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Effects of dietary fatty acid composition on the regulation of carnitine palmitoyltransferase (CPT) I in rainbow trout (*Oncorhynchus mykiss*)

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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 changes in gene expression of CPT I and PPARs is the main regulatory mechanism controlling CPT I function in fish using our experimental diet.

1. 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

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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 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 (H_{II}) 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 M-CoA 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 CPTI and 4) sensitivity of CPTI to its allosteric inhibitor M-CoA (IC_{50}). Taken all together, these data will give insight into the dietary regulation of mitochondrial fatty acid oxidation in the liver and red muscle.

2. Materials and methods

2.1. 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 1). Fatty acid composition of the different lipid sources are provided in Tapia-Salazar et al. (2006) and 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 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 anesthetised using MS-222 (0.5 g/L, buffered with sodium

Table 1
Composition of experimental diets

Ingredients (g/kg as is basis)	Diet		
	SFA	CTL	PUFA
Fish meal, herring 68% CP	400	400	400
Corn gluten meal, 60% CP	200	200	200
Wheat middlings, 17% CP	226	226	226
Fish oil, herring	–	25	50
Antioxidant (ethoxyquin)	0.5	0.5	0.5
Fish oil concentrate (55% DHA, 5% EPA)	–	20	40
Olive oil	60	60	60
Beef Tallow	90	45	–
Vitamin Premix	6	6	6
Lysine HCL (79% lysine)	5	5	5
DL-methionine	2	2	2
Choline chloride	3	3	3
Mineral premix	3	3	3
NaCl	3	3	3
Rovimix Stay-C (25% ascorbic acid)	1.5	1.5	1.5
Total	1000	1000	1000
Calculated diet composition, as is basis			
Dry matter (%)	92.9	92.9	92.9
Crude protein (%, *N*%×6.25)	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.

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.

2.3. 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 (Suarez and Hochachka, 1981; Moyes et al., 1988). 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 $MgCl_2$, 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 colleted 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.

2.4. 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).

2.4.1. CPTI 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 μCi/sample of L-[*methyl*-^{3}H]carnitine hydrochloride (specific activity 82.0 Ci/mmol: Amersham Biosciences, Baie d'Urfé, Quebec, Canada) was added and 70 μL of the assay mixture was placed in 1.5 mL Eppendorf tubes and incubated with 10 μL 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 μL 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 μL of 1 M HCl. The palmitoyl-[^{3}H]-carnitine was collected according to published methods (Starritt et al., 2000). 20 μL of the assay mixture with L-[*methyl*-^{3}H]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.

2.4.2. 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 N_2 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 N_2 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.

2.4.3. Protein content

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

2.5. Malonyl-CoA (M-CoA) content

M-CoA concentrations were determined using a modified method from Richards et al. (Richards et al., 2002). Briefly, frozen whole tissue samples were powdered using a liquid N_2-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 (pH 6.8) 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, Missisauga, 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% CH_3CN. 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.

2.6. 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× diluted cDNA. Primers were designed using a CPT I sequence from rainbow trout liver (Gutieres et al., 2003) (see Table 3 for specific primer sequences). The thermal program included 3 min at 95 °C, 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °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

Table 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
n-3	1.29	19.23	40.06
n-6	8.09	7.89	7.67
n-3/n-6	0.16	2.44	5.22

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

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).

2.7. 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 °C. The samples were allowed to cool for

Table 3
Forward (F) and reverse (R) primers used for real-time PCR analysis of mRNA expression in trout

Gene	Primer
Ef1α	F-5′ CAT TGA CAA GAG AAC CAT TGA 3′
	R-5′ CCT TCA GCT TGT CCA GCA C 3′
CPT I	F-5′ GCC GCA AAC TAG AGA GAG GA 3′
	R-5′ CCC GTA GTA CAG CCA CAC CT 3′
PPARα	F-5′ CCA AGT TCA GTT TGC CAT GA 3′
	R-5′ ATT GGG GAA GAG GAA GGT GT 3′
PPARβ	F-5′ CTG GAG CTG GAT GAC AGT GA 3′
	R-5′ GTC AGC CAT CTT GTT GAG CA 3′

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 °C and proceeded using the following temperature profile: 160 °C for 4 min, increased 2 °C/min for 30 min, 220 °C for 16 min, increased 10 °C/min for 2 min, 240 °C 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).

2.8. 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 $p<0.05$.

