

**EFFECTS OF TEMPERATURE AND THYROID HORMONE ON OXIDATIVE
AND LIPID METABOLISM IN JUVENILE LAKE WHITEFISH (COREGONUS
CLUPEAFORMIS)**

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Megan Alexandra Zak, candidate for the degree of Master of Science in Biology, has presented a thesis titled, ***Effects of Temperature and Thyroid Hormone on Oxidative and Lipid Metabolism in Juvenile Lake Whitefish (Coregonus Clupeaformis)***, in an oral examination held on December 11, 2017. The following committee members have found the thesis acceptable in form and content, and that the candidate demonstrated satisfactory knowledge of the subject material.

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ABSTRACT

Nearly every aspect of biological function is influenced by temperature, including metabolism. Factors such as seasonal change and climate change both contribute to environmental temperature variability. While many eurythermal fish species can acclimate to varying environmental conditions by adjusting metabolic processes, the cellular mechanisms and endocrine control of these shifts have not been fully elucidated. I examined the interactions between elevated temperature exposure and hyperthyroidism on various metabolic markers in the cool-water teleost, lake whitefish (*Coregonus clupeaformis*). Juveniles were exposed to 13 (control), 17 or 21 °C for 4, 8 or 24 days. Widespread changes in the abundance of mRNA transcripts were observed in enzymes associated with oxidative metabolism (*citrate synthase*, *cytochrome c oxidase subunit 1*), lipogenesis (*acetyl-coA carboxylase α* , *acetyl-coA carboxylase β*) and peroxisomal β -oxidation (*acyl-coA oxidase 3*) following exposure to elevated temperatures. However, the absence of effects on mitochondrial β -oxidation enzyme isoforms, *carnitine palmitoyltransferase α* and *carnitine palmitoyltransferase β* , combined with a decline in citrate synthase and cytochrome c oxidase activities, suggests that exposure to elevated temperature used in this study did not significantly increase overall metabolic demands in juvenile lake whitefish. To assess the role of hyperthyroidism in mediating thermal responses, thyroid status of fish was altered using thyroxine implants prior to the initiation of temperature change. Exogenous thyroxine treatment resulted in notable effects of on *citrate synthase*, *acetyl-coA carboxylase α* , *acetyl-coA carboxylase β* and *carnitine palmitoyltransferase β* mRNA abundance, but only in fish exposed to 17 °C or 21 °C. Furthermore, these effects were transient, occurring primarily within the first 8

days of temperature change. Pronounced short-term decreases in *carnitine palmitoyltransferase β* mRNA abundance in the presence of exogenous thyroxine at all three temperatures suggests thyroid hormones may drive towards a less oxidative phenotype in liver, perhaps in an effort to promote/protect lipid stores. Overall, these results suggest temperature-dependent thyroid hormone action on the transcription of metabolic enzymes in juvenile lake whitefish which may be particularly influential in the early stages of temperature response.

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LIST OF ABBREVIATIONS AND SYMBOLS

6PGD	6-phosphogluconate dehydrogenase
ϵ	molar absorptivity constant
$\times g$	times the force of gravity
ACC or <i>acc</i>	acetyl-coA carboxylase
ACOX or <i>acox</i>	acyl-coA oxidase
ANOVA	analysis of variance
ADP	adenosine diphosphate
ATP	adenosine triphosphate
%B/B ₀	$\%B/B_0 = \frac{\text{Sample CPM}}{\text{Total (Blank) CPM}} \times 100$
<i>β-actin</i>	beta-actin
CA	California
cDNA	complementary DNA
COX or <i>cox</i>	cytochrome c oxidase
CPM	counts per minute
CPT1 or <i>cpt1</i>	carnitine palmitoyltransferase
Cq	cycle number
Cq1	number of qPCR cycles required to detect a single target molecule
CS or <i>cs</i>	citrate synthase
CTMax	critical thermal maximum
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNTB	5,50-dithiobis-2-nitrobenzoic acid
dpf	days post fertilization
E	efficiency
EDTA	ethylenediaminetetraacetic acid
<i>efl</i> α	elongation factor 1 alpha
ETC	electron transport chain
EtOH	ethanol
FADH ₂	Flavin adenine dinucleotide

FAS	fatty acid synthase
G6PD	glucose 6-phosphate dehydrogenase
H ₂ O ₂	hydrogen peroxide
HOAD	3-hydroxyacyl-CoA dehydrogenase
HPT	hypothalamus-pituitary-thyroid
¹²⁵ I	Iodine-125 radioisotope
id	identity
IRC	inter-run calibrator
kDa	kilodalton
L	optical path length (cm)
LXR	liver x receptor
MCMC	Markov Chain Monte Carlo
MCT8	monocarboxylate transporter 8
ME	malic enzyme
MIQE	minimum information for publication of quantitative real-time PCR experiments
mRNA	messenger RNA
MWCO	molecular weight cut off
N ₂	nitrogen
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
NRF	nuclear respiratory factor
O ₂	oxygen
OAA	oxaloacetate
OD	optical density
ON	Ontario
PCR	polymerase chain reaction
PGC-1	peroxisome proliferator-activated receptor-gamma coactivator-1
PPAR	peroxisome proliferator-activated receptor

Q ₁₀	temperature coefficient
rA	rate of absorbance change
RIA	radioimmunoassay
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-qPCR	reverse transcription quantitative PCR
RXR	retinoid x receptor
SEM	standard error of the mean
si	similarity
T ₂	3,5-diiodothyronine
T ₃	3',5,3-triiodothyronine
T ₄	thyroxine
TCA	tricarboxylic acid
TGL	triacylglycerol lipase
TH	thyroid hormone
T _{opt}	optimal temperature
TR	thyroid hormone receptor
TRE	thyroid hormone response element
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone
USA	United States of America

1. GENERAL INTRODUCTION

1.1. Physiological consequences of a changing thermal environment

1.1.1. *Thermal plasticity and its predicted scope of impact in teleosts*

Temperature directly impacts nearly all aspects of physiology and is, consequently, one of the most pervasive and influential stressors encountered in the environment. Not only does temperature contribute to large-scale spatial distribution patterns such as species range and dispersal (Brander et al., 2003; Cline et al., 2013; Somero, 2010), it also influences organisms on the individual level by disrupting critical aspects of cellular function, such as protein integrity (Iwama et al., 1998) and metabolism (Johnson and Dunn, 1987; Schulte, 2015). Behavioural thermoregulation via physical avoidance of extreme temperatures is used by many organisms to reduce impacts of temperature on critical cellular processes (Hutchinson and Maness, 1979) and has been documented in a diverse range species (Barton et al., 2014; Hutchinson and Maness, 1979; Lopes and Bicca-Marques, 2017; Ludwig et al., 2015; Perry et al., 2005). However, when behavioural avoidance of unfavourable temperatures is not possible, alternative mechanisms must be used to cope with negative impacts of temperature on physiology.

Physiological plasticity can be observed following thermal challenge in most vertebrate species, ranging from fish (Egginton, 2002; Johnson and Dunn, 1987) to birds (Chaffee and Roberts, 1971) and mammals (Chaffee and Roberts, 1971; Egginton, 2002). For instance, long-term cold exposure leads to increases in the density of capillary networks in Syrian hamsters (*Mesocricetus auratus*) and the capillary/muscle fiber ratio in

both striped bass (*Morone saxatilis*) and rainbow trout (*Oncorhynchus mykiss*; Egginton, 2002). Similarly, phenotypic changes in response to temperature extend down to the cellular level (Egginton, 2002). Terminology to describe this phenomenon has been divided to capture effects of both one (acclimation) or more (acclimatization) stressors. Therefore, *acclimation* is typically used to describe the responses to a single environmental variable manipulated in a laboratory setting, while *acclimatization* is typically used to describe experiments involving multiple stressors, or those conducted in the field (Hutchinson and Maness, 1979). Plastic responses to temperature, often referred to as thermal acclimation, are typically reversible (Schulte et al., 2011) and allow the individual to become better-suited to the current environmental conditions, thereby increasing performance, fitness and survival.

Thermal acclimation in fish and other ectothermic species have been of great interest to researchers over the past several decades. This is, in part, due to their relationship between environmental and body temperatures. Fish lack endogenous mechanisms to regulate body temperature and, as such, their cells are exposed to the full spectrum of temperatures encountered in the environment. In temperate regions, natural circadian fluctuations in water temperature in streams can range between 1 and 15 °C (Contantz et al., 1994, Johnson, 2004), while seasonal fluctuations in surface water temperature of shallow lakes can exceed 20 °C (Bremer and Moyes, 2011, Dr. Rebecca North, 2017, personal communication). These variations in temperature on both short and long temporal scales have the potential to inflict significant thermal challenges on fish and other aquatic ectotherms inhabiting these regions. Although temperate fish are adapted to withstand a certain level of variability in their thermal environment relative to

stenothermal species (Gunderson and Stillman, 2015; Johnston and Dunn, 1987), they are still susceptible to temperature extremes. These effects can be particularly harsh in shallow river and lake systems that do thermally stratify and can result in summer and winter fish kills.

In addition to naturally occurring temperature fluctuations due to circadian or seasonal variability, anthropogenic activities can also contribute to thermal fluctuations observed in the environment. Localized thermal disturbances can be prevalent in river and lake systems adjacent to thermal effluents released from large-scale industrial and power generation plants. These plants are a potential source of repeated, transient heat stress, depending on proximity to the source. However, of more widespread significance to aquatic organisms is that of climate change. Mean global land and water temperatures have risen by 0.85 °C since 1880 with the most rapid incline observed within the last 30 years (IPCC, 2014). Furthermore, predicted increases in temperature over the next 65-85 years range between 1.4 and 3.1 °C without intervention to reduce greenhouse gas emissions (IPCC, 2014). Such temperature changes have the potential to negatively impact fish species. Northward shifts in the distribution of Atlantic cod (*Gadus morhua*) and snake blenny (*Ophidion barbatum*) in the North Sea have already been attributed to the effects of climate change on water temperatures (Perry et al., 2005) and similar distribution shifts have been observed for several freshwater fishes (Chu et al., 2005). Effects of climate change on species distribution patterns are only expected to continue (Chu et al., 2005). Overall, the effects of rapidly rising global temperatures on physiology has prompted questions as to whether animals in the most heavily impacted ecosystems will be able to adapt to changing climates and has encouraged the need for additional

research on the impact of long-term, sub-lethal temperature changes on critical aspects of physiology, including the activity and regulation of metabolism.

1.1.2. Thermal plasticity and energy metabolism

Metabolism is heavily influenced by temperature. This is largely due to the profound effects of temperature on enzyme catalytic rates. Enzyme activity typically responds to temperature in three distinct phases, whereby activity rate (1) increases with temperature, (2) briefly plateaus at the thermal optimum (T_{opt}) and (3) declines rapidly as temperature exceeds optimum (Campbell and Reece, 2005; Elliot and Elliot, 2005; Schulte, 2015; Figure A1). Furthermore, similar patterns of temperature sensitivity underlie other whole-body performance measures, such as routine and maximum metabolic rates (Huey 1979; Schulte, 2015; Schulte et al., 2011). For fish, whose body temperature reflects that of ambient conditions, enzyme activity rates constantly fluctuate between different points along the temperature-activity curve.

