not as an elevation at the onset of migration, but rather a decrease in these factors throughout migration as a result of the inability to oxidize lipids either due to their reduction in availability, or due to overall loss of muscle tissue and a reduction in transcription and activity in response to lack of energetic resources. It will be important in future studies to determine rates of lipid oxidation and the function of transcriptional machinery to address this issue. In addition,

it will be vital to address the mechanisms which may be signalling these shifts in transcriptional and metabolic activity during migration and also to assess whether these changes are playing in a role in differences in migration distances between populations of the same species. Indeed, there is evidence that salmon population which have longer migration distances are more energy efficient than coastal populations (Crossin, 2004; Gilhousen, 1980).

Table 5.1: Primers used to amplify and sequence PPAR α . S1, S2, S3 represent individual sections of the gene which were sequenced individually.

Primer	5' to 3' Forward Primer	5' to 3' Reverse Primer	T_m °C	Size (bp)
PPAR α S1	catctacgaggcctacctcaa	agacttggcgaactcggta	58	259
PPAR α S2	cgaggcctacctaagaact	tggagtgacgtgcctctgt	60	741
PPAR α S3	tcttctaacctggagtgtcg	agggtggagtgcctgtcttg	60	424

Table 5.2: Primer sequences used for real time PCR analysis

Gene	5' to 3' Forward Primer	5' to 3' Reverse Primer	T_m °C	Size (bp)
PPAR ^α	ccaagttcagttgccatga	attggggaagaggaaggtgt	60	173
PPAR ^β	ctggagctggatgacagtga	gtcagccatctgttgagca	60	195
CPT I ^β 1	gatgttccgtgagggtagga	ttgtcttgcattggctctgac	58	80
CPT I ^β 2	gccgcaaactagagagagga	cccgtagtacagccacacct	58	199
CPT I ^α 1a	atgaggaatgccctcaagtg	gcttctgccagagaacaac	58	120
CPT I ^α 1b	cgcttcaagaatggggtgat	caaccacctgctgtttctca	58	187
CPT I ^α 2	ccgttcctaacagaggtgct	acactccgtagccatcgtct	58	154
HK	ctgggacgctgaagaccaga	cggtgctgcatacctccttg	58	159
AST	gacctgtggcttgacttcc	gcaatctccttccactgctc	58	135
AMPK	actgtgttccgttgacagg	tcaatcatgaggcatcaaa	58	272
EF1 ^α	cattgacaagagaaccattga	ccttcagctgtccagcac	58	94

Table 5.3: Physical characteristics of salmon at each sampling point along the migration route. Values are means \pm S.E. n=5. Letters indicate significance between sampling sites.

Site	fork length (cm)	weight (kg)	condition factor	% lipid	% protein
Port Hardy	54.2 \pm 1.2 ^a	2.34 \pm 0.037	1.5 \pm 0.10 ^a	15.6 \pm 0.6 ^a	18.4 \pm 0.6
Whonnock	58.0 \pm 0.7 ^b	2.35 \pm 0.081	1.2 \pm 0.02 ^b	10.3 \pm 1.4 ^b	19.1 \pm 0.5
Savona	58.0 \pm 1.0 ^b	2.20 \pm 0.137	1.1 \pm 0.10 ^b	6.4 \pm 1.0 ^c	19.6 \pm 1.5
Adam's River	52.8 \pm 0.7 ^a	2.34 \pm 0.063	1.6 \pm 0.04 ^a	2.5 \pm 0.2 ^d	17.9 \pm 0.4

Table 5.4: Physical characteristics of trout before and after four weeks of exercise. Values are means \pm S.E. n=4. Asterisks indicates significance between initial and final values. CF = condition factor.

	Initial	Final
Control		
Length (cm)	16.10 \pm 0.20	16.17 \pm 0.39
Weight (g)	43.95 \pm 2.09	47.75 \pm 4.54
CF	1.05 \pm 0.02	1.11 \pm 0.05
Exercised		
Length (cm)	16.50 \pm 0.35	18.12 \pm 0.38*
Weight (g)	46.17 \pm 2.64	75.15 \pm 5.08*
CF	1.02 \pm 0.03	1.25 \pm 0.04

Table 5.5: Fold change in enzyme activity in red and white muscle between the beginning and end of migration in salmon and before and after exercise in trout. HOAD – β -hydroxyacyl-CoA dehydrogenase; CS – citrate synthase; HK – hexokinase.

Enzyme	Red Muscle		White Muscle	
	Migrating Salmon	Exercised Trout	Migrating Salmon	Exercised Trout
HOAD	4.6x	1.6x	no change	no change
CS	6.8x	2.4x	no change	no change
HK	1.7x	1.4x	2.7x	1.8x

Fig. 5.1:

Map of the Fraser river system with sampling location indicated by stars. Port Hardy = ocean site; Whonnock = river entry site; Savona = mid-river site; Adam's River = spawning grounds.

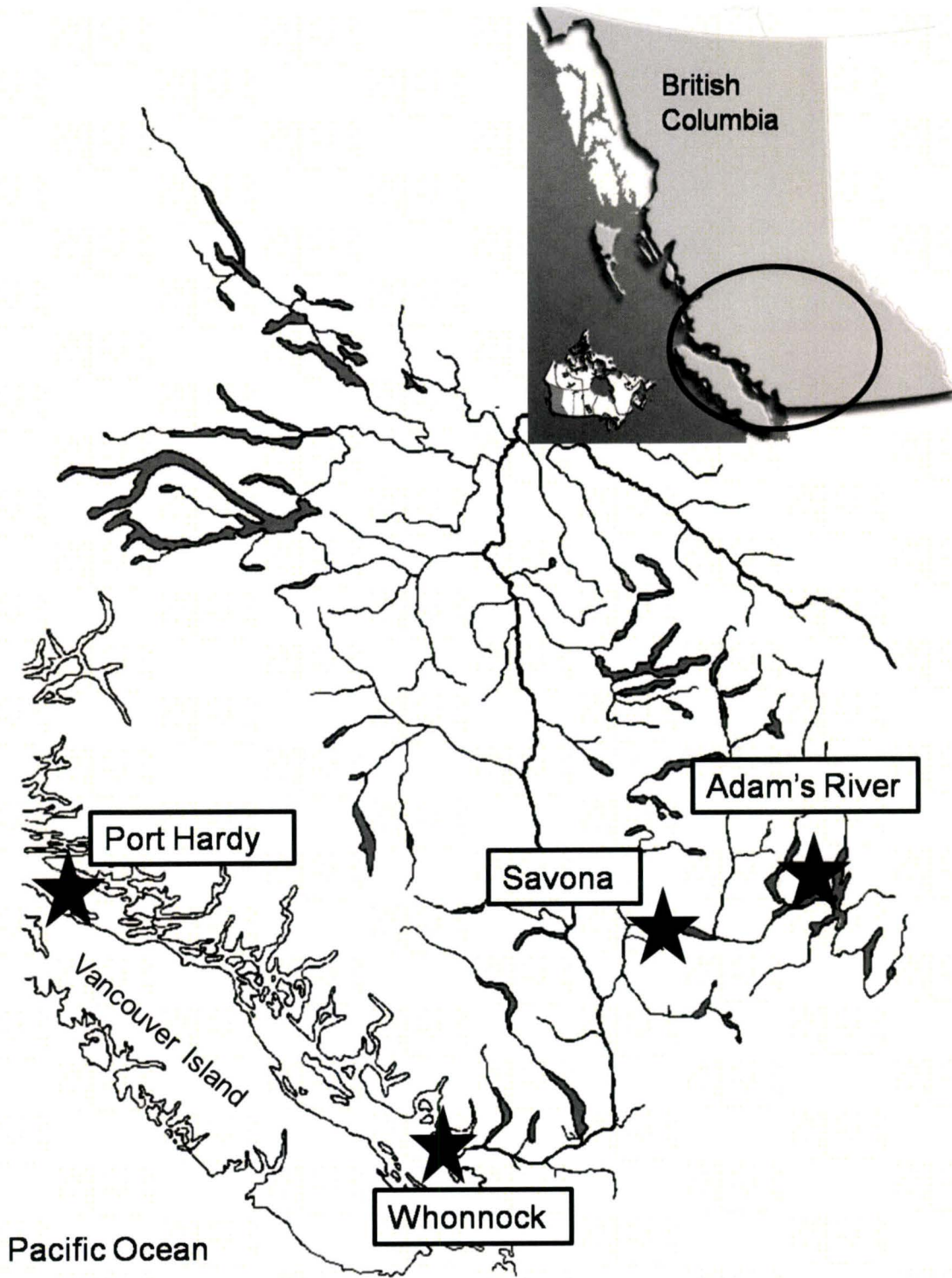


Fig. 5.2:

Carnitine palmitoyltransferase I isoform mRNA expression in salmon A) red and B) white muscle across 4 sampling sites during migration. Values are means \pm S.E. n=5. Letters indicate significance between sites for each individual isoform. PH - Port Hardy; WH - Whonnock; SA - Savona; AR - Adam's River.

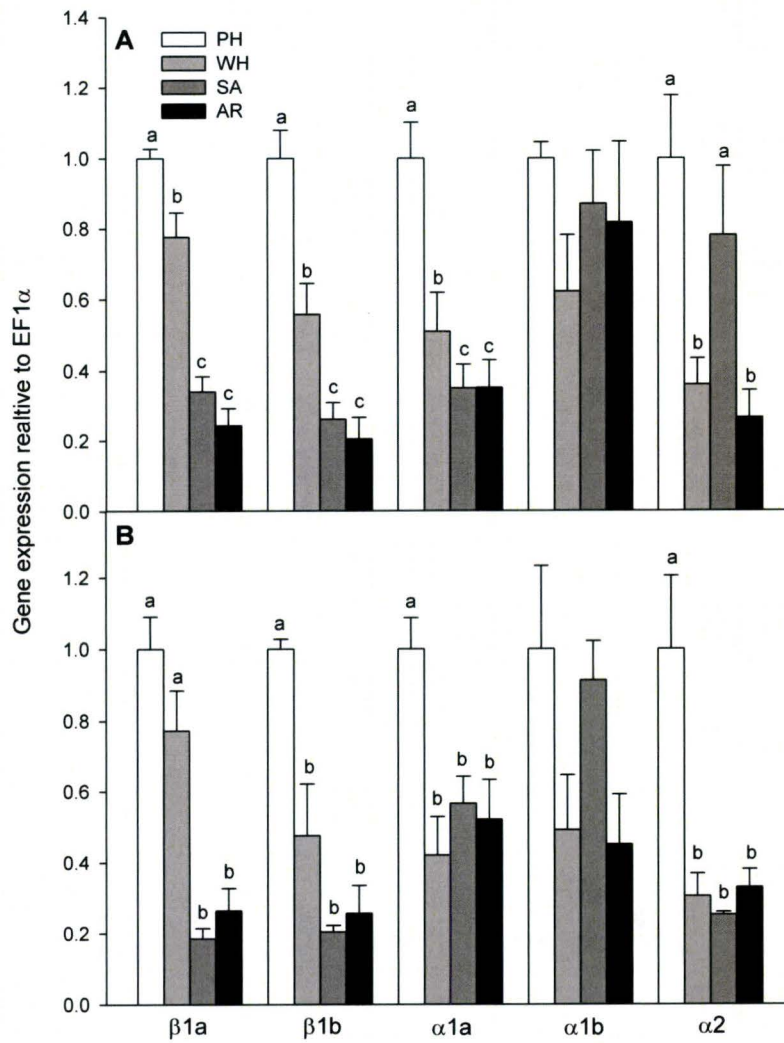


Fig. 5.3:

AMP kinase (AMPK), peroxisome proliferator-activated receptor (PPAR) α and β , hexokinase (HK) and aspartate amino transferase (AST) mRNA expression in salmon A) red and B) white muscle across 4 sampling sites during migration. Values are means \pm S.E. n=5. Letters indicate significance between sites for each individual isoform. PH - Port Hardy; WH - Whonnock; SA - Savona; AR - Adam's River.