3. Results

3.1. Growth Performance

No significant differences were observed in the growth of the fish fed the different diets ($p>0.05$) (Table 4). On average, fish weight increased 220% and fork length 110%. Mortality was low (<1%) and unaffected by treatment.

3.2. Mitochondrial membrane composition

Mitochondrial phospholipid fatty acid composition was determined in red muscle and liver of fish fed the three different diets

Table 4
Growth performance of experimental fish

Diet	Initial condition			Final condition			% Weight gain
	BW (g)	BL (cm)	CF	BW (g)	BL (cm)	CF	
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

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

Table 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*

	Red muscle			Liver		
Fatty acid	SFA	CTL	PUFA	SFA	CTL	PUFA
C14:0	1.1±0.1	1.0±0.1	0.8±0.4	0.5±0.3	1.2±0.3	1.4±0.1
C16:0	20.2±4.8	21.3±2.3	18.8±0.8	23.2±3.4	21.4±1.8	20.3±1.3
C16:1n7	0.8±0.3	0.8±0.2	0.8±0.3	0.6±0.2	1.0±0.1	0.8±0.4
C18:0	7.7±1.1	7.9±1.4	5.2±1.3	6.3±1.0	5.9±1.3	5.6±0.5
C18:1n7	9.3±2.9	9.9±1.2	8.7±0.8	11.4±2.0	9.8±1.5	8.9±1.2
C18:2n6	4.4±1.0	3.7±0.2	8.9±5.1	4.4±0.7	4.2±0.8	3.8±0.8
C18:3n3	4.9±3.0	2.8±1.4	2.4±1.9	2.9±1.7	2.5±2.5	1.7±1.7
C20:1n9	6.2±3.8	3.3±1.9	3.5±3.0	4.0±1.8	3.1±3.1	2.5±2.2
C20:4n6	7.1±3.7	3.3±1.7	3.7±3.0	5.6±1.8	4.1±3.0	3.6±2.2
C20:5n3	2.9±1.8	2.4±1.4	2.7±1.2	1.4±1.4	3.9±2.1	3.7±1.8
C22:5n3	6.5±2.1	2.8±0.7	3.3±1.7	3.8±1.1	3.0±2.1	1.8±1.2
C22:6n3	11.8±3.4[a]	29.4±5.8[ab]	40.4±7.6[b]	31.5±4.5	36.9±8.5	42.9±5.1
Saturates	29.0±4.9	30.4±3.4	24.8±0.9	29.9±2.2	28.5±0.4	27.3±0.7
Monounsaturates	16.3±2.1	14.0±1.8	13.0±2.9	16.0±0.5	14.0±1.4	12.3±1.3
Polyunsaturates	47.16±5.4	50.7±5.5	61.6±3.8	49.7±1.3	54.8±3.2	57.6±2.8
n-3 polyunsaturates	35.6±6.6	43.6±6.9	48.9±6.3	39.6±2.6	46.5±5.0	50.2±4.0
n-6 polyunsaturates	11.5±2.7	7.1±1.9	12.6±5.0	10.0±2.1	8.3±1.8	7.4±1.9
n-3/n-6	3.6±1.7	8.5±3.2	7.4±5.8	5.0±1.7	6.6±1.7	7.8±2.1
UI[†]	2.5±0.4	2.8±0.4	3.3±0.3	2.7±0.1	3.0±0.2	3.2±0.2

SFA=high saturated fat, CTL=control, PUFA=high polyunsaturated fat. Values are means±S.E., $n=4$. Different symbols denote significant difference ($p<0.05$).
*Values are means±SE; n.d.=not detectable.
[†]Unsaturation index=$\Sigma m_i \cdot n_i$; where m_i is the mole percentage and n_i is the number of C–C double bonds in the fatty acid "i".

Table 6
Mol% of mitochondrial membrane phospholipid classes in red muscle and liver after 8 weeks of an experimental diet*

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

SFA=high saturated fat, CTL=control, PUFA=high polyunsaturated fat. Values are means±S.E., $n=4$.
*Values are means±SE.
[†]PC=phosphatidylcholine, PE=phosphatidylethanolamine.

(Table 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 5). There appeared to be a similar pattern when looking at the individual phospholipid classes within the mitochondrial membrane (Table 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 6).