In addition to temperature-activity curves, the relationship between temperature and enzyme catalytic rate is frequently estimated using the Q_{10} temperature coefficient, which represents the factor by which enzyme activity changes with a 10 °C increase in temperature. Q_{10} values for metabolic enzymes typically range between 2-3 for both mammals (Reyes et al., 2008) and ectotherms (Hochachka and Somero, 2002), but vary among enzymes. For instance, Q_{10} values for cytochrome c oxidase (COX) fall below this range in several fish species (Hardewig et al., 1999; Schnurr et al., 2014), including lake whitefish (*Coregonus clupeaformis*; Blier and Guderley, 1988). However, despite the relative robustness of certain metabolic enzymes to thermal fluctuations, prolonged

changes in temperature still induce physiological adjustments to balance metabolic pathways regulating energy supply and demand (Blier and Guderley, 1988; Hardewig et al., 1999; Schnurr et al., 2014).

1.1.2.1. Oxidative metabolism and thermal acclimation

The regeneration of usable energy in the form of ATP is of utmost importance to cellular function. The energy contained in ATP can be released through the cleavage of a single phosphate group to form ADP and used to power critical cellular processes that require an input of energy. The electrons used during ATP regeneration can be derived from many different sources, including lipids, carbohydrates and proteins. However, under aerobic conditions, metabolized substrates are ultimately funneled through two key oxidative pathways: the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC; Cohen, 1991). The TCA cycle is composed of several enzymatic reactions that systematically strip electrons from the starting citrate molecule (Cohen, 1991). The final product generated by one turn of the TCA cycle is oxaloacetate (OAA), which is subsequently joined with acetyl-coA by citrate synthase (CS), thus completing the cyclical nature of this metabolic pathway. Control of the TCA cycle is predicted to occur at several points, but most notably through the allosteric inhibition of CS by excess ATP (Cohen, 1991). Electrons stripped from citrate during the TCA cycle are transferred to the various electron-carrying complexes of ETC and facilitate the pumping of protons from the mitochondrial matrix into the intermembrane space. The energy derived from the flow of protons down their electrochemical gradient into the mitochondrial matrix is coupled to the activity of ATP synthase which regenerates ATP from ADP (Cohen,

1991). Capacity of the ETC is often estimated through the activity of its terminal enzyme, cytochrome c oxidase (COX; Leary et al., 2003; Lyons et al., 2004), which transfers low-energy electrons to O₂, thereby removing them from the ETC and maintaining electron flow through the various electron-carrier complexes (Cohen, 1991; Figure A2).

Effects of temperature on oxidative processes in fish range from those on whole-body parameters to individual tissues and cells (Johnston and Dunn, 1987). However, irrespective of the level of biological organization, oxidative responses typically display a compensatory increase following long-term exposure to cold temperatures (Johnston and Dunn, 1987). This can be observed at several levels of biological organization, resulting in an increase in oxidative markers such as O₂ consumption rates (Guderley and Johnston, 1996; Little et al., 2013; Moon et al., 1985), metabolic scope (Little et al., 2013) and mitochondrial density (Dhillon and Schulte, 2011; Guderley, 2004) as well as the activity of oxidative enzymes (Batterby and Moyes, 1998; McClelland et al., 2006). These increases in oxidative markers following cold acclimation have been attributed to compensation for the loss of oxidative enzyme activity as temperatures decline, with the aim of maintaining cellular homeostasis (Johnston and Dunn, 1987).

Despite the ecological relevance of elevated heat stress and its potential impacts on metabolism, acclimation responses to temperatures above optimal have received less attention from the research community. Although it is reasonable to predict opposing effects of heat to those observed in response to cold acclimation at mildly elevated temperatures, there are several factors to consider as temperatures extend beyond the optimal thermal range of a species, including enzyme limitations associated with

denaturation. Elevations in red and white muscle COX activity and *coxI* mRNA abundance have been identified in rainbow trout (*Oncorhynchus mykiss*) acclimated to 18 °C, relative to those acclimated to 4 °C (Battersby and Moyes, 1998). However, Guderley and Gawlicka (1991) observed opposing responses to the same temperature stress in this species. Furthermore, certain studies in both Antarctic fish species (Hardewig et al., 1999; Windisch et al., 2011) and lake whitefish (Blier and Guderley, 1988), have demonstrated a depression in oxidative enzyme activity and transcript levels in warm-versus cold-acclimated fish. Discrepancies in the oxidative responses to warm acclimation warrant further investigation into regulatory pathways associated with thermal responses in fish, particularly as temperatures extend beyond the upper optimal thermal range of a species.

1.1.2.2. Lipid metabolism and thermal acclimation

Lipids are an important fuel source for downstream oxidative pathways and the regeneration of usable energy in the form of ATP. Unlike carbohydrates, which are rapidly depleted (Tarr, 1972), recover slowly to post-exercise exercise levels (Tarr, 1972) and only provide 4 kcal energy \times g⁻¹ (Sheridan, 1994), lipids contain nearly twice the amount of energy per unit weight (Sheridan, 1994) and can be used as a long-term fuel source for the generation of ATP during energy-intensive events such as migration, life cycle transitions, reproduction, overwintering and somatic growth. As such, lipids play a crucial role during energy-intensive events that require a constant and reliable source of ATP.

The oxidation of free fatty acids into components that can be used directly by the TCA and ETC is completed through a process called β -oxidation. This process, which occurs independently in both mitochondria and peroxisomes (Houten and Wanders, 2010; Leaver et al., 2008), generates NADH, FADH₂ and acetyl-CoA (Houten and Wanders, 2010). β -oxidation occurring in the mitochondria typically results in high energy output, since it is coupled directly to the ETC; however, it is unable to process very long-chain or complex fatty acids (Reddy and Hashimoto 2001). In contrast, peroxisomes are capable of accepting a wider variety of fatty acids, but produce less energy per molecule than their mitochondrial counter-parts (Leaver et al., 2008; Reddy and Hashimoto, 2001; Figure A3).

While both mitochondrial and peroxisomal β -oxidation result in fatty acid breakdown, these processes are completed by two distinct sets of enzymes (Leaver et al., 2008; Poirier et al., 2006) and are limited at different points in the oxidation pathway (Drynan et al., 1996; Leaver et al., 2008; Small et al. 1985). The rate of mitochondrial β -oxidation is limited by transport of fatty-acids into the matrix which is initiated by enzymatic linkage of acyl-CoA and carnitine by carnitine palmitoyltransferase-1 (CPT1) (Drynan et al., 1996). The resulting acyl-CoA/carnitine complex can be subsequently transported into the intermembrane space (Drynan et al., 1996; Houten and Wanders, 2010), on route to the matrix (Leaver et al., 2008). In peroxisomes, the rate-limiting step is the conversion of acyl-CoA molecules and O₂ to trans-2,3-dehydroacyl-CoA and H₂O₂ by acyl-CoA oxidase (ACOX) (Leaver et al., 2008; Small et al., 1985), which represents the first step in enzymatic breakdown of fatty acid molecules in this organelle (Poirier et al., 2006).

Temperature sensitivity at various points in the β -oxidation pathway have been identified in fish, but has led to conflicting interpretations on the overall effect of temperature on the catabolism of fatty acids. Heart *cpt1* mRNA abundance increases in Atlantic salmon (*Salmo salar*) following exposure to 14 to 19 °C (Jørgensen et al., 2014), but liver *cpt1* levels are highly depressed in warm acclimated medaka (*Oryzias latipes*) relative to their cold-acclimated counterparts (Kondo et al., 2010). Similarly, cold exposure has been shown to elevate liver *cpt1* mRNA abundance in American bullfrog (*Lithobates catesbeianus*) tadpoles (Suzuki et al., 2016), liver *cpt1* α mRNA abundance in gilthead sea bream (*Sparus aurata* L.; Bermejo-Nogales et al., 2014) and muscle CPT1 activity in striped bass (*Morone saxatilis*; Rodnick and Sidell, 1994). These results may reflect tissue-specific differences in the expression and regulation of *cpt1* isoforms. However, cross-tissue expression of *cpt1* α and *cpt1* β has been observed in salmonids (Morash et al., 2010), suggesting added complexity in the regulation of mitochondrial β -oxidation in these species. Information on the expression and activity of *acox* isoforms in fish in response to temperature change is scarce, but overall ACOX activity has been shown to increase following short-term exposure to cold in Atlantic salmon hepatocytes (Moya-Falcón et al., 2006).

Equally important in the understanding of temperature on lipid metabolism is the *de novo* synthesis of fatty acids and triglycerides. Accumulation and storage of fats is crucial to rebuilding energy stores and is necessary to support the diverse functions of lipids and their derivatives as structural components of cell membranes (Leaver et al., 2008) and signaling molecules (Hadley, 2000). This pathway is initiated by the carboxylation of acetyl-coA to malonyl-coA by acetyl-coA carboxylase (ACC) and

subsequent assembly and elongation of fatty acids is completed in the cytosol by the multifunctional enzyme, fatty acid synthase (FAS; Leaver et al., 2008). Reducing power, in the form of nicotinamide adenine dinucleotide phosphate (NADPH), is required for FAS activity and is primarily supplied by malic enzyme (ME) and glucose 6-phosphate dehydrogenase (G6PD; Leaver et al. 2008). The rate-limiting step of lipid biosynthetic pathways is the synthesis of malonyl-coA by ACC (Brownsey et al., 2006; Leaver et al., 2008; Figure A3). In mammals, the expression of the ACC isoforms, ACC α and ACC β , is influenced by nutritional status as well as hormonal factors such as insulin, leptin and thyroid hormones (THs; Brownsey et al., 2006). Little information on the potential influence of temperature on the expression of *acc* isoforms exists, suggesting it may not be a primary regulator of *acc* expression. However, this does not eliminate the possibility of indirect effects of temperature on *acc* expression such as through interactions with other temperature-sensitive enzymes (Brownsey et al., 2006) or impacts of temperature on regulatory hormone levels (Brownsey et al., 2006; Comeau et al., 2000; Eales and Fletcher, 1982; Johnston and Eales, 1995; Larsen et al., 2001).