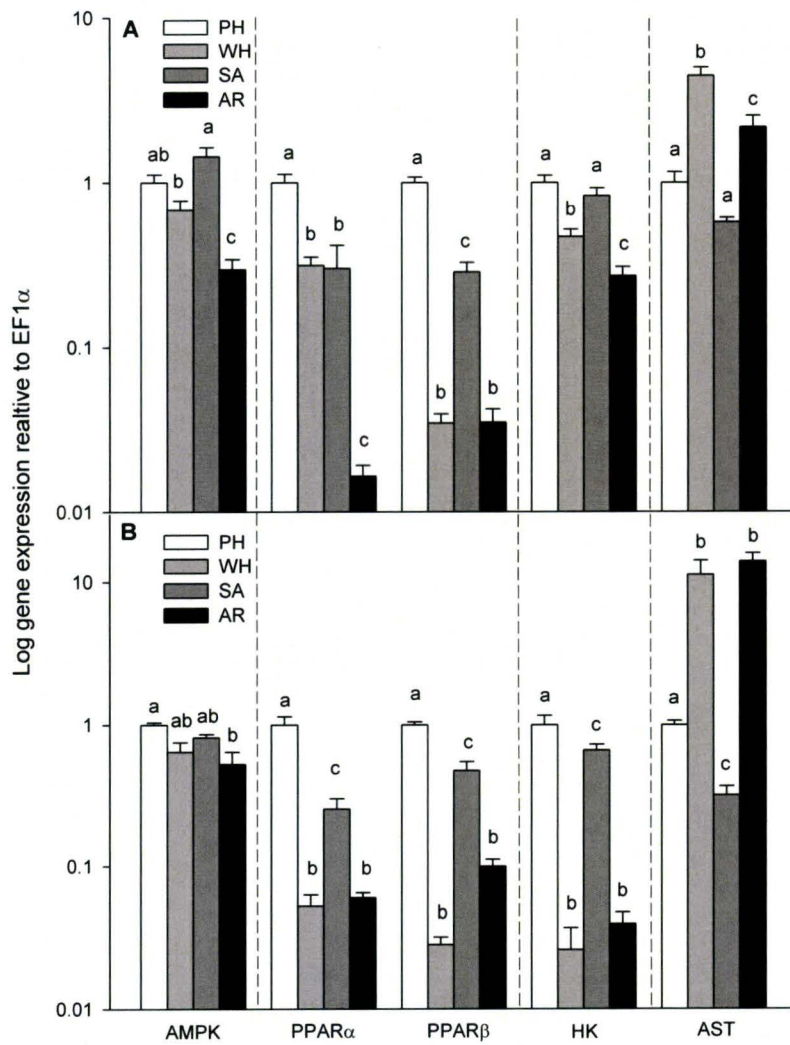


Fig. 5.4:

Carnitine palmitoyltransferase I isoform mRNA expression in trout A) red and B) white muscle across before and after 4 weeks of exercise. Values are means \pm S.E. n=4. Asterisk indicates significance between control and exercised fish for each individual isoform.

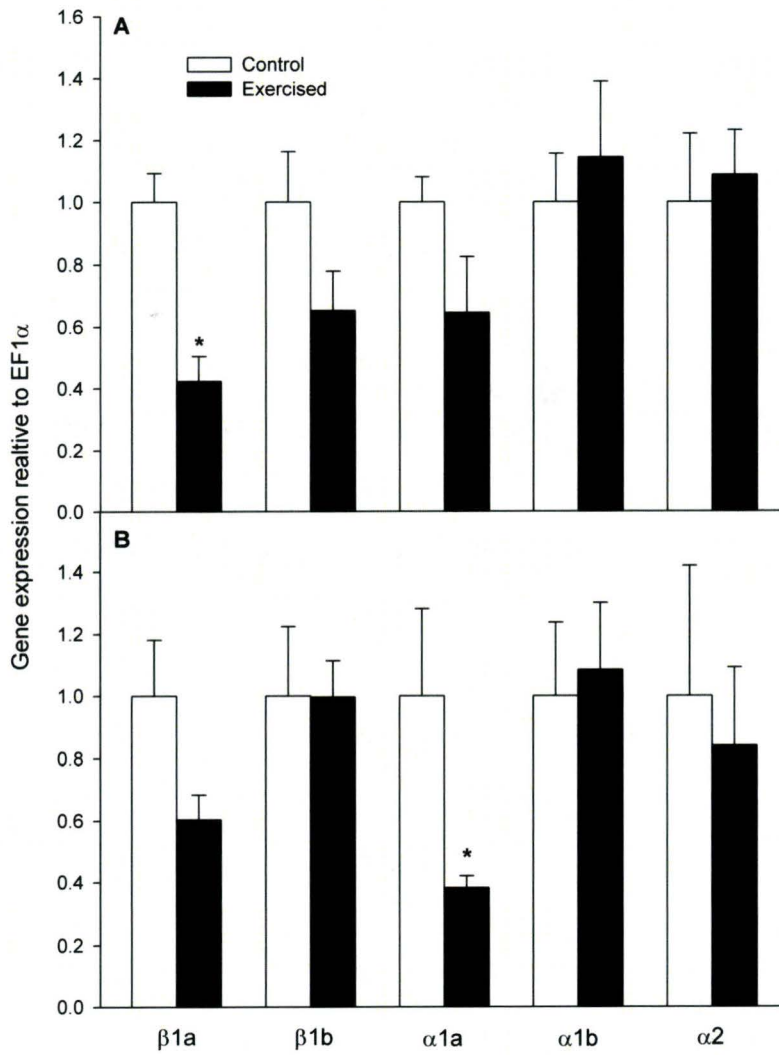


Fig. 5.5:

AMP kinase (AMPK), peroxisome proliferator-activated receptor (PPAR) α and β , hexokinase (HK) and aspartate amino transferase (AST) mRNA expression in trout A) red and B) white muscle before and after 4 weeks of exercise. Values are means \pm S.E. n=4. Asterisk indicates significance between control and exercised fish for each individual isoform.

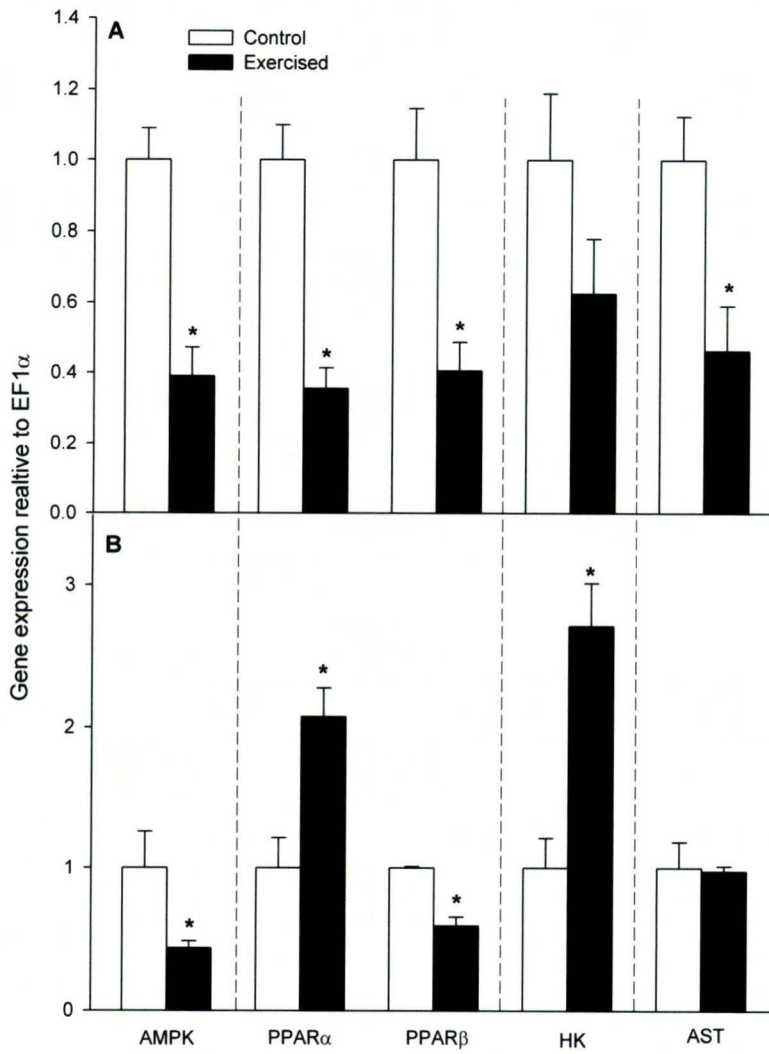


Fig 5.6:

Enzyme activity of β -hydroxyacyl-CoA dehydrogenase (HOAD) (A,D), citrate synthase (CS) (B,E) and hexokinase (HK) (C,F) expressed per g tissue in salmon red and white muscle. Values are means \pm S.E. n=5. Letters indicate significance between sites. PH - Port Hardy; WH - Whonnock; SA - Savona; AR - Adam's River.

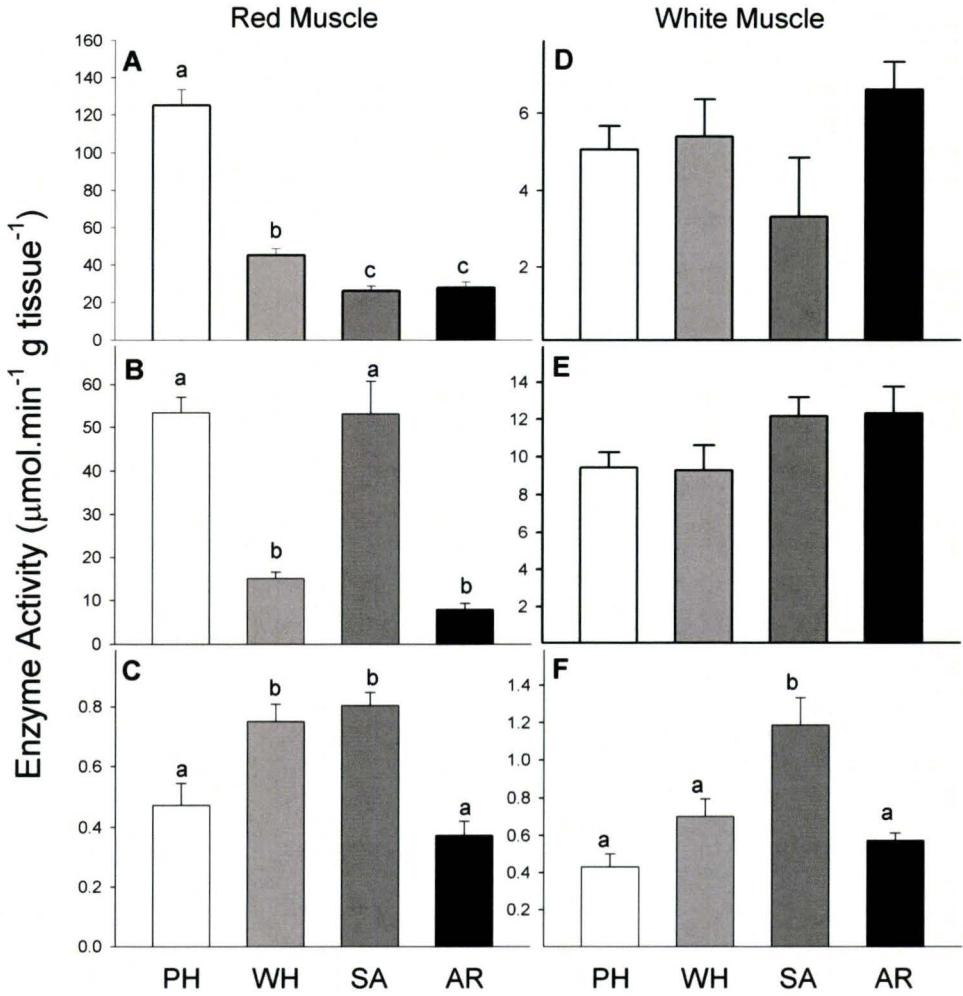


Fig. 5.7:

Enzyme activity of β -hydroxyacyl-CoA dehydrogenase (HOAD) (A,D), citrate synthase (CS) (B,E) and hexokinase (HK) (C,F) expressed per ng DNA in salmon red and white muscle. Values are means \pm S.E. n=5. Letters indicate significance between sites. PH - Port Hardy; WH - Whonnock; SA - Savona; AR - Adam's River.