3.3. 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 7). In the liver, all three diets produced similar IC_{50} values (SFA=0.11±0.03, CTL=0.12±

Table 7
The concentration of malonyl-CoA (μM) to reduce the activity of malonyl-CoA sensitive carnitine palmitoyltransferase (CPT) I activity by 50% (IC_{50}) under high saturated fat (SFA) diet, control (CTL) diet or high polyunsaturated fat (PUFA) diet

Diet	Liver	Red muscle
SFA	0.11±0.03	1.83±0.75
CTL	0.12±0.05	1.19±0.89
PUFA	0.07±0.02	0.35±0.11

Values are means±SE. Liver and white muscle, n=5; heart, $n=4$; red muscle, $n=3$.

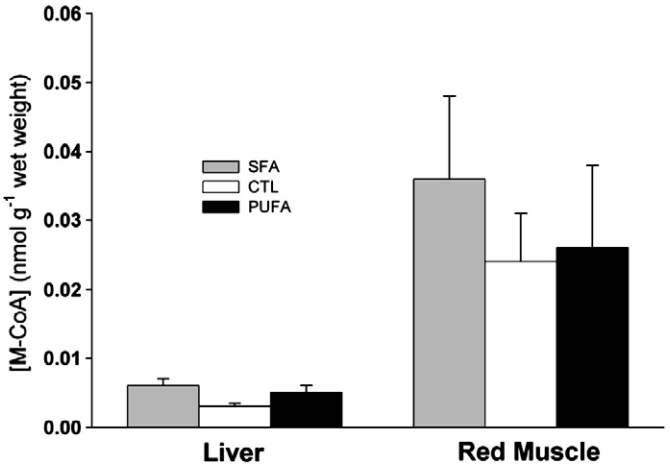

Fig. 1. Malonyl-CoA (M-CoA) content in red muscle and liver under high saturated fat (SFA) diet, control (CTL) diet or high polyunsaturated fat (PUFA) diet. Values are means±S.E. for four animals.

0.05, PUFA = 0.07 ± 0.03) with no statistically significant differences (Table 7).

3.4. 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. 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.