1.1.3. Cellular mechanisms of thermal plasticity

Many of the physiological endpoints associated with thermal plasticity, such as O₂ consumption, mitochondrial biogenesis and changes in the activity of key enzymes, have been characterized in teleosts, particularly in response to cold acclimation (Bremer and Moyes, 2011; Dhillon and Schulte, 2011; Guderley, 2004; Guderley and Johnston, 1996; Little et al., 2013; McClelland et al., 2006; Moon et al., 1985; Windisch et al., 2011). However, aspects of the mechanistic complexity controlling these physiological

adjustments are unclear (Schulte, 2011). Mammals have traditionally been used as model systems for determining the cellular mechanisms associated with thermal plasticity and metabolic remodeling in response to temperature change. This research has identified a complex network of transcription factors and signaling cascades involved in regulating mitochondrial biogenesis and secondary metabolic pathways (Bremer and Moyes, 2011; Drake et al., 2017). The chief transcription factor associated with these signaling cascades is peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α ; Cioffi et al., 2013; Hulbert, 2000; O'Brien, 2011; Puigserver et al., 1998), which is known to be heavily influenced by endocrine factors such as thyroid hormones (THs; Cioffi et al., 2013; Hulbert, 2000; Pucci et al. 2000; Weitzel and Iwen, 2011). PGC-1 α expression has been linked to the subsequent activation and/or transcription of additional DNA-binding proteins, including nuclear respiratory factors 1 (NRF1) and 2 (NRF2), peroxisome proliferator-activated receptors (PPARs) and the retinoid X receptor (RXR), among others (Drake et al., 2017). While analogs of all the critical transcription factors and accessory proteins involved in metabolic regulation following temperature change in mammals are also present in the fish genome (Bremer and Moyes, 2011; LeMoine et al., 2008; Windisch et al., 2011), there is conflicting evidence as to whether their role in the regulation of metabolic pathways have been conserved (Bremer et al., 2016; Bremer and Moyes, 2011; LeMoine et al., 2008; Little et al., 2013; Windisch et al., 2011). Studies in rainbow trout have suggested significant modifications in the metabolic temperature response in fish due to structural modifications in the PGC-1 α protein (Bremer et al., 2016) and suggest temperature-dependent modifications following thermal challenge may be, in part, funneled through the alternative transcriptional regulator, PGC-1 β (Bremer et

al., 2012). However, oxidative pathways are not the only aspect of metabolism that exhibit PGC-1-mediated thermal plasticity. Intermediary pathways such as lipid metabolism are modified by PGC-1 signaling pathways in mammals (Pucci et al. 2000). Overall, the complexity of metabolic regulation has left several gaps in its mechanistic understanding for fish and non-mammalian species, including endocrine influence of these responses.

1.2. Thyroid hormones and energy metabolism

1.2.1. Thyroid hormones

Communication between cells and coordination of cellular processes in multicellular organisms is complex. Large spatial distribution among cells comprising a single multicellular organism means that the integration of many cellular events is dependent on chemical messengers, such as hormones, secreted from specialized cells or glands. Several hormone families exist in the animal kingdom, and each exert unique physiological effects. Thyroid hormones are ubiquitous among vertebrate species (Hulbert, 2000). In addition to pronounced effects on cellular metabolism (Cioffi et al., 2013; Hulbert, 2000; Pucci et al. 2000; Weitzel and Iwen, 2011), they influence several aspects of growth (Hadley, 2000; Hulbert, 2000), development and metamorphosis (Hadley, 2000; Hulbert, 2000; Kao et al., 1997) and osmoregulation (McCormick, 2001). As with other hormones, THs are potent cellular regulators that induce physiological effects on target cells at low concentrations (Hulbert, 2000). Mammalian circulating TH levels are ultimately directed through the hypothalamic-pituitary-thyroid axis, whereby release of thyrotropin-releasing hormone (TRH) from the hypothalamus stimulates

thyroid-stimulating hormone (TSH) release from the pituitary. TSH, in turn, induces release of thyroxine (T_4) into the bloodstream (Blanton and Specker, 2007). The HPT axis in fish is thought to be very similar (Manzon and Manzon, 2017), with the exception that thyroid follicles are diffusely scattered throughout the ventral pharyngeal region rather than being contained within a discrete gland (Gupta and Thapliyal, 1991; Manzon and Manzon, 2017). Although circulating TH levels are largely regulated through negative feedback of THs on TSH secretion (Hadley, 2000), the neural connection established by the HPT axis still allows for environmental signals perceived by the brain, including temperature, to be reflected in circulating TH levels.

In addition to TH release, peripheral modification of T_4 to 3,5,3 -triiodothyronine, (T_3) or 3,5-diiodothyronine (T_2) is thought to play a large role in TH physiology in fish (Eales, 1985; Peter, 2011). Until recently, T_3 was regarded as the primary biologically active TH (Hulbert, 2000). However, physiological effects have also been observed with T_4 (Cioffi et al., 2013; Corderio et al., 2013; Peter and Oommen, 1993; Sheridan, 1994), which was previously thought to function primarily as a prohormone (Hulbert, 2000). Furthermore, T_2 , which was originally thought to be physiologically inert (Hulbert, 2000), has also been gaining respect as a functional TH (Little et al., 2013; Navarrate-Ramirez et al., 2014; Sayre and Lechleiter, 2012; Varghese and Oommen 1999). Taken together, the presence of multiple functional TH forms demonstrates the potential for fine-tuning of complex physiological responses initiated by this family of hormones and warrant further investigation into metabolic regulation via THs in fish species.

1.2.2. Relationship between thyroid hormones and metabolic pathways

Thyroid hormones are promising candidates in developing a mechanistic understanding of metabolic changes induced by thermal acclimation since they have been associated with numerous aspects of metabolic remodeling in mammalian species (Cioffi et al., 2013; Hulbert, 2000; Weitzel and Iwen, 2011). Furthermore, changes in thyroid status have been linked with changes in metabolic enzyme mRNA abundance (Chen et al., 2015; Little et al., 2013) and activity (Little et al., 2013; Zak et al., 2017) in teleosts, indicating they may play a role in mediating aspects of metabolic adjustments during thermal acclimation. A brief overview on the effects of TH on oxidative and lipid metabolism is outlined below.

1.2.2.1. Relationship of thyroid hormones with oxidative pathways

Thyroid hormones are generally regarded as stimulants of cell metabolism (Cioffi et al., 2013; Etkin et al., 1940; Hulbert, 2000; Pucci et al. 2000) and have been strongly linked to thermoregulation and a rise in oxidative metabolism in mammals (Cioffi et al., 2013; Hulbert, 2000; Weitzel and Iwen, 2011). Similarly, effects of THs have been associated with a number of metabolic effects suggestive of increased ATP regeneration in fish (Gupta and Thapliyal, 1991; Little et al. 2013; Peter and Oommen 1993), although these effects are unpredictable and inconsistently observed among, and even within, species (Etkin et al., 1940; Gupta and Thapliyal, 1991; Little et al. 2013; Peter and Oommen 1993; Umminger, 1978). For instance, neither thyroid feeding (Etkin et al., 1940) nor radiothyroidectomy (Umminger, 1978) altered *in vivo* O₂ consumption rates in goldfish (*Carassius auratus*). However, Leary et al. (1996) observed an increase in O₂

consumption of mitochondria isolated from goldfish liver and red muscle, following exposure to T₂ and T₃. Variable responses within a single species suggests metabolic adjustments may be influenced by additional internal and/or environmental cues.

Hypothyroidism has also been shown to depress active metabolic rate in zebrafish (*Danio rerio*; Little et al., 2013) and both T₃ and T₄ injection in hypothyroid climbing perch (*Anabas testudineus* Bloch) stimulate O₂ consumption rates (Peter and Oommen, 1993). Overall, these results suggest species-specific sensitivities to TH with respect to oxidative metabolic pathways that are likely influenced by external environmental factors (Gupta and Thapliyal, 1991).

1.2.2.2. Relationship of thyroid hormones with lipid pathways

While clear, directional effects of THs exist for oxidative metabolism in mammals, ultimately driving towards increased ATP regeneration, the effects of TH on lipid metabolism are less clear. THs appear to simulate simultaneous increases in both lipid biosynthetic and breakdown pathways (Mullur et al., 2014; Pucci et al., 2000). For instance, mRNA abundance of both liver *acc* and *fas* are reduced in hypothyroid rats and stimulated by T₃ injection (Blennemann et al., 1995) in addition to increases in both mRNA abundance (Mullur et al., 2014; Thakran et al., 2013) and activity (Mullur et al., 2014) of CPT1. However, due to their primary role as oxidative stimulants (Cioffi et al., 2013; Etkin et al., 1940; Hulbert, 2000; Pucci et al. 2000), promotion of catabolic β -oxidation pathways likely dominates overall metabolic effects by THs in mammals (Pucci et al., 2000) and the stimulation of lipogenesis is thought to primarily compensate for loss of fat stores (Mullur et al., 2014).

As in mammals, THs appear to stimulate several aspects of lipid metabolism in fish (Sheridan, 1994; Varghese and Oommen, 1999). Fatty acid mobilization following treatment with THs has been observed in gilthead seabream (*Sparus aurata* L.; Vargas-Chacoff, 2016), climbing perch (*Anabas testudineus* Bloch; Varghese and Oommen, 1999) and the common carp (*Cyprinus carpio*; Plisetskaya et al., 1983), suggesting some consistency of TH action of lipid metabolism in mammals and fish. Other documented effects of THs on lipid metabolism in fish include increases in triacylglycerol lipase (TGL) activity and declines in ACC activity in hypothyroid sea lamprey (*Petromyzon marinus*; Kao et al., 1999) and an increase in 3-hydroxyacyl-CoA dehydrogenase (HOAD) activity in dogfish shark (*Squalus acanthias*) following T₃ treatment (Battersby et al., 1996). Furthermore, Chen et al. (2015) observed a decrease in both the transcript abundance and activity of the lipogenic enzymes 6-phosphogluconate dehydrogenase (6PGD), glucose 6-phosphate dehydrogenase (G6PD) and fatty acid synthase (FAS) following induction of hypothyroidism, but an increase in carnitine palmitoyltransferase 1 (CPT1). The synchronicity between transcript abundance and enzyme activity observed in this study suggest important enzymes in lipid metabolism pathways could be regulated transcriptionally by THs.

1.2.3. Cellular mechanisms of thyroid hormone action

The mechanisms of TH action on cells is very complex, but can be broadly grouped into nuclear pathways and non-nuclear pathways. The TH-induced stimulation of basal metabolic rate was demonstrated in mammals (Barker and Kiltgaard, 1952) over 10 years before Tata et al. identified that these effects could be abolished in the presence

of the transcriptional inhibitor, actinomycin-D (Hulbert, 2000). The results of the study completed by Tata et al. suggested that at least some of physiological effects of THs on oxidative metabolism were transcriptionally-mediated and, therefore, likely to act through a mechanism tightly associated with the nucleus. Subsequent research identified the "classical" nuclear pathway of TH action. Under this mechanism, T₃ enters the nucleus where it interacts with TH receptors (TRs) bound to specific locations on the chromosome known as TH response elements (TRE; Cheng et al. 2010; Hulbert, 2000). Binding of T₃ to the TR alters receptor conformation and causes a change in transcription rate, either repressing or activating gene transcription. Despite its relatively simplistic sequence of events, there are several aspects in the classical TH pathway that can influence physiological function (Cheng, 2000) including two distinct isoforms of the TR (TR α and TR β), which elicit different physiological effects in fish (Flores-Morales et al., 2002; Navarrate-Ramirez, 2014), and a suite of coregulatory proteins that control aspects of T₃-TR complex function (Cheng, 2000).