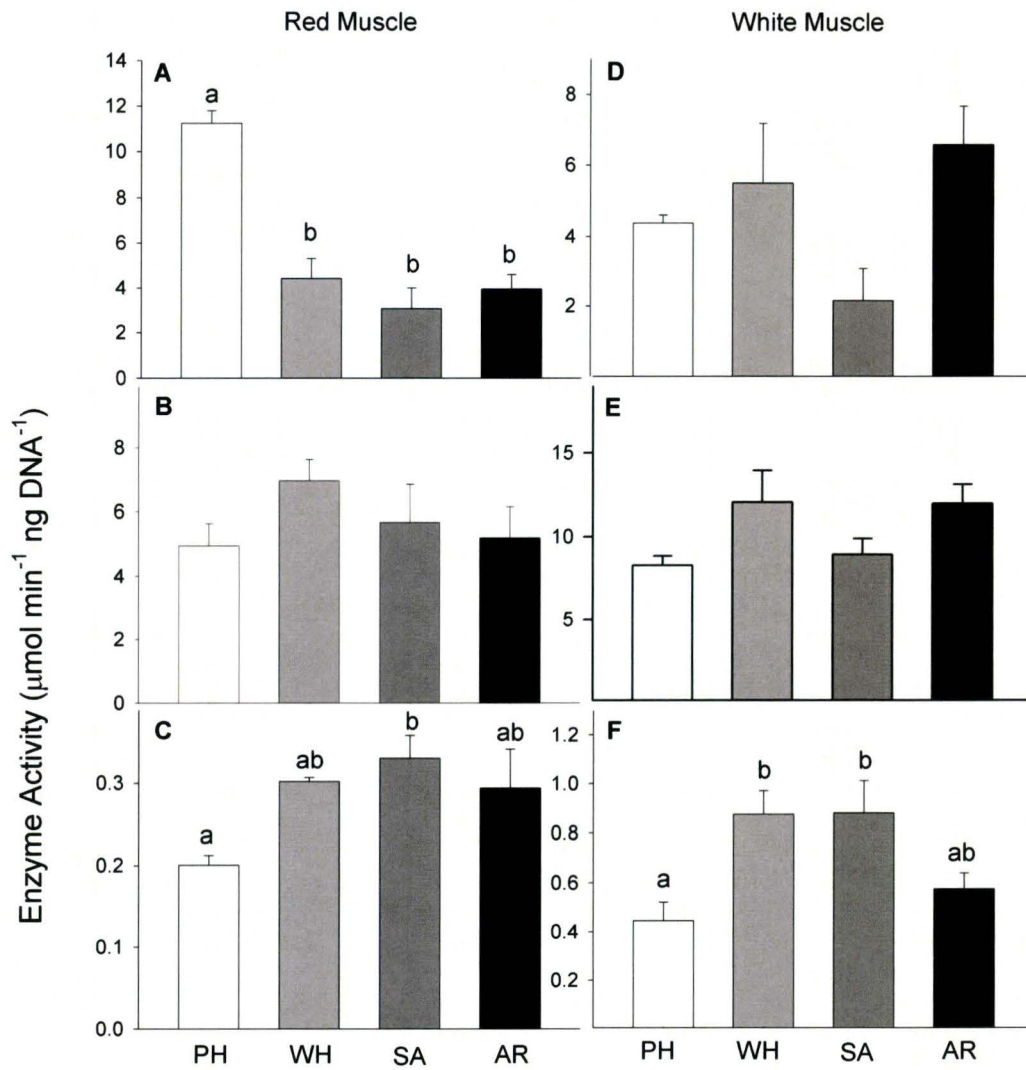
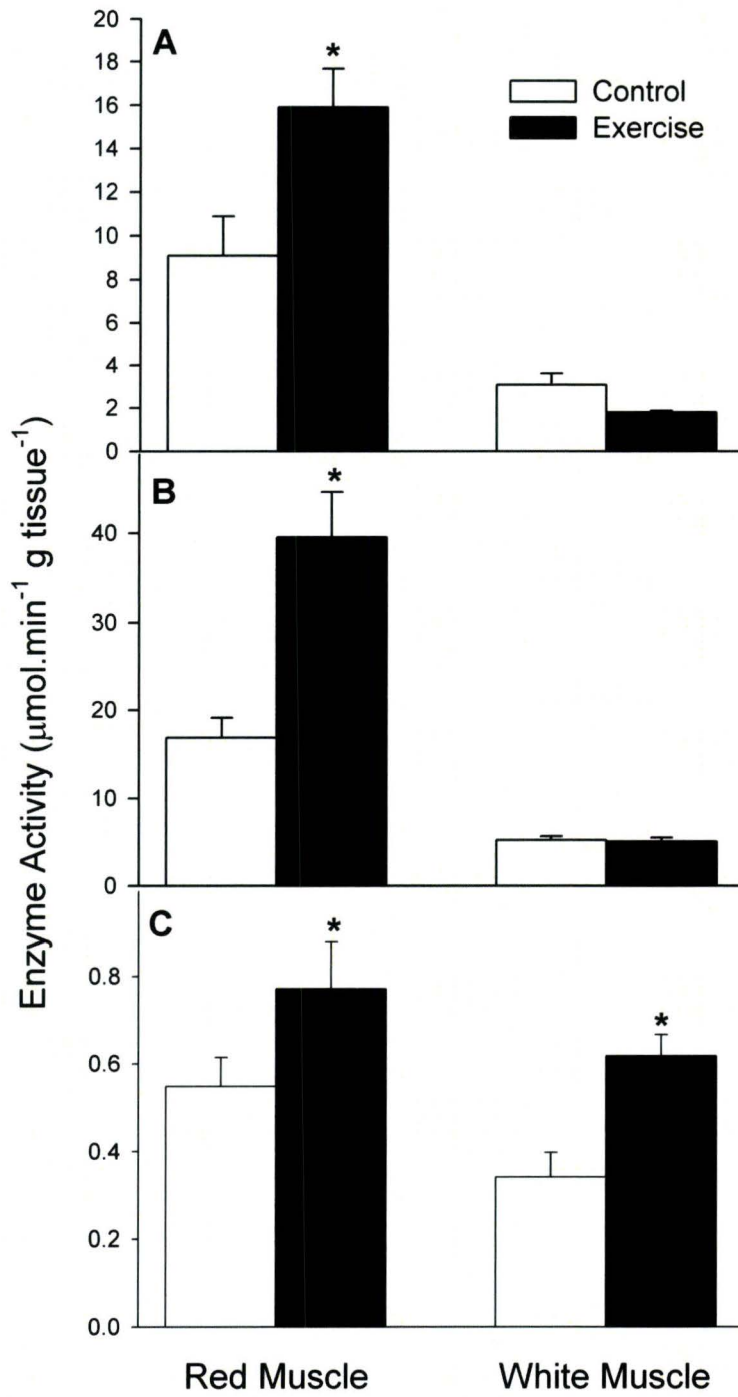


Fig. 8:

Enzyme activity of A) β -hydroxyacyl-CoA dehydrogenase (HOAD), B) citrate synthase (CS) and C) hexokinase (HK) in trout red and white muscle before and after 4 weeks of exercise. Values are means \pm S.E. n=4. Asterisk indicates significance between control and exercised fish.



REGULATION OF CARNITINE PALMITOYLTRANSFERASE (CPT) I DURING
FASTING IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) PROMOTES
INCREASED MITOCHONDRIAL FATTY ACID OXIDATION

Abstract

Periods of fasting, in most animals, are fuelled principally by fatty acids, and changes in the regulation of fatty acid oxidation must exist to meet this change in metabolic substrate selection. We examined the regulation of carnitine palmitoyltransferase (CPT) I, to help explain changes in mitochondrial fatty acid oxidation with fasting. After five weeks of fasting, mitochondria were isolated from red muscle and liver of rainbow trout (*Onchorhynchus mykiss*) to determine 1) mitochondrial fatty acid oxidation rate, 2) CPT I activity and the concentration of malonyl-CoA to inhibit this activity by 50% (IC₅₀), 3) mitochondrial membrane fluidity, 4) tissue malonyl-CoA content, and 5) CPT I (5 known isoforms) and PPAR (α and β) mRNA expression. In isolated mitochondria from both tissues fatty acid oxidation increased during fasting by four- and ten-fold in liver and red muscle, respectively. Fasting also decreased CPT I sensitivity to malonyl-CoA (increased IC₅₀) by two- and eight-times in red muscle and liver, respectively, suggesting this facilitates fatty acid oxidation. In the liver, there was also a significant increase in isolated mitochondria CPT I activity and in whole tissue PPAR α and PPAR β mRNA expression. However, there were no changes in mitochondrial membrane fluidity in either tissue, indicating that the decrease in CPT I sensitivity to malonyl-CoA is not due to bulk changes in mitochondrial membrane fluidity. However, there were significant differences in CPT I mRNA expression during fasting. These data indicate that there are important changes in the regulation of CPT I which promote increased mitochondrial fatty acid oxidation during fasting in trout.

Introduction

There are a variety of animal species that experience bouts of fasting as part of their natural life history. In most animals, these periods of fasting are fuelled principally by fatty acids. Indeed, the use of lipids as fuel is advantageous because they are highly reduced and can be stored in large quantities without hydration. The enzyme carnitine palmitoyltransferase (CPT) I is an important regulator of mitochondrial fatty acid oxidation, and may become important during fasting, since it regulates long chain fatty acid entry into the mitochondria (McGarry et al., 1983). Indeed, current evidence suggest that when food is unavailable or intake is suspended (e.g. during migration in fish) lipids are preferentially oxidized over other fuel sources in both mammals and fish. In contrast, carbohydrates are spared to provide glucose to the brain and central nervous system (Neumann-Haefelin et al., 2004), while proteins are usually only oxidized when lipid stores have been almost completely depleted (Frayn, 1996). There are a host of metabolic modifications that must take place to increase lipid oxidation and decrease carbohydrate oxidation. As a first step to uncovering the regulatory changes that occur with fasting in fish we chose to focus on various aspects of mitochondrial lipid oxidation and the regulation of CPT I.

Regulation of mitochondrial fat oxidation involves both genetic and non-genetic regulation of CPT I activity (McClelland, 2004; Morash et al., 2008). Regulation can be isoform-specific and in mammals, liver and muscle CPT I isoforms are encoded by two different genes, CPT I α in the liver and CPT I β in the muscle (Britton et al., 1997). These isoforms are differentially sensitive to regulation by malonyl-CoA (M-CoA). Muscle CPT I has been shown to be approximately 100 times more sensitive to M-CoA than the liver isoform in mammals (McGarry and Brown, 1997). Previously there was thought to be only one CPT I gene in fish (Gutierrez et al., 2003). Our recent research has demonstrated that that trout liver and muscle CPT I are differentially sensitive to M-CoA. However, unlike mammals, trout liver CPT I is more sensitive to M-CoA than CPT I in muscle (Morash et al., 2008). This phenomenon is most likely due to the differential expression of five distinct CPT I isoforms now known to be expressed in both liver and red muscle of rainbow trout .

CPT I mRNA and protein expression in mammals is, in part, controlled by the transcription factor peroxisome proliferator-activated receptor (PPAR) (Price et al., 2000). It is assumed that fish CPT I genes also contain a PPAR response element (PPRE) although the situation may be more complicated as multiple isoforms of PPAR α and β are known to exist, at least in some fish species (Leaver et al., 2007; Robinson-Rechavi et al., 2001a). Currently, only single isoforms of PPAR α and β have been identified in trout. The effects of fasting on gene expression in fish muscle and liver is currently unclear. If these nuclear receptors act similarly in fish as they do in mammals, increases in cytosolic free fatty acids, which occur with fasting, could act as ligands for PPARs thereby increasing transcription of many genes of the fatty acid oxidation pathway (Price et al., 2000). Also, differences in regulation of CPT I between fish and mammals suggest that there may be species-specific responses to fasting at the level of mitochondrial fatty acid oxidation.