3.5. Gene expression profiles

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

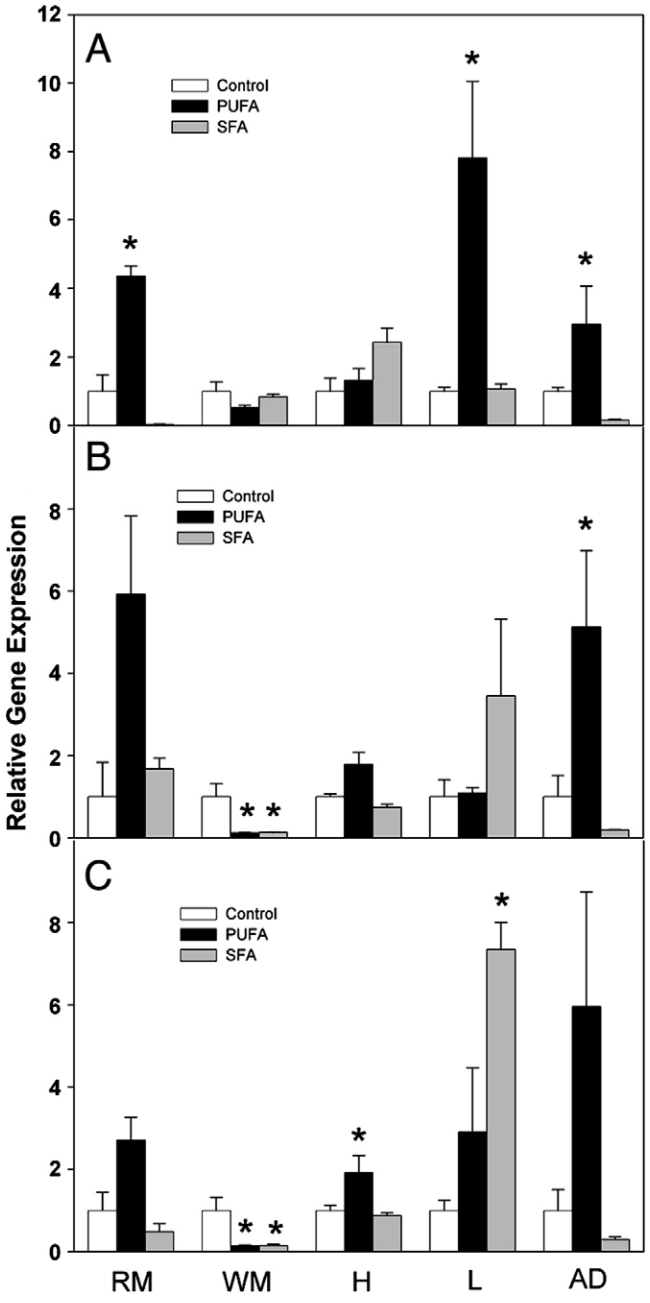

Fig. 2. Real time PCR gene expression profiles of A) carnitine palmitoyltransferase I (CPT I), B) peroxisome proliferator activated receptor α (PPARα) and C) peroxisome proliferator activated receptor β (PPARß) in red muscle (RM), white muscle (WM), heart (H), liver (L), and adipose tissue (AD). Values are expressed relative to EF1α and are means ± S.E. $n = 4$ for each tissue. Asterisks denotes significance between diets, $p < 0.05$.

3.5.1. CPT I

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. 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. 2A). No changes were seen in the expression of CPT I in the white muscle or heart between diets (Fig. 2A).

3.5.2. PPARα

The expression of PPARα mRNA did not follow the same pattern as CPT I. In heart tissue, PPARα expression was significantly higher in fish fed the PUFA diet compared to the control and SFA diets ($p = 0.032$, 0.005 respectively; Fig. 2B). Adipose tissue also had significantly higher PPARα expression in fish on the PUFA diet when compared to the SFA diet ($p < 0.05$; Fig. 2B). In contrast, the expression of PPARα in white muscle was significantly lower in the PUFA and SFA diets when compared to the control diet ($p < 0.05$; Fig. 2B). No changes were seen in the liver and red muscle expression of PPARα (Fig. 2B).

3.5.3. PPARβ

The pattern of PPARβ expression was different across tissues and diets. In heart tissue the expression of PPARβ was significantly higher in fish fed the PUFA diet than those fed the SFA diet ($p = 0.029$) while in the liver the opposite occured ($p = 0.020$; Fig. 2C). In white muscle, the same pattern was observed for PPARβ expression as for PPARα expression. In both the PUFA and SFA diets PPARβ expression was significantly lower ($p < 0.05$). No significant differences were found in red muscle and adipose tissue ($p > 0.05$; Fig. 2C).

4. 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 PPARα and PPARβ. 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, PPARα and PPARβ, 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.

4.1. 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. 2A-C). There is evidence in mammals that feeding high fat diets will increase CPT I expression compared to low fat diets (Thumelin et al.,

1994; Tabarin et al., 2005) presumably through activation of PPARα. However, other PPARα-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 (Robinson-Rechavi et al., 2001; Leaver et al., 2005; Leaver et al., 2007) but studies on their functional roles are very limited. In mammals, PPARα and β are more highly involved in regulating fatty acid oxidation in most tissues, whereas PPARγ 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 lead to multiple isoforms of PPARβ and PPARα previously shown in zebrafish and Atlantic Salmon (Robinson-Rechavi et al., 2001; Leaver et al., 2007). 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 PPARα (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. 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 Vmax in fish fed the PUFA diet (data not shown). Only in the adipose tissue did we see the corresponding increase in PPARα expression (Fig. 2B). Conversely, in the heart, we saw a significant increase in PPARα expression but no corresponding increase in CPT I expression. In fact, feeding the high SFA diet increased PPARα expression in the liver while feeding the PUFA diet did not. In mammals, PPARα 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 PPARα 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 PPARα may have been expressed earlier during the treatment, 3) there is an isoform specific difference in PPAR response elements across tissues, or 4) other signalling mechanisms exist up- or down-stream of PPAR affect CPTI expression in trout. In fact, in rat hepatoma cells inhibition of PPARα expression has no effect on the long chain fatty acid induced CPT I expression (LeMay et al., 2005). A PPARα-independent pathway of induction may also be present in fish but requires further investigation.

PPARβ expression was quite variable across tissues in trout. We found that feeding the high PUFA diet increased expression of PPARβ in the heart but not in any other tissue (Fig. 2C). Interestingly, like PPARα, the feeding of the SFA diet resulted in an increase of PPARß expression in the liver. In mammals, PPARβ 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 PPARβ expression with an increase in PUFAs in the diet suggesting that in fish, similar to mammals, that PPARβ 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.

4.2. 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 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.

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 PPARα 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 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 maybe 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 (Wong et al., 1984; Brady et al., 1989). 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.

4.3. 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 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.

5. 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.

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