Although many effects of TH on mitochondrial biogenesis and oxidative metabolism are thought to be mediated via nuclear TRs and the classical TH mechanisms (Cioffi et al., 2013; Hulbert, 2000; Weitzel and Iwen, 2011), it is likely that the majority of physiological responses elicited by THs, and particularly those involved in lipid metabolism, occur via non-nuclear signaling pathways (Cheng, 2000; Corderio et al., 2011; Hulbert, 2000). Early definitive evidence of non-nuclear TH signaling pathways was demonstrated on rat erythrocytes lacking a nucleus (Davis et al., 1983; Galo et al., 1981). Studies on this model system identified stimulatory effects on Ca²⁺-ATPase activity by both T₃ and T₄ (Davis et al., 1983; Galo et al., 1981). Several diverse

pathways of non-nuclear TH action have since been discovered, but are often referred to as non-classical TH signaling to distinguish them from effects mediated by direct association of THs with TR and TRE (Corderio et al., 2013). Non-nuclear effects of THs have been associated with a multiple site of action on target cells, including those both on the plasma membrane and within the cytosol (Cheng, 2000; Cheng et al., 2010). For instance, TH treatment has been associated with phosphorylation of effector proteins, activation of secondary messenger systems and modification of mRNA stability (Corderio et al., 2013). However, despite the wide range of TH-induced cellular effects, initiation of non-nuclear pathways can still ultimately have impacts on gene expression and transcription rates.

1.3. Integration and opportunities for study

1.3.1. Predicted relationships between temperature and thyroid hormones in teleosts

Several associations between temperature and THs in teleosts are present in the literature, ranging from system-level responses such as O₂ consumption (Gupta and Thapliyal, 1991; Little et al., 2013) to the mechanistic level such as the involvement of distinct transcription factors (Bremer et al., 2012; Bremer et al., 2016; Gupta and Thapliyal, 1991). In fish and other ectotherms, TH demonstrates an innate relationship with temperature. Serum TH levels vary seasonally and following temperature change in several fish species (Comeau et al., 2000; Eales and Fletcher, 1982; Johnston and Eales, 1995; Larsen et al., 2001; Levin and Bolotovskiy, 2015; O'Brien, 2010) and are elevated in populations of the common roach (*Rutilus rutilus*) at higher latitudes (Levin and Bolotovskiy, 2015). Furthermore, temperature has been shown to alter cellular hormone

uptake via monocarboxylate transporter 8 (MCT8), responsible for T₃ and T₄ transport across the cell membrane (Cioffi et al., 2013), in zebrafish (*Danio rerio*) cells (Arjona et al., 2011; Muzzio et al., 2014) as well as deiodination activity in other fish species (Cyr et al., 1998, Eales and Fletcher, 1982). Combined, these results suggest temperature impacts TH synthesis and/or release from thyroid follicles, increases overall tissue TH sensitivity and contributes to the activation and/or deactivation of THs within the peripheral tissues. As such, temperature has the potential to play a pivotal role in cellular responses to THs.

Further to their role on basic cellular sensitivity mechanisms, temperature-TH interactions have been observed on metabolic function in teleosts. Modification of thermogenesis and O₂ consumption rates are a hallmark of TH function in mammals (Cioffi et al., 2013; Hulbert et al., 2000; Weitzel and Iwen, 2011), but attempts to identify a similar relationship on oxidative processes in ectothermic species were initially met with inconsistent and conflicting results (Gupta and Thapliyal, 1991). These discrepancies remained unresolved until Maher and Levedahl uncovered the importance of acclimation temperature on TH effects in the Carolina anole lizard, *Anolis carolinensis* (Gupta and Thapliyal, 1991). Subsequent studies have demonstrated further evidence that TH exposure can initiate metabolic reactions in warm acclimated ectotherms, but becomes essentially ineffective at cold temperature. These results have been repeated on certain aspects of metabolic function in several teleost species (Gupta and Thapliyal, 1991; Little et al., 2013), including lake whitefish (Zak et al., 2017), but have also been met with skepticism and conflicting results.

The full extent of temperature-TH interactions in fish, as well as their mechanistic underpinnings, are still unresolved. However, temperature-mediated pathways are known to share a number of similarities to those traditionally activated by TH, including the master metabolic regulator peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1 α), nuclear respiratory factors (NRFs) as well as liver and retinoid X receptors (LXR; RXR; Bremer et al., 2012; Bremer and Moyes, 2011; Cioffi et al., 2013; Gao and Moyes, 2016; Hulbert et al., 2000; Morash et al., 2010; Weitzel and Iwen, 2011). Commonalities between the temperature and TH mechanistic pathways could, in part, be responsible for their combined effects on metabolic function and presents the possibility that TH could play some role in natural temperature responses of fish.

1.3.2. Thesis Objectives

This thesis is an investigation into thermal and endocrine influence on lake whitefish metabolism. The long-term objective of this project was to understand the cellular mechanisms used by fish to compensate for elevated temperature exposure and to gain a basic understanding for the potential of THs as metabolic regulators. To this end, lake whitefish were exposed to elevated temperatures either in the presence or absence of exogenous T₄ and mRNA abundance and activity of various enzymes were quantified. Specifically, the objectives of my thesis were to:

(1) characterize changes in mRNA transcript abundance and activity of key oxidative and lipid enzymes following exposure to elevated temperatures

(2) determine if elevated serum TH levels alter temperature-induced changes in mRNA transcript abundance and activity of key enzymes involved in oxidative and lipid metabolism in juvenile lake whitefish

Several sources of temperature variability exist in aquatic systems, ranging from natural environmental factors to those triggered by human influence. Since energy metabolism and the subsequent synthesis of ATP are critical for cellular function, understanding the effects of long-term temperature variability and regulatory factors controlling these processes will be valuable in assessing widespread impacts on physiological processes. The understanding of long-term elevated temperature stress and how it pertains to metabolism is currently understudied in cool-water fish, and the effects of temperature on TH physiology and the regulation of metabolic processes are only beginning to be uncovered. My research will provide insight into the plasticity of metabolic regulation in lake whitefish after exposure to long-term temperature changes and the role of TH in mediating this process.

2. CHAPTER ONE: EFFECTS OF TEMPERATURE AND THYROID HORMONE ON OXIDATIVE METABOLISM IN JUVENILE LAKE WHITEFISH

2.1. Introduction

Temperature is a dynamic abiotic factor that heavily influences several aspects of physiology, including metabolism. In temperate regions, seasonal variability alone can result in annual surface water temperature fluctuations of 20 °C (Bremer and Moyes, 2011) or greater (Dr. Rebecca North, 2017, personal communication), but is not the only source of temperature variability in the environment. In recent decades, climate change has become a topic of global concern, which can have far-reaching effects on both local and global species distribution patterns in fish and other aquatic ectotherms (Brander et al., 2003; Britton et al., 2010; Cline et al., 2013; Perry et al., 2005), as well as their physiological responses (Somero, 2010; Woodward et al., 2010). The most recent climate change models predict an overall increase in global air temperatures between 1.4 and 3.1 °C in the absence of intervention to reverse the current rates of greenhouse gas emissions (IPCC, 2014). Stenothermal species, which have historically lived thermally-stable environments that do not experience temperature fluctuations, are predicted to be most affected by the impacts of climate change on water temperatures due to their limited capacity to physiologically adapt to changing temperatures (Gunderson and Stillman, 2015). In contrast, eurythermal species, which reside in temperate regions and are routinely exposed to fluctuating temperatures, tend to have more robust thermal responses and can acclimatize to varying environmental conditions (Battersby and

Moyes, 1998; Blier and Guderley, 1988; Duggan et al., 2011; Little et al., 2013; Lucassen et al., 2006; Sidell, 1980). However, if seasonal fluctuations observed in temperate regions are combined with additional temperature increases associated with climate change, it is likely to place further demands on critical cellular processes of temperate-dwelling fish, including those on metabolism.

Since ectothermic species such as fish do not actively regulate body temperature through endogenous mechanisms, metabolic enzymes are directly influenced by ambient thermal conditions. However, despite this direct association with environmental temperatures, these enzymes often maintain similar relationships between temperature and catalytic rate to those observed in endothermic species, as indicated by Q_{10} values (Hochachka and Somero, 2002; Reyes et al., 2008). As a result, metabolic plasticity is necessary to cope with these thermal fluctuations and has been observed in several eurythermal fish species (Battersby and Moyes, 1998; Johnston and Dunn, 1987; Kullgren et al., 2013; Larsen et al., 2001), including lake whitefish (Blier and Guderley, 1988; Zak et al., 2017; Zak et al., unpublished). With respect to oxidative metabolism, these changes typically reflect a compensatory increase in enzyme activity (Battersby and Moyes, 1998; Blier and Guderley, 1988; Duggan et al., 2011; Little et al., 2013; Lucassen et al., 2006; Sidell, 1980) at colder temperatures (Campbell and Reece, 2005; Elliot and Elliot, 2005; Schulte, 2015). However, little is known about how oxidative processes in fish are affected following exposure to elevated temperature, and even less about the potential endocrine regulation of these responses.

Thyroid hormones (THs) are involved in the regulation of mitochondrial biogenesis in mammalian systems (Cioffi et al., 2013; Weitzel and Iwen, 2011). As a

result, oxidative metabolic pathways, which are principally located within the mitochondria, are also tightly linked to THs and can be partially assessed through the activity of key enzymes such as citrate synthase (CS) and cytochrome c oxidase (COX). The majority of TH effects on mammalian cellular mitochondrial content and oxidative metabolism are thought to occur through TH-dependent changes of the expression of key transcription factors, which initiate downstream pathways controlling transcription of metabolic genes. Traditional views of TH physiology consider 3',5,3-triiodothyronine (T₃) to be the primary bioactive TH, which is synthesized from its precursor, thyroxine (T₄), via enzymatic cleavage of a single iodine residue (Cioffi et al., 2013; Hulbert, 2000). Modification of oxidative metabolism is thought to occur through T₃-mediated transcription of peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1 α), which coordinates transcription of downstream transcription factors and DNA-binding proteins such as peroxisome proliferator activated receptors (PPARs) and nuclear respiratory factor 1 (NRF1) and 2 (NRF2).

The presence of a similar PGC-1 α -mediated signaling pathway regulating metabolic adaptation in fish and other ectotherms is not unequivocally accepted. Certain studies in rainbow trout (*Oncorhynchus mykiss*) have proposed substitutions in the amino acid sequence of PGC-1 α which could impede functional interactions with other DNA-binding proteins such as NRF1 (Bremer et al., 2016; LeMoine et al., 2010). However, changes in CS and COX activity correlated with changes in PGC-1 α expression have been observed in some fish species (LeMoine et al., 2008; Little et al., 2013; Sheehan et al., 2004). Additional studies have proposed that effects on mitochondrial biogenesis and oxidative metabolism may instead be regulated through the alternative transcriptional

regulator, PGC-1 β (Bremer et al., 2012). Furthermore, investigations into ectothermic metabolism have identified the importance of acclimation temperature on TH-dependent modification of oxidative metabolic pathways, such that effects are present at warmer temperatures, but not at cold (Gupta and Thapliyal, 1991). However, the full extent of TH involvement in the regulation of oxidative processes in fish is still largely unknown.