In addition to genetic control, CPT I activity is also regulated allosterically by M-CoA which is produced during the first committed step of fatty acid synthesis in the liver (McGarry and Brown, 2000). M-CoA in the cytosol can then inhibit CPT I activity and prevent the oxidation of any newly formed fatty acids. M-CoA levels can fluctuate in cells depending on the nutritional state of the animal and energetic status of the tissue. In both fish and mammals the liver is the primary site of lipogenesis and during fasting lipogenesis can be reduced in mammals by up to 80% thereby reducing the generation of M-CoA and promoting fat oxidation by reducing the inhibition on CPT I (Jansen et al., 1966). In muscle, where lipogenesis is low, M-CoA is believed to be present only to regulate CPT I (McGarry et al., 1983). Here, M-CoA is regulated by the balance in activity between acetyl-CoA carboxylase (ACC) and malonyl-CoA decarboxylase (MCD) (Alam and Saggerson, 1998).

Regulation of fatty acid oxidation is not only dependent on levels of tissue M-CoA, but also the sensitivity of CPT I to this modulator. Most notably, changes in sensitivity can occur by changes in mitochondrial membrane fluidity (Kolodziej and Zammit, 1990). This has been theorized to occur because the active site for M-CoA is located on the cytosolic side of CPT I and is adjacent to the mitochondrial membrane (Jackson et al., 2000). Increases in the fluidity of the mitochondrial membrane reduce the ability of M-CoA to bind, as key amino acids at the N- and C-termini of the M-CoA binding site of the CPT I protein become separated (Faye et al., 2005). In mammals, mitochondrial membrane fluidity can be altered by a number of physiological states including diabetes and starvation (Zammit et al., 1997) as well as by ingesting a diet high in polyunsaturated fatty acids (Power et al., 1994). In rat liver, starvation causes an increase in mitochondrial membrane fluidity (Zammit et al., 1998) as well as a decrease in CPT I sensitivity to M-CoA (Drynan et al., 1996; Zammit et al., 1998). In fish, membrane composition and fluidity are highly malleable by temperature (Hazel, 1984), and diet (Guderley et al., 2008; Morash et al., 2008), but the effect of fasting is currently unclear.

To date, most research on the effects of fasting on lipid oxidation has focused on a single tissue (liver) and taxon (mammals). However, many fish species experience bouts of fasting as part of their natural life history which makes them prime group for investigating the physiology of fasting. We have previously shown that trout liver and red muscle CPT I show differential regulation and activity, and that this regulation is quite different from that seen in mammals (Morash et al., 2008).

Our goal was to determine the responses in liver and red muscle mitochondria on rainbow trout to prolonged food deprivation. More specifically we examined the genetic and non-genetic regulation of CPT I as a means to increase fatty acid oxidation capacity with fasting. After 5 weeks of fasting, mitochondria were isolated from red muscle and liver to determine 1) changes in mitochondrial fatty acid oxidation, 2) CPT I activity and sensitivity to M-CoA 3) membrane fluidity, 4) tissue concentrations of M-CoA, and 5) CPT I isoforms and PPAR (α , β) mRNA expression.

Materials and Methods

Experimental Fish and Conditions

Rainbow trout, *Oncorhynchus mykiss*, (~200g) were obtained from a local hatchery (Humber Springs, Orangeville, ON) and maintained in 500 L tanks with circulating dechlorinated Hamilton tap water kept at 12°C and fed a commercial fish diet (Profishent Classic Floating Trout Grower, Martin Mills, Elmira, ON) until the start of the experiment. Upon the start of the experiment, fish were randomly separated into two 500L tanks. For five weeks, one group of fish were fed the same commercial diet daily to satiation while the other group was fasted.

Mitochondrial Isolation

Mitochondria were isolated from red muscle and liver according to established methods (Moyes et al., 1988; Suarez and Hochachka, 1981) and previously described in elsewhere (Morash et al., 2008). Briefly, each tissue was immediately excised (red muscle ~4 g and whole liver) and placed, on ice, in mitochondrial isolation buffer (MIB) containing (in mM) 140 KCl, 10 EDTA, 5 MgCl₂, 20 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 0.5% BSA (pH 7.0) for red muscle and 250 sucrose, 1 EDTA, 20 HEPES and 0.5% BSA (pH 7.4) for liver. Tissues were homogenized three times, first using a wide clearance Teflon pestle on a chilled glass homogenizer, then three times with a narrow clearance Teflon homogenizer to lyse cells. Homogenates were centrifuged at 800 ×g for 10 min at 4 °C. The supernatant was then spun at 9000 ×g for 10 min at 4 °C. Pellets were resuspended in the appropriate MIB lacking BSA and spun again at 9000 ×g for 10 min at 4 °C. The supernatant was discarded and the mitochondrial pellet was resuspended in an appropriate volume of MIB lacking BSA and kept on ice for use in a CPT I assay and fatty acid oxidation assay.

Enzyme and Protein Assays

Fatty Acid Oxidation Assay. Fatty acid oxidation was assayed using a modified protocol for human skeletal muscle (Bezaire et al., 2006) and respiration buffer for fish (Leary et al., 2003). Palmitate oxidation was determined at room temperature by measuring trapped ¹⁴C from labelled CO₂ production after a 30 minute incubation of viable mitochondria in a sealed system. An 900 µl aliquot of respiration buffer (in mM, 140 KCl, 20 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 5 Na₂HPO₄, 0.5% BSA supplemented with 5 ATP, 1 NAD⁺, 0.5 carnitine, 0.1 coenzyme A, 0.5 malate) was added to a 20 mL glass scintillation vial which contained 150 µl of benzethonium hydroxide in a tube suspended in the reaction vial to trap ¹⁴CO₂ produced during oxidation. Mitochondria (100 µl ~2 mg·ml⁻¹) were added to the system and then sealed with a rubber cap. The reaction was then started with the addition of 1 µCi of [1-¹⁴C]palmitate (specific activity 56 mCi/mmol: Amersham Biosciences, Baie d'Urfé, Quebec) via a syringe through the rubber cap. After 30 minutes at room temperature the reaction was terminated by the addition of 50 µL of HClO₄. The remaining reaction mixture was acidified using 1 mL of H₂SO₄. Gaseous ¹⁴CO₂ produced from the oxidation of [1-¹⁴C]palmitate was trapped in the benzethonium hydroxide for 90 minutes at room

temperature. The suspended tube containing the benzethonium hydroxide and trapped $^{14}\text{CO}_2$ was then transferred to a new scintillation vial and radioactivity was counted.

CPT I Assay. Radioactive CPT I assay followed a published protocol (Morash et al., 2008) which was modified from previous methods for mammals (McGarry et al., 1983) to incorporate the appropriate changes for fish mitochondria (Rodnick and Sidell, 1994). Briefly, 1 μCi /sample of L-[methyl- ^3H] carnitine hydrochloride (specific activity 82.0 Ci/mmol) (Amersham Biosciences, Quebec) was added and 70 μl of the assay mixture (in mM, 20 HEPES, 40 KCl, 1 EGTA, 220 sucrose, 0.1 DTT, 0.04 palmitoyl-CoA, 1 carnitine and 1.3 mg/ml BSA, pH 7.0) in 1.5 ml Eppendorf tubes and incubated with 10 μl of 0.5–500 mM malonyl-CoA (for a final concentration of 0.05–50 μM) or H_2O in place of mitochondria for blanks or in place of malonyl-CoA and maximum activity. The reaction was initiated by the addition of 20 μl of mitochondria in MIB ($\sim 2 \text{ mg}\cdot\text{ml}^{-1}$), and incubated at room temperature for 8 min. The reaction was stopped by the addition of 60 μl of 1M HCl. The palmitoyl-[^3H]-carnitine was collected according to (Starritt et al., 2000). 20 μl of the assay mixture with L-[methyl- ^3H]-carnitine hydrochloride was also counted in duplicate for determination of individual specific activity. Background counts were determined from a blank sample containing aqueous counting scintillation fluid. The decays per minute (DPM) were read for 5 minutes per sample on a Tricarb 2900 TR Liquid Scintillation Analyzer (PerkinElmer) using QuantaSmart 1.31 (Packard Instrument Company) analysis software. The assay was performed at room temperature.

Protein Content. Protein concentrations were determined by the Bradford method (Bradford, 1976) using a BioRad kit (BioRad, Mississauga, Ontario).

Mitochondrial Membrane Fluidity

Aliquots of frozen mitochondria were thawed on ice and diluted in 3 mL of mitochondrial isolation buffer to a concentration of 50 $\mu\text{g}\cdot\text{ml}^{-1}$ in a 3 mL quartz cuvette. Diluted mitochondria were then incubated in the dark with 1.5 μL 1,6-diphenyl 1,3,5-hexatriene (DPH) in 2 mM N',N'-dimethyl formamide for 10 minutes at 10°C. Samples were stirred throughout the duration of the incubation and the assay with a micro stir bar in a water jacketed cell holder. Fluorescence polarization of DPH was measured at 10°C on a QuantaMaster model C-61 T-format scanning spectrofluorometer equipped with polarizing filters (Photon Technology International, London, Ontario). Excitation of DPH was at 358 nm and fluorescence emissions were measured at 428nm. Each sample was measured for 120 seconds at a rate of one reading per second using FeliX version 1.41 software. Samples were measured in triplicate. Fluorescence polarization, an index of membrane fluidity was calculated according to published methods (Litman and Barenholz, 1982).

Malonyl-CoA Content

Malonyl-CoA was measured following published protocols (Morash et al., 2008) which was modified from a previous protocol (Richards et al., 2002a). Briefly, ground tissues were lyophilized for 24 h and kept at -80°C until analysis. 50 mg of lyophilized tissue was homogenized at 4 $^\circ\text{C}$ for 20 sec using a Teflon pestle in 200 μl of 0.5 M perchloric acid with 50 μM DTE and 10 $\text{mg}\cdot\text{ml}^{-1}$ propionyl-CoA used as an internal

standard. Homogenized samples were centrifuged at 20,000 ×g for 10 min at 4 °C and 200 µl of the supernatant was transferred and adjusted to pH 3 using 4 M NaOH while being vortexed. The sample was transferred to an autosampler vial containing 20 µl of MOPS (pH 6.8) and the final pH was determined (always less than 5). Autosampler vials were placed in a Waters 717 Plus autosampler (Waters, Mississauga, ON) at room temperature and M-CoA was separated using reverse-phase HPLC based on a method from (Demoz et al., 1995). 200 µl of the sample was injected onto a Zorbax ODS Rx C-18 column (25 cm×0.46 mm) (Agilent Technologies, Mississauga, ON). The elution gradient was created using a Waters Model 510 pump controller. Mobile phase A was 100 mM sodium phosphate and 75 mM sodium acetate in deionized water (pH 4.6). Mobile phase B was the same as A except that it contained 30% CH₃CN. The elution gradient was as follows: 0 min, 90% A; 17 min, 50% A; and 17.6 min, 90% A. Baseline conditions were established after 5 min of 90% A. The flow rate was 1.5 ml/min and absorbance measurements were made at 254 nm on a Lambda Max 481 LC spectrophotometer (Waters, Mississauga, ON). Peaks were manually identified by comparisons to known M-CoA standards and quantified using the internal standard (propionyl-CoA).

mRNA quantification by real time PCR

Total RNA was extracted from red muscle and liver using TRIzol Reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. RNA was quantified by UV spectroscopy at 260nm and then diluted to 0.5µg·µl⁻¹. cDNA was synthesized using 1 µg of DNase (Invitrogen, Carlsbad, CA) treated mRNA with SuperScript RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA). Real time PCR was conducted using SYBR green with ROX as a reference dye in a Stratagene Mx3000P real-time PCR system. Each 25 µl reaction contained 12.5 µL SYBR green mix, 1 µL each of forward and reverse primer (5µM), 5.5 µL of DNase/RNase free water and 5 µL of 5× diluted cDNA. Primers were designed using Primer3 software (Rozen and Skaletsky, 2000) or previously published (Morash et al., 2008) and the specific primer sequences appear in Table 6.1. The thermal program included 3 minutes at 95°C, 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec for PPARα and β and 3 minutes at 95°C, 40 cycles of 95°C for 15 sec, 58°C for 30 sec and 72°C for 30 sec for the five CPT I isoforms. A no-template control and dissociation curve was performed to ensure only one PCR product was being amplified and stock solution were not contaminated. Standard curves were constructed for each gene using serial dilutions of stock cDNA to account for any differences in amplification efficiencies. All samples were normalized to the housekeeping gene, EF1-α a housekeeping gene used extensively in fish research inclosing other fasting studies (Gabillard, 2006), which was not significantly different between tissues or between treatments (p>0.05).