From an ecological perspective, studies on the effects of environmentally-relevant temperatures and their potential regulation via THs are best carried out for temperate species that routinely experience large annual thermal fluctuations. Lake whitefish (*Coregonus clupeaformis*) is a cool-water temperate species inhabiting freshwater systems across North America (Bernachez and Dodson, 1991; Holmes et al., 2002), many of which are geographically isolated. As a member of the salmonid family, lake whitefish are closely related to well-studied model species such as rainbow trout and Atlantic salmon (*Salmo salar*). However, compared to other cool-water species that inhabit a similar geographic range such as northern pike (*Esox lucius*) and coho salmon (*Oncorhynchus kisutch*), young lake whitefish have a lower lethal temperature limit (Wismer and Christie, 1987). The optimal temperature for juvenile lake whitefish typically ranges between 12.7 and 16.8 °C (Holmes et al., 2002; Wismer and Christie, 1987), with lethal limits ranging between 24 and 27 °C (Edsall and Rottiers, 1976; Zak et al., unpublished; Table A1). However, lake whitefish is known to exhibit phenotypic plasticity following long-term exposure to temperatures outside the thermal optimum, both with respect to cellular stress responses (Zak et al., unpublished) and metabolism (Blier and Guderley, 1988; Zak et al., 2017). The aim of the current study was to characterize the extent of hepatic oxidative metabolic remodeling induced by long-term

exposure to elevated temperatures in this species and assess the potential role of TH in mediating this process. To this end, transcript abundance and activity of key oxidative enzymes were quantified in juvenile lake whitefish exposed to elevated temperatures either in the presence or absence of exogenous T₄ treatment. My results demonstrate limited effects of elevated temperatures and manipulation of TH status on COX, but highlight changes in the transcription and activity of CS in response to temperature, which are further modified in the presence of exogenous T₄.

2.2. Materials and Methods

2.2.1. Animal husbandry

Spawning adult male and female lake whitefish (*Coregonus clupeaformis*) were captured from Lake Huron, ON Canada in the winter of 2014 using 12-16 h gill net sets. Ova and milt were stripped from multiple individuals and pooled for fertilization. Ova were dry-fertilized *in vitro* for 4 minutes and disinfected for 30 min using a 0.5 % iodine solution. Fertilized ova were subsequently rinsed three times in fresh lake water, placed in 1 L plastic bottles (~ 10 000 embryos L⁻¹) and shipped on ice by same-day air transport to the University of Regina aquatics facility. Upon receipt, embryos were housed at 4 °C in 2 L mini-hatching bell jars (Aquatic Ecosystems Inc. Apopka, Florida, USA) until hatch. Aerated, dechlorinated City of Regina water was pumped through the system continuously at a rate sufficient to maintain embryos at a gentle roll. Dead embryos were removed from the system daily and water changes were performed weekly. Synchronous hatch was initiated at 86 dpf by increasing water temperature to 8 °C at a rate of approximately 0.5 °C min⁻¹. Post-hatch, fish were placed in 60 L glass aquaria and tank

temperature was slowly raised to 10 °C. Hatchlings were fed Otohime fish feed (Reed Mariculture, California, USA) *ad libitum* several times daily. Seven months post-hatch, fish were transferred to 1700 L fiberglass holding tanks maintained at 13 °C. Water changes to fiberglass tanks were performed three times per week using a flow through system and dechlorinated City of Regina water. Fish were fed *ad libitum* with Aqueon mini cichlid sinking pellets (PetSmart) beginning 14 months post-hatch and food was withheld for a minimum of 12 h prior to each experimental sampling point. All husbandry and experimental procedures were carried out in accordance with the Canadian Council on Animal Care (CCAC) guidelines and were approved by the University of Regina President's Committee on Animal Care.

2.2.2. Experimental design, hormone treatments and tissue collection

A total of 269 14-month-old juvenile lake whitefish were divided into three TH status groups (TH control, low T₄ and high T₄) receiving exogenous T₄ to manipulate circulating TH levels. Final T₄ dosages were 0 µg T₄ g⁻¹ body weight (TH control), 1 µg T₄ g⁻¹ body weight (low T₄) and 10 µg T₄ g⁻¹ body weight (high T₄) and were administered via coconut oil (President's Choice, Extra Foods, Regina) implants at a volume of 15 µl g⁻¹ fish using 4.4 % DMSO (BDH Chemicals, VWR International, Mississauga, ON, Canada) as vehicle. Seven days post-injection, 10 fish from each TH status group were sampled to provide baseline data on effects of hormone manipulation alone on metabolic activity. Remaining fish in each TH status group were further divided into three temperature groups of 13 (temperature control), 17 or 21 °C. Tank temperature was raised to the desired temperature at a rate of 0.3 °C h⁻¹ and fish from each treatment

group (TH status x acclimation temperature) were sampled 4, 8 and 24 days following the initiation of temperature change. Sample size for each treatment group ranged between 7-10.

Length and weight measurements were collected following anesthesia with 0.03% 2-phenoxyethanol (Sigma) and blood was collected either from the caudal vein or via caudal severance and permitted to clot at 4 °C for a minimum of 4 h. Serum was isolated from whole blood samples via two centrifugation steps (4000 x g for 5 min and 3000 x g for 3 min) and stored at -80 °C for measurement of serum T₄ concentration. Whole livers were excised rapidly from animals following euthanasia via transection of the spinal cord, snap frozen on liquid N₂ and stored at -80 °C until mRNA and protein analyses.

2.2.3. Serum thyroxine (T₄) quantification

Total serum T₄ concentrations in juvenile lake whitefish were quantified in duplicate using the T₄ Monoclonal Solid Phase Radioimmunoassay kit from MP Biomedicals (Catalog No. 06B-254030) according to the manufacturer's directions, using 50 µl serum for sham and low T₄-treated fish and 25 µl serum for high T₄-treated fish. This kit uses a competitive binding assay to quantify unknown total T₄ concentrations in serum samples against a standard curve. Kit range was expanded by adding 1 and 40 µg dl⁻¹ to the standard curve as they maintained conformity with kit standards (Figure A4). Equal parts 0 and 2 µg dl⁻¹ kit standards were loaded to a final volume of 25 µl tube⁻¹ to achieve 1 µg dl⁻¹ and input volume of 20 µg dl⁻¹ kit standard was doubled to 50 µl tube⁻¹ to achieve 40 µg dl⁻¹. ¹²⁵I activity was read in counts per minute (CPM) using a Wallac

1470 Wizard® automatic gamma counter (Perkin Elmer Life Sciences, Turku, Finland). Read duration for all runs was chosen to ensure total ($0 \mu\text{g dl}^{-1}$) CPM were $> 20,000$.

To confirm kit reactivity with lake whitefish serum, a linearity check was performed between kit standards and TH-stripped lake whitefish test serum spiked with equivalent concentrations of T_4 prior to completing experimental samples. Test serum was collected from a healthy, adult lake whitefish using the serum isolation procedure described above. Endogenous THs were stripped by suspending test serum in 50 mg ml^{-1} water-equilibrated AG-1-X8 resin (BioRad) and shaking for 5 h at room temperature. Serum was then collected by centrifugation ($1000 \times g$ for 10 min), transferred to a fresh tube of water-equilibrated resin and shaken overnight at $4 \text{ }^\circ\text{C}$. Stripped serum was collected via a final centrifugation step ($21000 \times g$ for 30 min) and stored at $-86 \text{ }^\circ\text{C}$ until use. Stripped serum was spiked with an appropriate volume of 0.04 mg ml^{-1} stock T_4 solution in 0.22 M NaOH to achieve final concentrations of 1, 2, 4, 8, 12, 20 and $40 \mu\text{g dl}^{-1}$ and run simultaneously with comparison kit standards. $\text{Logit}(B/B_0)$ values were calculated for each set of standards by taking the logit of observed counts (CPM) divided by total counts and plotted against $\log[\text{Concentration } (\mu\text{g dl}^{-1})]$ to confirm linearity parallelism between the two curves (Figure A5).

2.2.4. RNA extraction, cDNA preparation and real-time quantitative-PCR (RT-qPCR) of metabolic enzyme mRNA transcripts

Total RNA was isolated from liver samples using TRIzol® Reagent (Invitrogen Life Technologies, Burlington, ON, Canada) according to the manufacturer's instructions and re-suspended in 20-100 μl molecular-grade water. RNA concentration and

absorbance ratios (A260/280 and A260/230) were obtained using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and A260/280 and A260/230 values > 1.8 were used to confirm absence of salt, phenol and protein contamination. RNA quality was assessed by performing agarose gel electrophoresis to detect the presence of distinct 28S and 18S rRNA bands and stored at -86 °C until required. mRNA transcripts were transcribed to cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen, Mississauga, ON, Canada), according to the manufacturer's directions, using 1 µg total RNA and a final reaction volume of 20 µl.

Partial lake whitefish cDNA sequences for target genes *cs* and *cox4* were amplified, sub-cloned and sequenced using PCR-based sequencing methods. Degenerate primers used for initial amplification of target sequences were generated using BlockMaker and CODEHOP designer from teleost sequences available on the NCBI database. Predicted amino acid sequences for each cloned cDNA fragment were generated using EMBOSS Sixpack (EMBL-EBI) and compared to sequences available on the NCBI database for several teleost species to confirm clone specificity (Table 1). Gene-specific primers to be used in RT-qPCR were designed using Primer3 (Koressaar and Remm, 2007; Untergasser et al. 2012) and NetPrimer software (Premier Biosoft, Palo Alto, CA, USA) using cloned partial cDNA sequences *cs* and *cox4*, and partial lake whitefish cDNA sequence information available on the NCBI database for *cox1* (GenBank accession no. JX960883.1, Table 2). All primers used for RT-qPCR were validated to meet Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009) prior to use on experimental samples. Appropriate cDNA and primer concentrations were determined empirically by

Table 1. Percent amino acid identity (id) and similarity (si) of predicted amino acid sequences of cloned lake whitefish oxidative enzyme cDNA fragments with those of select teleost species. Accession numbers of NCBI sequences used for each comparison are provided below id/si percentages.

Percent amino acid identity and similarity (id/si)					
Target	<i>Salmo salar</i>	<i>Oncorhynchus mykiss</i>	<i>Esox lucius</i>	<i>Danio rerio</i>	<i>Oreochromis niloticus</i>
<i>cs</i>	94.0/98.5 (XM_014136182.1)	94.1/98.5 (CDQ59991.1)	91.8/98.5 (XM_010890560.2)	97.0/99.3 (NM_199598.1)	93.3/99.3 (XM_003438897.2)
<i>cox4</i>	98.0/99.0 (ACN12665.1)	98.0/99.0 (ACO08259.1)	85.7/98.0 (NP_001291071.1)	78.6/94.9 (NP_999866.1)	81.6/95.9 (XM_003442244.3)

Table 2. RT-qPCR primers for target oxidative lake whitefish transcripts

Target	Sequence (5'–3')	Amplicon Length (bp)	cDNA Volume (μl)	[Final Primer] (nM)	Annealing Temperature (°C)	Primer Efficiency (%)
<i>cs</i>	F: CAC AGA GGA ACA GGT GAA CT R: GGA GGT TGG TGG GGA AGT TA	101	0.5	250 250	58	103.7
<i>cox1</i>	F: AAT AGT CGG CAC AGC CCT AA R: GTT TCC AAA CGG TCC AAT CA	140	0.01	250 250	63	91.8
<i>cox4</i>	F: CCA TAG CAC GAC CAG TCC G R: AAC GAG GGC ACA AAG GAG T	81	0.5	250 250	62	96.7

constructing a standard curve for each target sequence using serially diluted liver cDNA from a reference animal (Table 2). Standard curves were also used to ensure amplification efficiency of each primer pair ranged between 90-110 %. mRNA transcript abundance of target and reference genes was quantified in triplicate using 2x SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Mississauga, ON, Canada) in a final reaction volume of 20 µl. All RT-qPCR quantifications were performed using a CFX Connect Real-Time detection system (BioRad) using CFX Manager 3.1 software (BioRad) under the following reaction conditions: 1 cycle of 95 °C for 2 min and 40 cycles of 95 °C for 5 s followed by a 30 s extension phase at 58, 62 or 63 °C (Table 2). The absence of genomic DNA contamination was confirmed using melt curve analysis. Inter-run variation between RT-qPCR runs was monitored using an inter-run calibrator (IRC) consisting of a cDNA pool from several treatment groups.

2.2.5. Quantification of citrate synthase and cytochrome c oxidase enzyme activities

The activity of CS and COX were determined spectrophotometrically as described by Zak et al., 2017 using chemicals purchased from Sigma Aldrich (Oakville, ON, Canada), unless otherwise noted. Crude homogenates were prepared in 10 volumes of homogenization buffer (50 mM Hepes, 1 mM EDTA, 0.1 % Triton X-100, pH 7.4) using chilled 5 ml Wheaton glass/Teflon homogenizers and ~ 40-60 mg liver tissue. Samples were homogenized on ice for 2 min. Prior to running experimental samples, assays were optimized to 0.06-0.12 change in optical density per minute ($\Delta OD \text{ min}^{-1}$) for 0.03-0.06 $\Delta OD \text{ min}^{-1}$ for CS and COX, respectively, using serial dilutions to ensure traces were

linear for the entire assay period. Reactions were prepared in triplicate in chilled 96-well plates and read at 25 °C using a Synergy HTX multi-mode microplate reader (BioTek).

Cytochrome c oxidase assays were completed within 30 min of homogenization using 10 µl of 1:12-diluted liver homogenate. Homogenates were incubated on ice for 5 min in 100 µl COX incubation solution (50 mM Tris, pH 8.0, 0.5 % Tween 20) immediately prior to running the assay to aid in membrane solubilization. Reduced cytochrome c solution was added to the wells at a final concentration of 0.048 mM and reactions were read immediately at 550 nm for 5 min to detect enzymatic oxidation of cytochrome c. Cytochrome c solution (1.8 mM) was reduced by combining cytochrome c (Calyzme Laboratories, San Luis Obispo, CA, USA) and an excess of ascorbate in 50 mM Tris, pH 8.0. Ascorbate was removed by exhaustive dialysis in 50 mM Tris, pH 8.0 with 6-8 kDa MWCO standard cellulose dialysis tubing (Spectrum Laboratories, CA, USA) at 4 °C. A280/550 and A550/565 ratios ranging between 1.1-1.3 and 10-20, respectively, were used to confirm complete reduction of cytochrome c prior to storage at -86 °C.

Citrate synthase assays were completed on homogenates that had gone through two freeze thaw cycles following confirmation that it did not result in a loss of activity (Figure A6). Oxaloacetate (OAA; 20 mM OAA, 50 mM Tris, pH 8.0) was added to the homogenate at a final concentration of 0.47 mM and the assay was initiated by addition of 200 µl CS batch solution (0.1 mM 5,5'-dithiobis-2-nitrobenzoic acid in 95% EtOH, 0.15 mM Acetyl CoA, 50 mM Tris, pH 8.0) for a final assay volume of 215 µl. CoA(SH) +DNTB complex formation detection was performed at 412 nm for 5 min. Background

deacetylase activity was determined at 412 nm for each homogenate using a no-OAA control well.

2.2.6. Data handling and statistical analysis

2.2.6.1. Serum T₄ concentrations

Interpolation and statistical analysis of serum T₄ concentrations was completed in GraphPad Prism 6 statistical software (La Jolla, CA, USA). Total T₄ concentration of unknown serum samples were determined from serum standards using a four parameter logistic (sigmoidal) equation and no special handling of outsider values. Values that fell outside the range of the standard curve were eliminated from the data set. Serum T₄ levels in juvenile lake whitefish treated with hormone implants did not meet parametric assumptions of normality and homoscedasticity, so were analyzed using Kruskal-Wallis (non-parametric) statistical tests. No significant differences in serum T₄ levels were detected among sham-injected fish at any temperature or time point, so values were pooled into a single sham reference group for subsequent analyses. Increases in hormone levels due to T₄ treatment were analyzed separately for low- and high-T₄ groups using Kruskal-Wallis tests followed by Dunn's multiple comparison tests and the reference sham treatment group as a global control. Additional tests were completed at each time point in low- and high-T₄ treatment groups to detect significant differences in serum T₄ levels among temperature groups. All differences were considered significant when $P \leq 0.05$.

2.2.6.2. RT-qPCR mRNA transcript abundance

Statistical analysis of liver *cs*, *cox1* and *cox4* mRNA abundance was completed using the MCMC.qPCR package (Matz et al., 2013) in R Studio (R version 3.3.1; R Studio version 1.0.136; R core team, R Foundation for Statistical Computing, Vienna, Austria). This package predicts posterior mean estimates of mRNA transcript levels within a Bayesian framework using a Poisson-lognormal generalized linear mixed model and a Markov Chain Monte Carlo (MCMC) sampling scheme (Matz et al., 2013). Random effects representing variation among technical replicates due to uneven template loading are also incorporated into the model, acting to normalize the data without the use of reference genes. Raw quantification cycle (Cq) values for target genes were exported from CFX Manager 3.1 software (BioRad) and converted into molecular counts with the `cq2counts()` function using a Cq1 value of 37. The Cq1 value represents the number of qPCR cycles required to detect a single target molecule. Previous tests in our laboratory have shown no difference in molecule count approximation or model fit using either a Cq1 value of 37, as recommended by Matz et al. (2013), or empirically determined values for single-molecule amplification (Stefanovic et al. 2016). All models were run in R with the `mcmc.qpcr()` function through 45000 iterations using a thinning value of 20 and a burn-in value of 5000. Impacts of temperature were modeled using temperature, time and temperature \times time as fixed factors. Impacts of TH manipulation were modeled separately for each temperature using hormone treatment (sham, low T₄ or high T₄), time and treatment \times time as fixed factors. Models were run without priors since use of the reference genes *ef1 α* and *β -actin* as priors did not alter model fit or data interpretation. Posterior mean estimates of target transcript levels are plotted as log₂ mRNA abundance

and error bars represent 95 % credible intervals surrounding the posterior mean estimate. Estimates were considered significant when 95 % credible intervals did not overlap.

2.2.6.3. CS and COX activities

Activity rates for CS and COX activity were determined using the equation,

$$Activity (\mu\text{mol} \times \text{mg tissue}^{-1} \times \text{min}^{-1}) = \left(\frac{rA}{L \times \varepsilon \times t} \right) \times \left(\frac{V_a}{V_s} \right)$$

where rA is the rate of absorbance change ($\text{OD} \times \text{min}^{-1}$) L is optical path length (cm), ε is molar absorptivity ($\text{OD} \times \text{mM}^{-1} \times \text{cm}^{-1}$), t is tissue concentration of the homogenate ($\text{mg} \times \text{ml}^{-1}$), V_a is total assay volume (ml) and V_s is homogenate volume (ml). For CS, activity was calculated using the rate of absorbance change in the final 120 s of each assay trace. Background activity was calculated for each sample and subtracted from the triplicate average. For COX, activity was calculated using the maximal rate of absorbance change in a 120 s period of the assay trace. Molar absorptivity values used for CS and COX enzyme activity calculations were 13.6 and 28.5 $\text{OD} \text{ mM}^{-1} \times \text{min}^{-1}$, respectively.

Significant effects of temperature on CS and COX activities in juvenile lake whitefish was determined separately for each enzyme using a two-way ANOVA (GraphPad Prism 6) to detect effects of temperature over time followed by Bonferroni's multiple comparison tests to detect pair-wise differences in enzyme activity among temperature groups. Similarly, significant effects of TH treatment on CS and COX activities were determined within each temperature group using two-way ANOVA to

detect effects of TH treatment over time, followed by Bonferroni's multiple comparison tests to detect pair-wise difference in enzyme activity among TH treatment groups. All results were considered significant when $P \leq 0.05$. Plots represent mean activities \pm SEM.

2.3. Results

2.3.1. Time-course of oxidative metabolic remodeling in response to elevated temperature exposure in juvenile lake whitefish liver

Temperature-induced effects on oxidative metabolism in juvenile lake whitefish were assessed by quantifying mRNA abundance and activity of oxidative enzymes in liver at 0, 4, 8 and 24 days following exposure to 13 (control), 17 or 21 °C. Overall, elevated temperature had mild effects on oxidative enzymes in liver, but favoured a depression of mRNA and enzyme activity levels following exposure to 21 °C. Relative to control fish maintained at the control temperature of 13 °C, mean *cs* mRNA abundance declined 2.35-fold on *day 4* following the onset of 21 °C (Figure 1A; Table 3). However, this effect was short-lived, as *cs* mRNA levels converged with controls by *day 8* and did not sustain long-term change in steady state at *day 24*. In addition to *cs*, two COX subunits were quantified as markers of respiratory chain capacity. Transcript abundance of the mitochondrial-encoded subunit, *cox1*, exhibited a 3.68-fold increase at *day 8* following the onset of 21 °C exposure (Figure 1B), showing that elevated temperature increases transcription rates and/or stability of mitochondrial-encoded transcripts in lake whitefish liver. However, like *cs*, there were no long-term changes in steady state *cox1* mRNA abundance observed at *day 24*. In contrast to *cox1*, transcript abundance of the nuclear-encoded subunit, *cox4*, was not altered by temperature at any time point (Figure