Statistical Analysis

All statistical analyses were performed using SigmaStat (Systat Software Inc., San Jose, CA). A Student's T-test was used to test for significance between control and fasted fish. Significance level was set at $\alpha = 0.05$.

Results

Fatty acid oxidation

Palmitate oxidation was measured in isolated mitochondria and found to be significantly higher in both red muscle and liver of fasted fish when compared to fed controls ($p < 0.05$; Fig. 6.1A and B). Furthermore, red muscle mitochondria of both control and fasted fish oxidized palmitate approximately 13- and 26-fold greater rate than those of liver ($p < 0.05$).

CPT I Activity

CPT I activity (U mg^{-1} mitochondrial protein) was significantly higher in red muscle compared to liver ($p < 0.05$; Fig. 6.2). Fasting however, did not increase CPT I activity in red muscle to a level significantly different than fed controls (Fig. 6.2). In contrast, fasting resulted in a significant increase in liver CPT I activity ($p < 0.05$; Fig. 2).

CPT I inhibition by M-CoA (IC_{50})

The IC_{50} (concentration of inhibitor to decrease enzyme activity by 50%) was determined in liver and red muscle to establish if fasting modified the sensitivity of CPT I to M-CoA. The IC_{50} in both red muscle and liver was found to be significantly higher in fasted fish when compared to fed controls indicating a decrease in sensitivity of the enzyme to its allosteric inhibitor ($p < 0.05$; Fig. 6.3A and B).

Mitochondrial membrane fluidity

Mitochondrial membrane fluidity was measured using DPH anisotropy where fluidity is inversely proportional to anisotropy. Anisotropy values for red muscle and liver of fasted and control fish are presented in Fig. 6.4. There were no statistically significant changes in mitochondrial membrane fluidity in either red muscle or liver tissue of fasted fish when compared to fed controls. However, red muscle mitochondrial membranes are significantly more fluid than those of the liver ($p < 0.05$).

Malonyl-CoA content

Total tissue homogenate M-CoA content did not change during fasting. Tissue concentrations were similar between red muscle and liver (Fig. 6.5).

Gene expression

PPAR α expression remained constant in both red muscle and liver with fasting (Fig. 6A-B). However, fasting affected the expression of other genes examined differentially in red muscle and liver. PPAR β mRNA expression significantly decreased in red muscle (Fig. 6.6A), whereas, in the liver, PPAR β significantly increased its

expression during fasting ($p < 0.05$; Fig. 6.6B). CPT I β 1a and b expression did not change in either tissue (Fig. 6.7) but there was a significant induction of CPT I α 1a expression in liver during fasting ($p < 0.05$; Fig. 6.7B). Moreover, there was a significant reduction of CPT I α 1b in red muscle during fasting ($p < 0.05$; Fig. 6.7A).

Discussion

In most animals, energy metabolism during fasting is fuelled primarily through upregulating mitochondrial fatty acid oxidation. One mechanism towards this increase is modulation of CPT I regulation, an enzyme which controls fat entry into the mitochondria (McGarry et al., 1983). After five weeks of fasting, rainbow trout increased mitochondrial fatty acid oxidation in both liver and red muscle (Fig. 6.1). At the same time there was a decrease in sensitivity of CPT I to M-CoA in both tissues and an increase in CPT I activity in liver but not in red muscle (Figs. 6.3 and 6.2, respectively). Previously we observed a correlation between indices of mitochondrial membrane fluidity and IC_{50} of CPT I for malonyl-CoA (Morash et al., 2008). Thus, we anticipated mitochondrial membrane fluidity would increase with fasting and help explain the increase in IC_{50} . However, we observed no change in mitochondrial membrane fluidity in either tissue with fasting (Fig. 6.4). In contrast, fasting did affect mRNA expression of specific CPT I isoforms and PPAR β in fasted fish. This data suggests that mitochondrial fatty acid oxidation increases in fish during fasting through a variety of genomic and non-genomic mechanisms which act on CPT I to increase mitochondrial fatty acid uptake and ultimately oxidation.

Fatty acid oxidation

The rate of palmitate oxidation in isolated mitochondria increased after five weeks of fasting by four- and ten-fold in liver and red muscle, respectively (Fig. 6.1). Fatty acid oxidation becomes paramount in the liver during fasting for a number of reasons. Firstly, fatty acids are a source of energy for the liver. Secondly, partially oxidized fatty acids yield acetyl-CoA which can condense to form ketone bodies, an essential fuel for the brain when glucose becomes depleted (Owen, 2005; Soengas et al., 1998; Soengas et al., 1996). Lipids are also important to fuel muscle functions, especially during fasting, because of their relative abundance compared to scarce carbohydrate and valuable protein stores. Moreover, it has been noted in other fish species that liver mitochondria oxidize palmitoleic acid (C16:1) at a significantly higher rate than C16:0 (Crockett and Sidell, 1993). Therefore, *in vitro*, palmitic acid (C16:0) may be oxidized at a faster rate in red muscle mitochondria than in liver mitochondria. Indeed, our previous work indicates that, in general, red muscle has a greater capacity for fat oxidation (Morash et al., 2008).

CPT I activity and sensitivity to M-CoA

The kinetics of CPT I and the cellular concentration of its allosteric modulator, M-CoA, are two of the major factors determining entry of fatty acids into the mitochondria. During fasting, we found that the activity of CPT I was significantly increased by 2.5-fold in liver, but did not significantly increase in red muscle (Fig. 6.2). The change in CPT I

activity in the liver during fasting may be due, in part, to increased expression of CPT I mRNA (Fig. 6.6B). Despite modest increases in CPT I activity in liver and no increase in muscle, there was a substantial decrease in the sensitivity of CPT I to M-CoA in both tissues by approximately two- and eight-times, in red muscle and liver, respectively (Fig. 6.3). A similar response has been noted for mammalian liver CPT I isoform (CPT I α) in response to 24 hours of fasting (Drynan et al., 1996; Zammit et al., 1998). Although there have been few mechanistic explanations for changes in IC₅₀, one intriguing hypothesis is that increased mitochondrial membrane fluidity decreases CPT I sensitivity to M-CoA. However, we saw no change in fluidity in either tissue with fasting (Fig. 6.4). Therefore, other mechanisms must be responsible for the fasting-induced changes in CPT I kinetics, such as, changes in cytosolic pH (Bezaire et al., 2004), covalent modulation (Harano et al., 1985), or the differential expression of isoforms or splice variants with altered M-CoA sensitivities as seen in rat red muscle (Kim et al., 2002) and recently in trout (Morash, 2010).

Tissue M-CoA concentration

M-CoA content is a reflection of the anabolic or catabolic state of a tissue. Levels of this allosteric inhibitor will also reflect the cellular environment in which mitochondria exist and also the level of inhibition of mitochondrial fat oxidation *in vivo*. CPT I activity rapidly declines at M-CoA concentrations between 0 - 5 μ M in trout mitochondria *in vitro* (Morash et al., 2008). This suggests that *in vivo* concentrations of M-CoA (Fig. 6.5) are in a range that would tightly regulate mitochondrial fatty acid oxidation and that small changes in M-CoA in this concentration range can have a large effect of CPT I activity. In fasted fish, the rate of lipogenesis is greatly reduced, thus, we anticipated a decrease in M-CoA content in the liver. In fact, decreases in M-CoA have been noted in rat liver tissue, and soleus and gastrocnemius muscles after 48 hours of fasting (Drynan et al., 1996; Winder et al., 1995). In contrast, we found no change in liver or red muscle M-CoA content during fasting in rainbow trout (Fig. 6.5), although there was an increase (non-statistically significant) in red muscle. The data presented here are in contrast to our previously published M-CoA data for trout red muscle and liver (Chapter 2&3). We believe the current data is a more accurate reflection of the true M-CoA content of the tissues as it is within a physiological range which would inhibit CPT I, unlike the previously published data.

There are several possibilities to explain why M-CoA concentrations do not change in fish during fasting. Firstly, it has been proposed in mammals that M-CoA may be bound to proteins or compartmentalized within muscle cells (King et al., 2005; McGarry and Brown, 1997). This may explain why the overall concentration of M-CoA does not change during fasting, and perhaps only the unbound fraction changes and is available to inhibit CPT I. Secondly, muscle M-CoA is regulated differently than the liver, and is controlled by ACC activity. It may be that fasting has no effect on this enzyme and thus has no effect on M-CoA concentration. Finally, there may be species and tissue specific regulation of M-CoA that may act differently during physiological stresses requiring increased fatty acid oxidation. For example, there is a decrease in M-CoA levels in rats (Winder et al., 1995) but not in humans with exercise (Odland et al.,

1996) or after exercise training (Starritt et al., 2000). Even though M-CoA is not changing, the reduced sensitivity of CPT I should be sufficient to increase fat oxidation in fasted fish.

Gene Expression

The induced expression of CPT I in mammals is regulated, in part, by the transcription factors and nuclear receptors, PPAR α and β (Price et al., 2000). During fasting, free fatty acids are elevated (Pottinger et al., 2003) and could act as a ligand to activate PPAR α and induce expression of CPT I in both liver and muscle. However, we saw no change in either liver or red muscle PPAR α mRNA expression after five weeks of fasting (Fig. 6.6A-B). Despite this, there was an increase in CPT I α 1a mRNA expression in the liver while in red muscle CPT I α 1b expression decreased (Fig. 6.7). Based on previous analysis, these isoforms have amino acid substitutions that may potentially affect their sensitivity to M-CoA. Unlike mammals, which only express one isoform per tissue, fish have the ability to express all five CPT I isoforms within the same tissue. This allows for greater spatial and temporal control of expression during physiological perturbations where an increase in fatty acid oxidation is required.

In contrast to PPAR α , PPAR β mRNA expression was significantly up-regulated in the liver, while in the red muscle it was significantly down-regulated (Fig. 6.6A-B). The exact role of PPAR β has yet to be elucidated, but the ubiquitous expression across tissues suggests that it functions at a basal level of metabolism in both rats and fish (Batista-Pinto et al., 2005; Braissant and Wahli, 1998). In addition, fish appear to have multiple isoforms of both PPAR α and PPAR β , as shown in a variety of species including zebrafish (Robinson-Rechavi et al., 2001a) and Atlantic salmon (Leaver et al., 2007). These isoforms have not yet been identified in rainbow trout but it is possible that liver and red muscle of trout express different isoforms of PPAR α and β at different times during fasting, and that these isoforms have very specific roles in the regulation of lipid metabolism. For a more comprehensive picture of fasting-induced gene expression in this species, identification of specific isoforms, their tissue distribution and inducibility needs to be fully established.

Fasting in mammals vs. fish

It appears that reliance on fatty acids during fasting is conserved across vertebrate taxa, but with distinct regulatory mechanisms. We show here that increases in mitochondrial fatty acid oxidation most likely result of a variety of different mechanisms that are distinct between liver and muscle and indeed between fish and mammals. Decreasing the sensitivity of CPT I to M-CoA appears to be a conserved mechanism to increase mitochondrial fatty acid oxidation in both liver and muscle of fish and mammals in response to fasting (Drynan et al., 1996; Zammit et al., 1998). Distinct from mammals, the genomic control of fatty acid oxidation is quite different in fish, particularly between the tissues studied here. This has broad ecological relevance as many fish species experience periods of food limitation, or undergo voluntary fasting during migration.

Conclusions

Fish are excellent models for examining the response to fasting as they experience bouts of anorexia during their natural life history. We have shown here that there are many physiological differences between tissues of the same animal and response to fasting. Although these two tissues respond differently, ultimately they achieve the same goal; an increase the ability of the mitochondria to increase the use of fats as a fuel source. Here we showed that fasting induced increased mitochondrial fatty acid oxidation in both tissues and this was achieved partially through modifications in CPT I allosteric modulation and changes in gene expression. Interestingly, changes in CPT I sensitivity to M-CoA were not due to modifications in mitochondrial membrane fluidity as predicted. This suggest the possibility of induced expression of other less sensitive CPT I isoforms. Furthermore, in liver, but not red muscle, fasting appears to also increase expression of CPT I.

Table 6.1: Forward (F) and reverse (R) primers used for real-time PCR analysis of mRNA expression in rainbow trout during fasting.

Gene	5' to 3' Forward Primer	5' to 3' Reverse Primer	T_m °C	Size (bp)
PPAR α	ccaagttcagtttgccatga	attggggaagaggaaggtgt	60	204
PPAR β	ctggagctggatgacagtga	gtcagccatcttgtagca	60	
CPT I β 1	gatgttcgtagggtagga	ttgtcttgcattgctctgac	58	80
CPT I β 2	gccgcaaactagagagagga	cccgtagtacagccacacct	58	199
CPT I α 1a	atgaggaatgccctcaagtg	gcttctgccagagaacaac	58	120
CPT I α 1b	cgettcaagaatggggtgat	caaccacctgctgtttctca	58	187
CPT I α 2	ccgttctaacagaggtgct	acactccgtagccatcgtct	58	154
EF1 α	cattgacaagagaaccattga	ccttcagctgtccagcac	58	94

EF1 α – Elongation factor 1 α

CPT I – Carnitine palmitoyltransferase I

PPAR – peroxisome proliferators-activated receptor

Fig. 6.1

Palmitate oxidation in isolated mitochondria from (A) red muscle and (B) liver of control and fasted rainbow trout. Values are means \pm S.E., n=3. Letters denotes significance ($p < 0.05$) between control and fasted fish; numbers denote significance between red muscle and liver ($p < 0.05$).

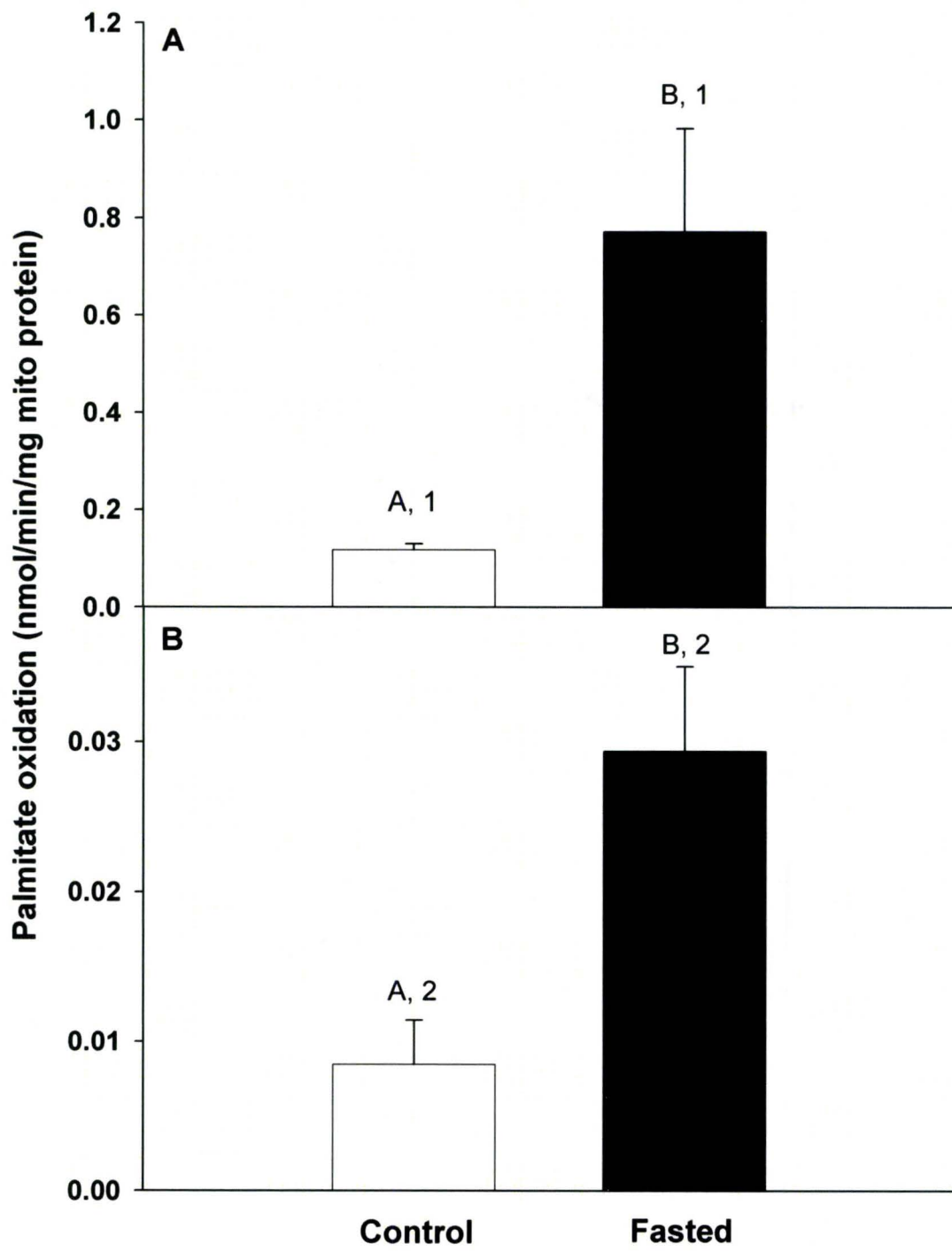


Fig. 6.2

Carnitine palmitoyltransferase (CPT) I activity in red muscle and liver of control and fasted rainbow trout. Values are means \pm S.E., n=4. Letters denotes significance ($p < 0.05$) between control and fasted fish in each tissue; numbers denote significance between red muscle and liver ($p < 0.05$).

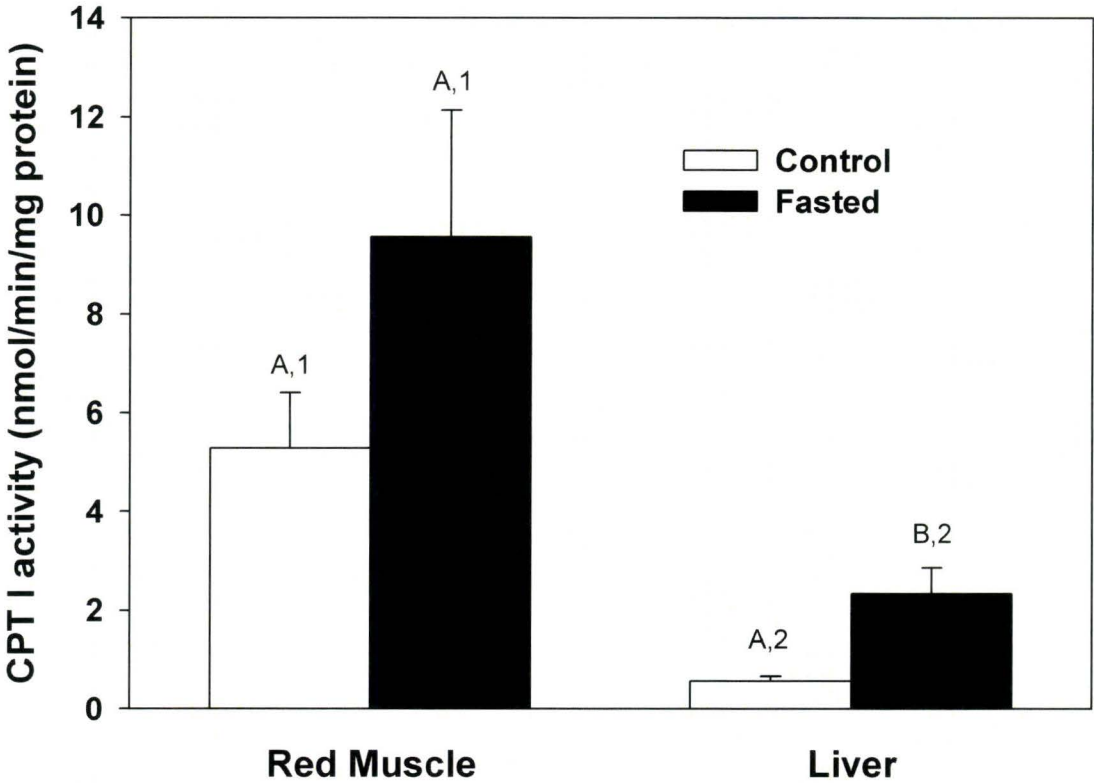


Fig. 6.3

The concentration of malonyl-CoA (M-CoA) that reduces carnitine palmitoyltransferase (CPT) I activity by 50% (IC₅₀) in (A) red muscle and (B) liver of control and fasted rainbow trout. Values are means \pm S.E., n=4. Asterisk denotes significance ($p < 0.05$) between control and fasted fish.

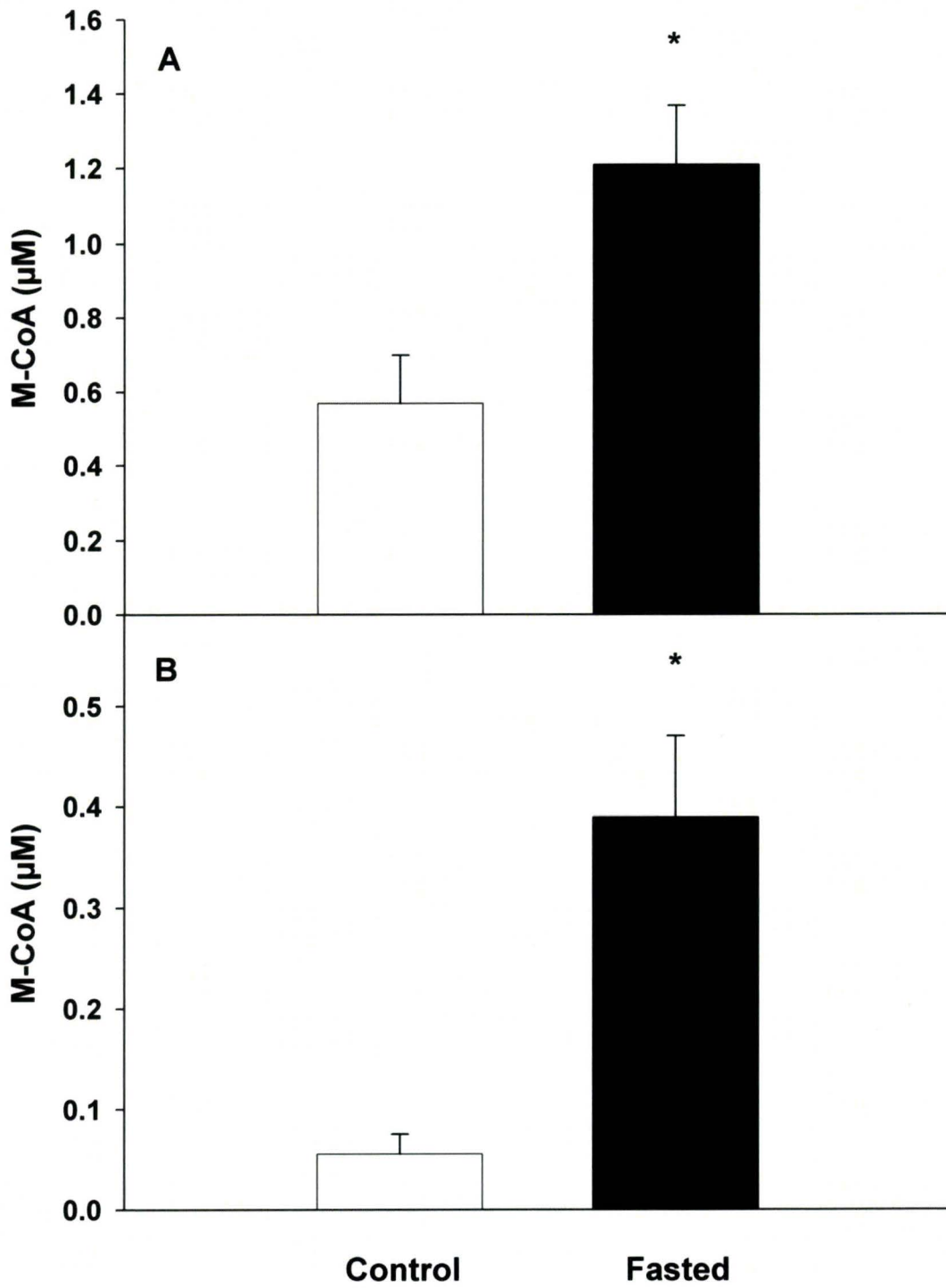


Fig. 6.4

1,6-diphenyl 1,3,5-hexatriene (DPH) anisotropy values from red muscle and liver of control and fasted rainbow trout mitochondria. Mitochondrial membrane fluidity is inversely proportional to anisotropy. Values are means \pm S.E., n=4. Asterisk denotes significance between red muscle and liver ($p < 0.05$).