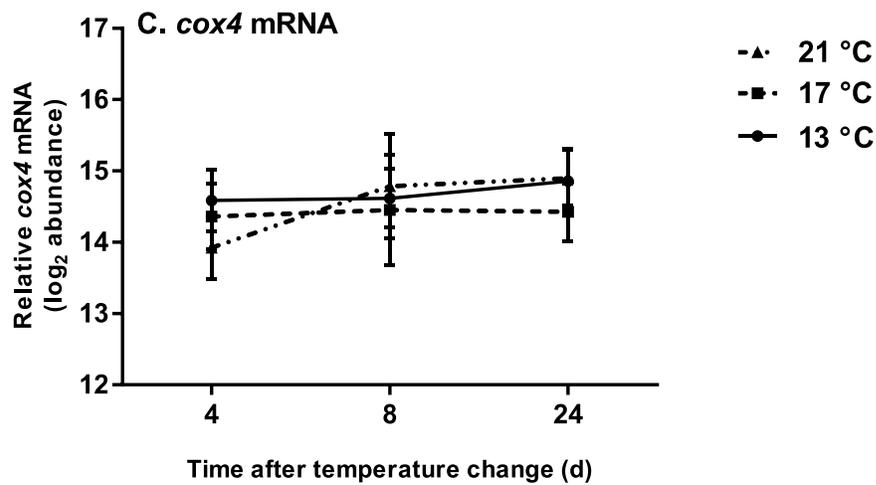
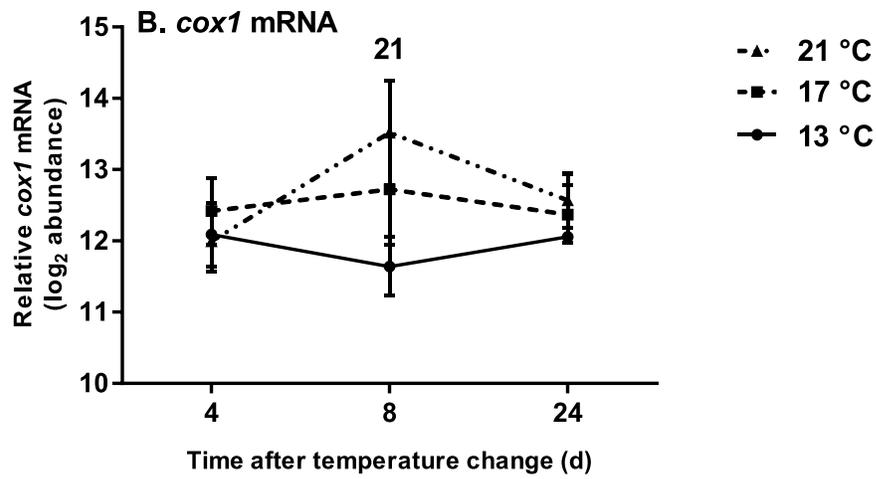
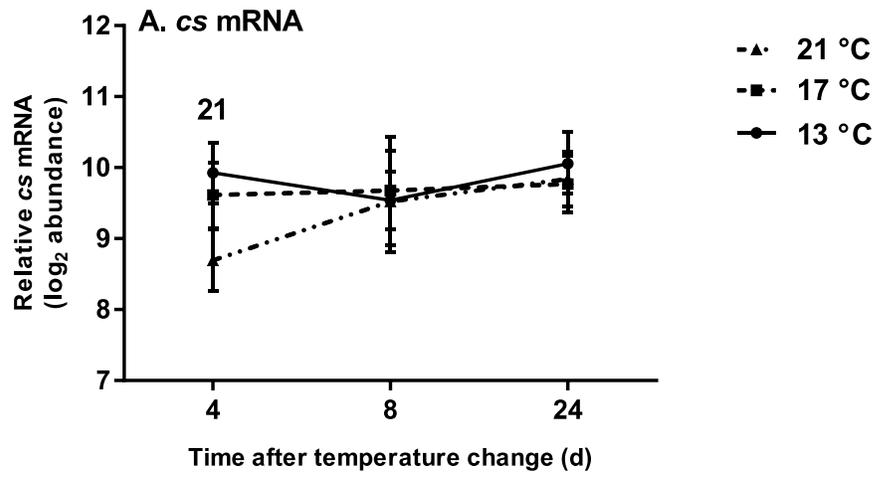


Figure 1. Effects of elevated temperature exposure on *cs* (A), *cox1* (B) and *cox4* (C) mRNA abundance (log₂ abundance arbitrary units) in juvenile lake whitefish. mRNA abundance of oxidative enzyme was quantified in liver 0, 4, 8 or 24 d following exposure to 13, 17 or 21 °C. Data was modeled using a Bayesian MCMC sampling scheme and the MCMC.qPCR package in R (Matz et al. 2013) with temperature, time and temperature × time as fixed factors. Plots represent posterior mean estimates from 7-10 fish ± 95 % credible intervals. Numbers above treatment groups indicate the temperature at which 95 % credible intervals differed from fish maintained at 13 °C within each time point.

Table 3. Summary statistics for the effects of elevated temperature exposure on *cs*, *cox1* and *cox4* mRNA abundance in juvenile lake whitefish. Different lowercase letters indicate non-overlapping 95 % credible intervals between time points within a temperature. Bolded values indicate significant non-overlapping 95 % credible intervals relative to 13 °C controls within a time point.

Gene		Time		
		4 d	8 d	24 d
<i>cs</i>	13 °C	13/4	13/8	13/24
	17 °C	17/4	17/8	17/24
	21 °C	21/4^a	21/8 ^{ab}	21/24 ^b
<i>cox1</i>	13 °C	13/4 ^{ab}	13/8 ^a	13/24 ^b
	17 °C	17/4	17/8	17/24
	21 °C	21/4 ^a	21/8^b	21/24 ^{ab}
<i>cox4</i>	13 °C	13/4	13/8	13/24
	17 °C	17/4	17/8	17/24
	21 °C	21/4	21/8	21/24

1C), demonstrating that not all COX subunits are consistently affected by elevated temperature exposure.

Capacities of the oxidative enzymes, CS and COX, were also investigated to detect functional shifts in metabolism in response to temperature change. Unlike *cs* mRNA levels, which were only altered by elevated temperature at *day 4*, CS activity did not experience short-term changes in response to temperature change. Rather, CS activity declined gradually over the 24 day period and was significantly lower at *day 24* following onset of 21 °C than activity levels observed in all three temperatures groups on *day 4*. (Figure 2A, Table 4). Similarly, COX activity, which was used as a marker for overall functional capacity of the electron transport chain, exhibited long-term effects of elevated temperature exposure. Here, mean COX activity remained stable in response to temperature until *day 24* following temperature change where it was 1.28- and 1.52-fold lower in 17 and 21 °C fish than in 13 °C control fish, respectively (Figure 2B). However, this effect was only significant for fish exposed to 21 °C. Overall, these results demonstrate that exposure to 21 °C influences oxidative enzymes in juvenile lake whitefish liver at both the mRNA and activity level. However, whereas effects on mRNA abundance of oxidative enzymes were transient, elevated temperatures lead to long-term reduction in metabolic function at the activity level.

2.3.2. Combined effects of elevated temperature exposure and TH manipulation on oxidative metabolism in juvenile lake whitefish liver

To examine the role of THs in mediating the temperature responses in juvenile lake whitefish liver, coconut oil implants containing either low (1 µg T₄ × g body

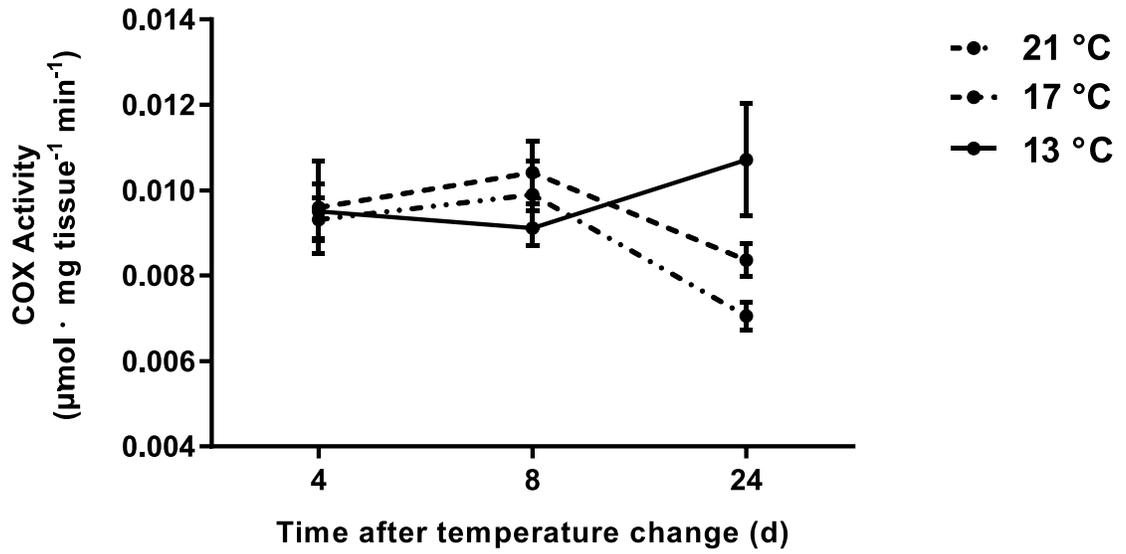
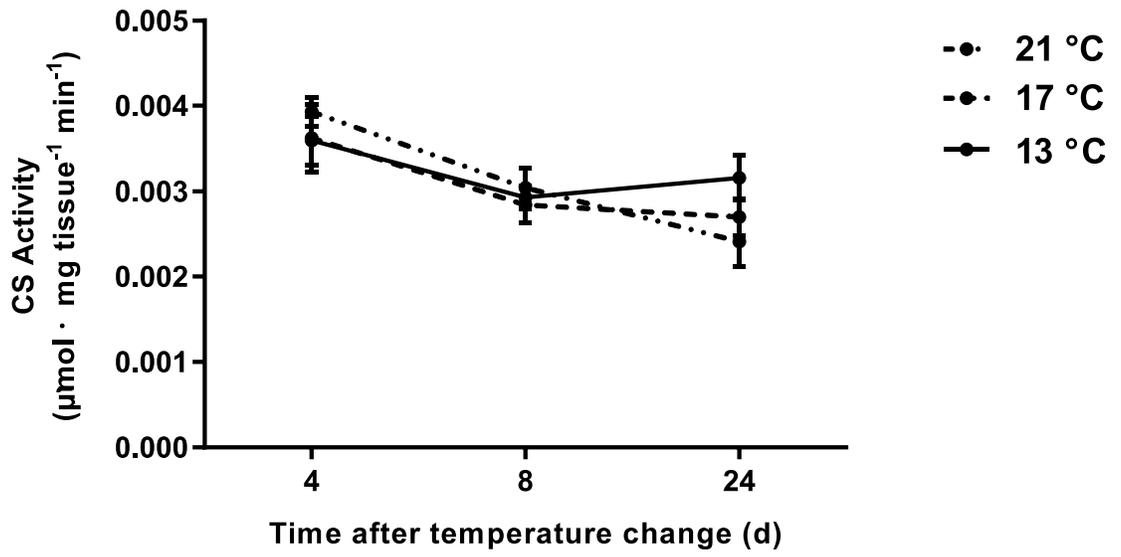


Figure 2. Effects of elevated temperature on CS (A) and COX (B) activities ($\mu\text{mol} \cdot \text{mg} \text{ tissue}^{-1} \text{ min}^{-1}$) in juvenile lake whitefish at 4, 8 and 24 d following exposure to 13, 17 or 21 °C. Data was analyzed using two-way ANOVA to examine effects of temperature, time and temperature \times time on enzyme activity followed by Tukey post-hoc comparisons. Significant pair-wise comparisons ($P < 0.05$) between 13 °C and elevated temperature groups within a time point are indicated by the corresponding temperature value above the treatment group. Plots represent mean activities of 7-10 fish \pm SEM.