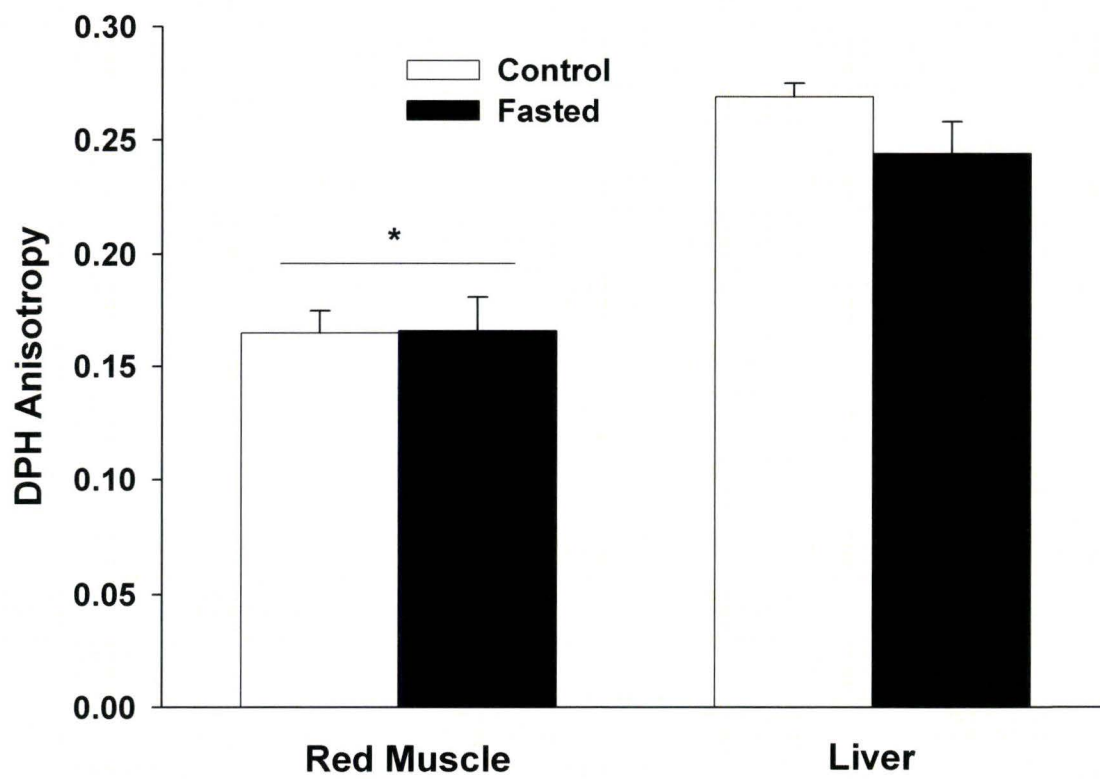


Fig. 6.5

Malonyl-CoA concentration in red muscle and liver of control and fasted rainbow trout. Values are means \pm S.E., n=4. Asterisk denotes significance ($p < 0.05$) between control and fasted fish.

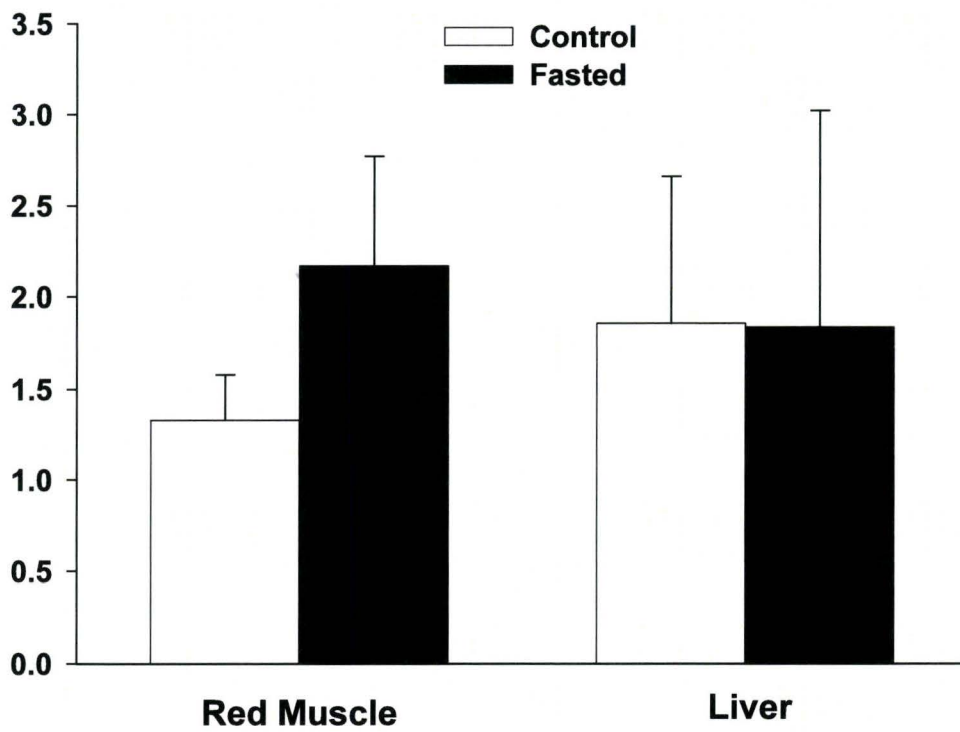


Fig. 6.6

Real time quantitative PCR gene expression of peroxisome proliferator activated receptor alpha (PPAR α) and PPAR β in (A) red muscle and (B) liver. Expression is relative to Efl α . Values are means \pm S.E., n=4. Asterisk denotes significance (p<0.05) between control and fasted fish for each gene.

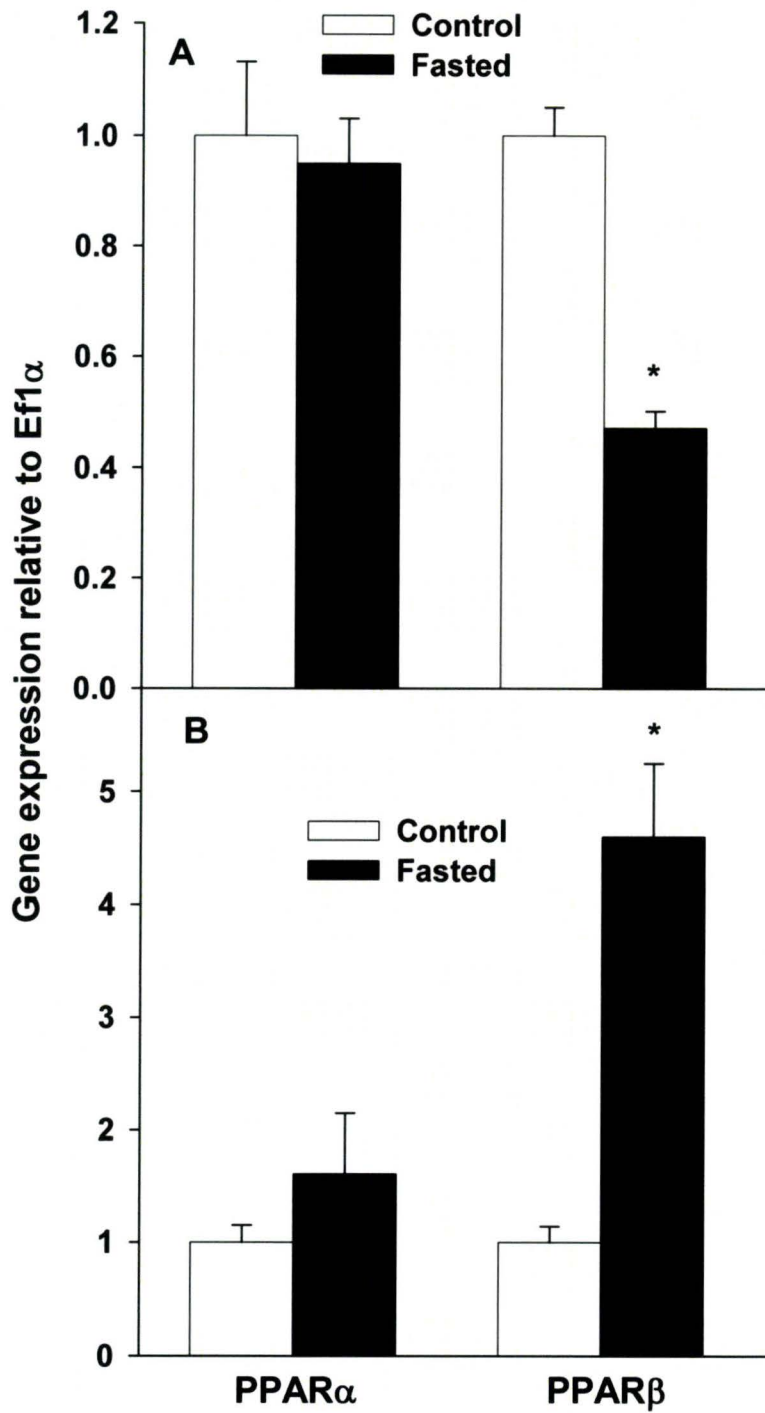
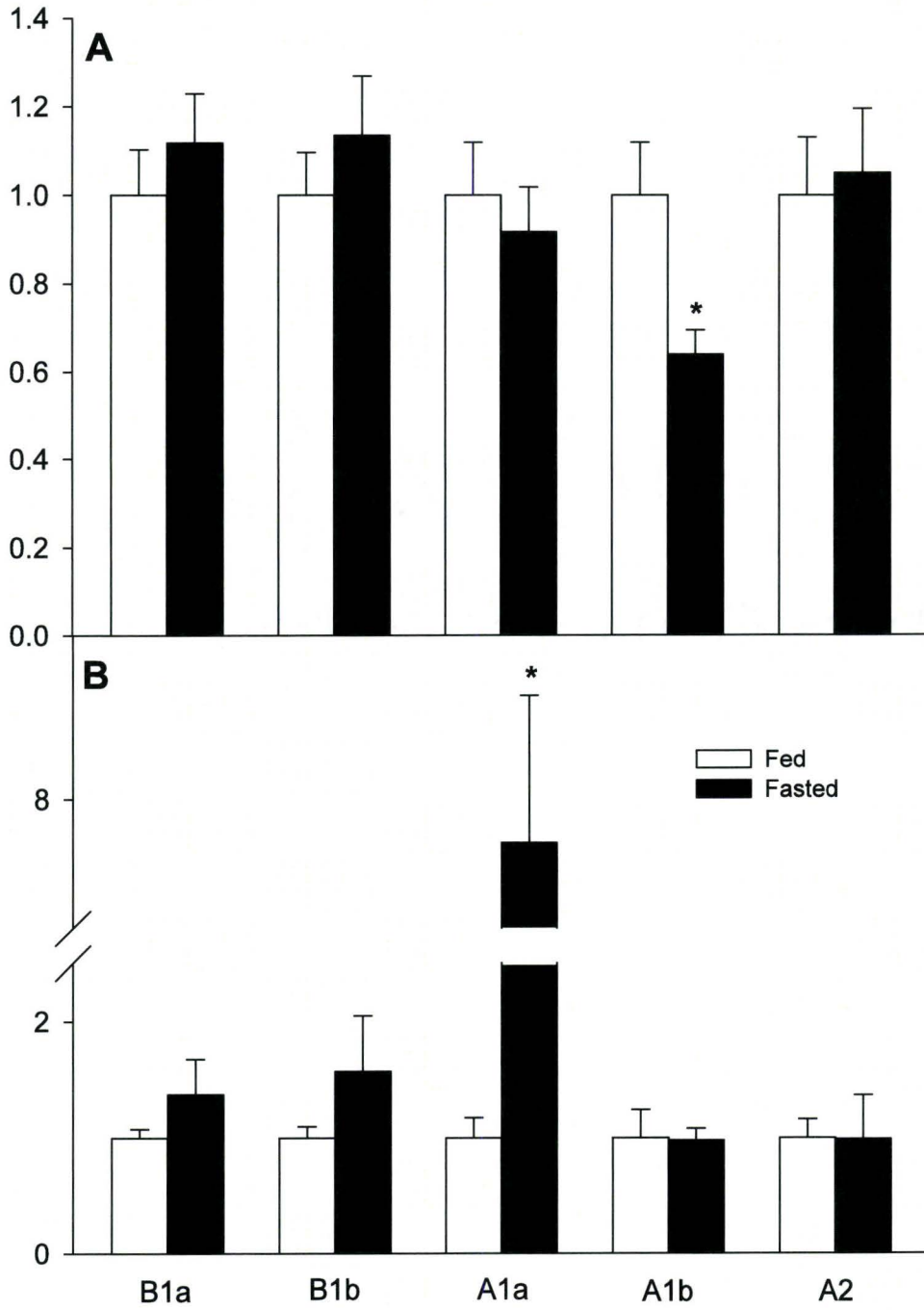


Fig. 6.7

Real time quantitative PCR gene expression of carnitine palmitoyltransferase (CPT) I isoforms in (A) red muscle and (B) liver. Expression is relative to $Ef1\alpha$. Values are means \pm S.E., n=4. Asterisk denotes significance ($p < 0.05$) between control and fasted fish for each gene.



GENERAL SUMMARY AND CONCLUSIONS

At the onset of this thesis, little was known regarding the regulation of lipid metabolism in fish, particularly across tissues or during various physiological states. This thesis has used an integrative approach to characterize the regulatory mechanisms controlling mitochondrial lipid metabolism and highlights the distinct differences between tissues and taxa. Specifically, this work draws attention to the enzyme carnitine palmitoyltransferase (CPT) I and its regulation during changes in environmental and physiological conditions.

Differences in the regulation of mitochondrial lipid metabolism between tissues

Much of what we knew about the regulation of lipid oxidation came from investigations on liver tissue, as it plays a predominant role in lipid metabolism and homeostasis. However, as outlined in **chapter 1**, muscle is also an important tissue in regulating overall metabolic homeostasis as it makes up a large proportion of the body weight in most animals and has a high oxidative capacity (Froyland et al., 1998; Hochachka and McClelland, 1997; Leary et al., 2003; McClelland, 2004). **Chapter 2** investigated the capacity for lipid oxidation between liver and muscle tissues as well as the regulation of CPT I function, fulfilling the aims of **objective 1**. Evidence from this work suggests that, in fish, these two types of tissues have varying capacities for lipid oxidation which may be partially due to the differential regulation of CPT I between tissues. Specifically, I found that red muscle tissue CPT I is much less sensitive to its inhibitor, M-CoA, than the liver; a finding which is opposite to what we see in mammals. Furthermore, I found that the differences in the IC_{50} between tissues correlated with differences in mitochondrial membrane composition indicating that membrane fluidity may be an important regulator of lipid metabolism in fish as it is in mammals. However, the data also suggested that there may be more than one CPT I isoform in fish which may also be contributing to the overall tissue differences in lipid metabolism.

In **chapter 3**, I further investigated the role that mitochondrial membrane composition may be playing on the inhibition of CPT I by M-CoA. To accomplish this, and the goals of **objective 2**, I fed trout diets high in polyunsaturated or saturated fatty acids in attempt to modify the mitochondrial membrane composition and alter the inhibitory kinetics of CPT I. After 8 weeks of the specific diet, I found no change in mitochondrial membrane composition, which was unexpected given that this has been shown in mammals. However, I did find a significant increase CPT I and PPAR expression in liver and muscle. Taken together, this data may suggest that in trout, changes in dietary fatty acids may affect the genomic control of CPT I rather than changes in the sensitivity of CPT I to M-CoA.

Based on the previous two chapters it became evident that there was more than one isoform of CPT I in rainbow trout. Therefore, to complete **objective 3**, I used a phylogenetic approach to investigate the evolution of this enzyme in fish compared to mammals. I found, in fact, that there are 5 isoforms of CPT I in rainbow trout, but that

this is not the case for all fish, indicating that the retention of specific CPT I isoforms after genome duplications was species specific. In mammals, CPT I expression is very tissue specific with one isoform present in the muscles and another present in the liver. In trout, however, I found that all 5 isoforms are expressed in both tissue types. While there are some indications of both β isoforms and the $\alpha 1a$ isoform being more highly expressed in muscle, there is no clear cut pattern of expression between tissues. I further investigated the expression of these isoform during development in rainbow trout and found that there are quite drastic changes in expression from fry to juvenile to adult trout. These differences in tissue distribution and expression during development may be indicative of differences in kinetics of the individual isoforms. Therefore, I investigated the protein structure of each isoform to determine if there were structural changes which may affect M-CoA binding and inhibition. The data demonstrate that there are many differences in amino acid sequence as well as shifts in the transmembrane domains between isoforms, both of which will effect M-CoA inhibition. The data presented in **chapter 4** suggest that the evolution of CPT I in fish may allow a degree of specificity for its regulation, both at the protein and tissue level that is not available in mammals. Furthermore, this chapter highlights the need for tissue and taxa specific investigations of lipid metabolism.

Effect of physiological condition on the regulation of mitochondrial lipid metabolism

After determining the tissue specific mechanisms of CPT I regulation, I was able to accomplish the main goal of this thesis; investigate modifications in the regulation of lipid metabolism under varying physiological conditions, which is presented in **chapters 5 and 6** and completes **objectives 4 and 5**. In these chapters I investigated the individual effects of fasting and endurance exercise on the regulation of lipid metabolism as well as their combined effects in a closely related species, sockeye salmon, during migration, where they experience both fasting and exercise concurrently.

Migrating salmon face a host of environmental and physiological changes during migration making it difficult to discern the individual effects of any particular stress. In **chapter 5**, I investigated the capacity and regulation of lipid metabolism at various points along the migration route to determine how salmon remodel their muscles for an increase in fatty acid oxidation. I have clearly shown that these salmon transcriptionally and metabolically remodel their muscles at the onset of migration and they are therefore primed for the high rates of lipid oxidation to fuel the energetic demands of the tissues. Transcriptionally, there is an increase in CPT I, AMPK and PPAR mRNA expression at the onset of migration, all of which have an important role in the regulation of lipid metabolism. Furthermore, metabolically, there is an increase in both mitochondrial content, as indexed by the activity of citrate synthase, and fat oxidation capacity as indexed by HOAD activity, at the start of migration. As the lipid stores become depleted, I have shown that these same processes are downregulated and that HK and AST, enzymes involved in carbohydrate and protein oxidation, respectively, are upregulated during the latter stages of migration.

The most likely factors to influence the rate of lipid oxidation in migrating salmon are endurance exercise and fasting, both of which induce greater rates of lipid oxidation

individually. Therefore, in **chapters 5 and 6**, I investigated the individual effects of these two states on the regulation of lipid metabolism in rainbow trout. After 4 weeks of chronic exercise, trout show similar increases in the activity of HOAD and CS as I found in the migrating salmon. However, the extent of the increase after exercise alone was only approximately one third of the change in migrating salmon, indicating that there are other factors contributing to this increase. I found relatively few changes in the expression CPT I, and decreases in the expression of AMPK and PPAR after 4 weeks of exercise. I would expect to see increases in the expression of these enzymes and this likely occurs at the onset of the exercise training. This highlights the need for temporal measurements as there is a specific timeline and lag between gene and protein expression and functional consequences in the animal.

Lastly, in **chapter 6**, I have shown that fasting causes an increase in lipid oxidation in both red muscle and liver, and that this increase is mediated by a decrease in the sensitivity of CPT I to M-CoA; an approach also used by mammals during fasting. I hypothesized that there may be changes in mitochondrial membrane fluidity to mediate this change in sensitivity, however, there were no changes after 5 weeks of fasting. Alternatively, if fluidity does not change, it is possible to express a less sensitive isoform of CPT I and obtain the same tissue change in sensitivity to M-CoA. However, when I investigated the expression of the 5 CPT I isoforms, I found only slight changes in CPT I after 5 weeks of fasting. However, as indicated above, I expect that changes in gene expression may have taken place at an earlier time point during fasting.

In the future it will be vital to systematically determine the kinetics and inhibitory properties of each individual isoform. While this will be a difficult task given the ubiquitous expression of the isoforms, it will be a fruitful area for future research regarding lipid metabolism in fish. This information would allow us to completely determine the role of these isoforms in development and during changes in energetic demand such as during fasting and exercise. Furthermore, differences in carnitine concentrations across tissues and between differing physiological states will be important in determining the overall function of CPT I in fish.

Perspectives & Significance

The objectives of this thesis arose from the need for more in depth knowledge of lipid metabolism as data on non-mammalian vertebrates and the diversity of lipid metabolism regulation was lacking. Throughout this thesis I have thoroughly identified and assessed several of the main regulatory mechanisms controlling lipid metabolism in various fish tissues and provided a framework for future studies in this area.

Initially, there was very little information regarding fatty acid oxidation in fish, particularly in muscle or under various physiological states. I have demonstrated that there are tissue-specific responses in the regulation of lipid metabolism, particularly in regards to CPT I, and that these responses are distinct compared to mammals. The evolution of CPT I in non-mammalian vertebrates is complex in nature and has added another degree of potential plasticity, unique to fish. I have shown that the use of both genomic and non-genomic mechanisms of regulation allows a degree of specificity in response to varying physiological states. By understanding the particular effects of any

given physiological state on lipid oxidation, one can assess the potential impacts of environmental condition on whole animal lipid metabolism. As outlined in the introduction, fish naturally experience a large variety of environmental challenges throughout their lifetime. In many instances, the ability of fish to adapt to these situations, particularly to energetic demands has overall consequences on their ecology and population dynamics. Furthermore, fish, predominantly salmonid species, have also become economically important to many countries. The rise in salmonid aquaculture facilities has demanded cost- and growth-efficient diets for their fish production, and this is highly dependent on the way these diets are metabolized by the fish. The data I have presented in this thesis regarding the regulation of lipid metabolism has potential impacts on both the economics and ecology of salmonids. Data from chapters 1,2 and 3 have potential relevance to the future research on the effects of diet on the growth and metabolism of farmed fish, while data from chapters 4 and 5 are the beginning of important metabolic research on salmonids and their ability to successfully migrate. In this thesis I have laid the basic foundation for future studies in the area of fish lipid metabolism. However, I have also demonstrated the complexity and plasticity of mitochondrial lipid oxidation in fish. This area promises to be a profitable subject for future research.

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