Table 4. Two-way ANOVA statistics (F) on CS and COX activity in lake whitefish exposed to 13, 17 or 21 °C

	Temperature			Time			Temperature × Time		
	F	df _n , df _d	P	F	df _n , df _d	P	F	df _n , df _d	P
CS	0.367	2, 67	0.695	12.72	2, 67	< 0.001*	1.334	4, 67	0.266
COX	1.600	2, 70	0.209	1.848	2, 70	0.165	2.990	4, 70	0.024*

weight⁻¹) or high (1 µg T₄ × g body weight⁻¹) T₄ doses were used to manipulate TH status in juvenile lake whitefish. T₄ implants were administered 7 days prior to the onset of temperature change and serum T₄ levels were quantified to confirm manipulation the TH status. While circulating serum T₄ levels were stable in sham-injected fish across all treatment groups (Table 5), levels in low- and high-T₄ treated fish exhibited variability with respect to both temperature and time (Figure 3). Serum T₄ levels were significantly elevated relative to sham-injected controls at all three temperatures in high-T₄ treated fish and were also elevated above sham levels in low-T₄ treated fish. However, elevations in T₄ levels among the low-T₄ fish were much more pronounced in 17 and 21 °C temperature groups on *day 4* and *day 8* post temperature change. Low-T₄ did not affect serum T₄ levels in any 13 °C groups or the 17 and 21 °C groups when sampled on *day 24*.

The observed changes in TH status had temperature-dependent effects on *cs* mRNA abundance in juvenile lake whitefish liver. While neither low nor high T₄ treatment altered *cs* mRNA abundance of fish maintained at 13 °C (Figure 4A; Table 6), transient effects of TH manipulation were observed in response to temperatures in both 17 and 21 °C fish. At 17 °C, low T₄ increased *cs* mRNA levels 2.44-fold over sham-injected controls, 8 days after the onset of temperature change (Figure 4B). However, no effects of TH manipulation were observed at any other time point. The strongest effects of TH manipulation were observed in fish exposed to 21 °C. Here, *cs* mRNA abundance declined 3.26-fold on *day 4* in sham-injected fish, but remained elevated in fish treated with high T₄ (Figure 4C), showing that elevated serum T₄ levels act in the opposite

Table 5. Kruskal-Wallis statistics (H) on effects of hormone treatment group on serum T₄ levels in juvenile lake whitefish within each temperature group (A) and effects of temperature on serum T₄ levels in juvenile lake whitefish within each treatment group (B).

	Day 4		Day 8		Day 24	
A. Treatment effects within temperature						
	H	P-value	H	P-value	H	P-value
13 °C	10.65	0.0005*	10.13	0.0008*	14.65	< 0.0001*
17 °C	11.08	0.0001*	7.5	0.0097*	14.52	< 0.0001*
21 °C	12.10	< 0.0001*	12.27	0.0002*	13.73	< 0.0001*
B. Temperature effects within treatment						
	H	P-value	H	P-value	H	P-value
Sham	2.601	0.2883	1.077	0.6298	0.299	0.8695
Low-T₄	4.711	0.0924	7.221	0.0194*	3.545	0.1728
High-T₄	1.804	0.4186	0.535	0.7897	1.773	0.4292

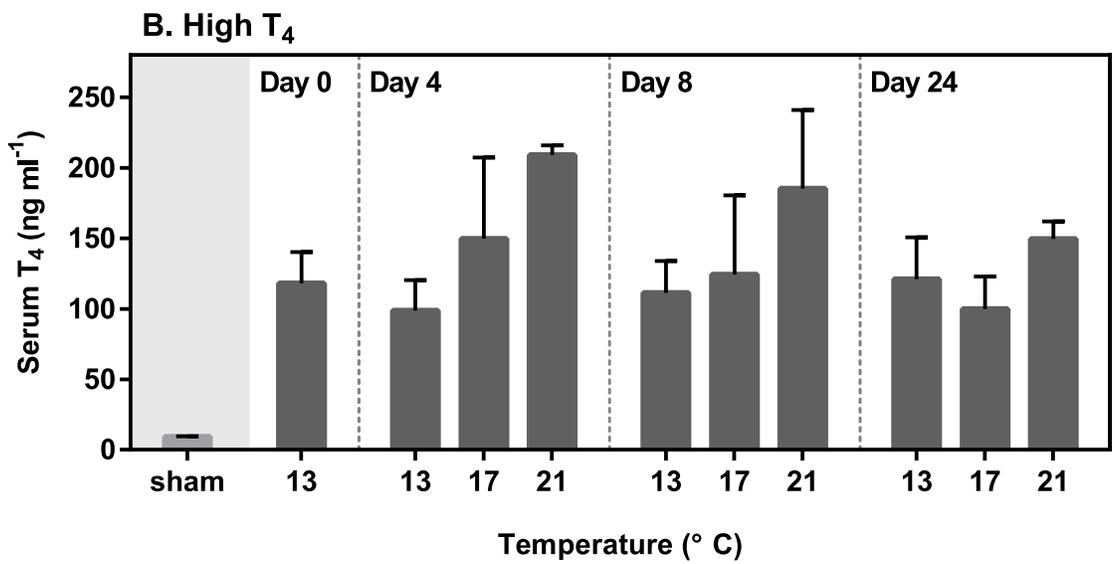
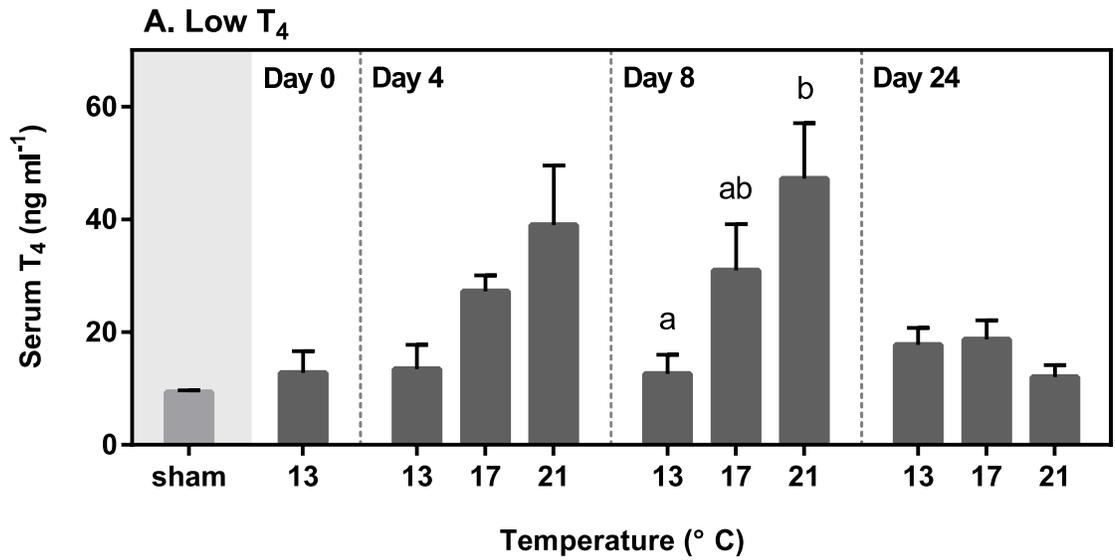


Figure 3. Serum T₄ levels (ng ml⁻¹) in juvenile lake whitefish treated with low (A; 1 µg T₄ g body weight⁻¹) and high (B; 10 µg T₄ g body weight⁻¹) T₄ coconut oil implants at 4, 8 and 24 d following exposure to 13, 17 or 21 °C. Data were analyzed using Kruskal-Wallis (non-parametric) tests to identify effects of temperature on hormone implants within each time point, followed by Dunn's multiple comparison tests. Significant pairwise comparisons ($P < 0.05$) between temperatures within a time point are indicated by different lower-case letters above treatment groups. Plots represent mean of 3-8 serum samples \pm SEM. Serum T₄ levels in sham-injected fish were not significantly different between temperature or time points (Table 5) so were pooled and included in each plot for reference.

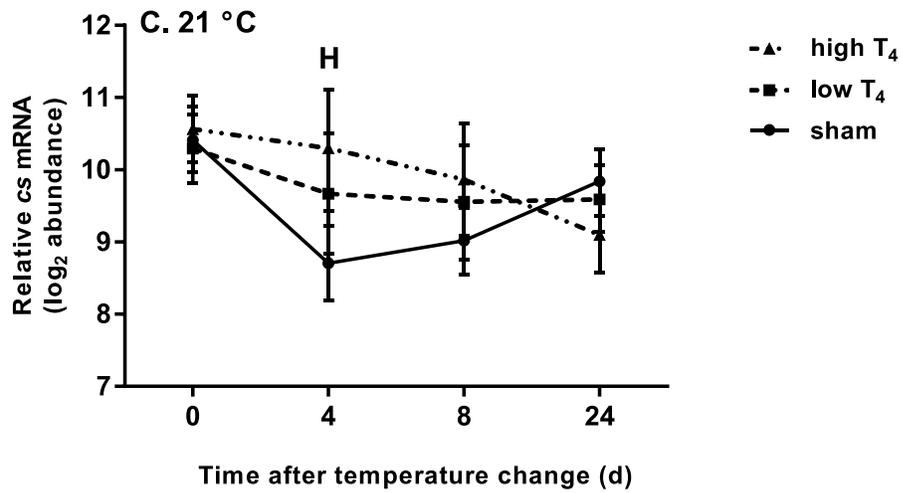
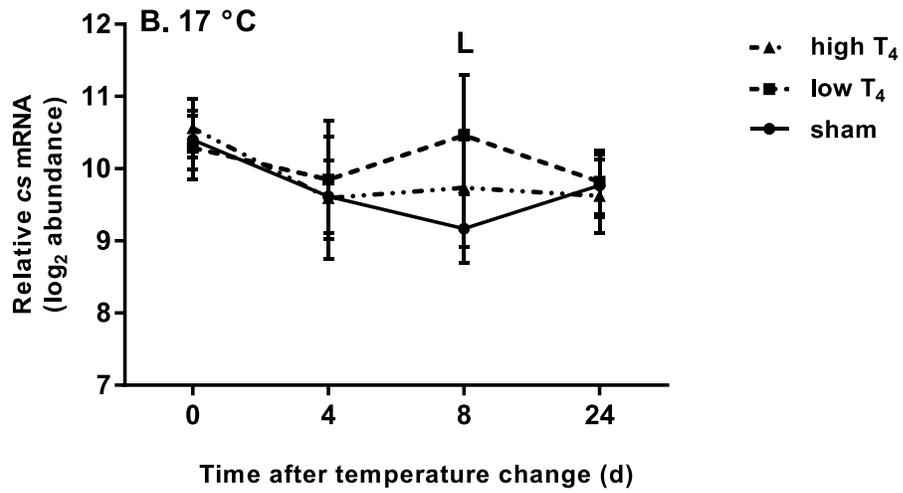
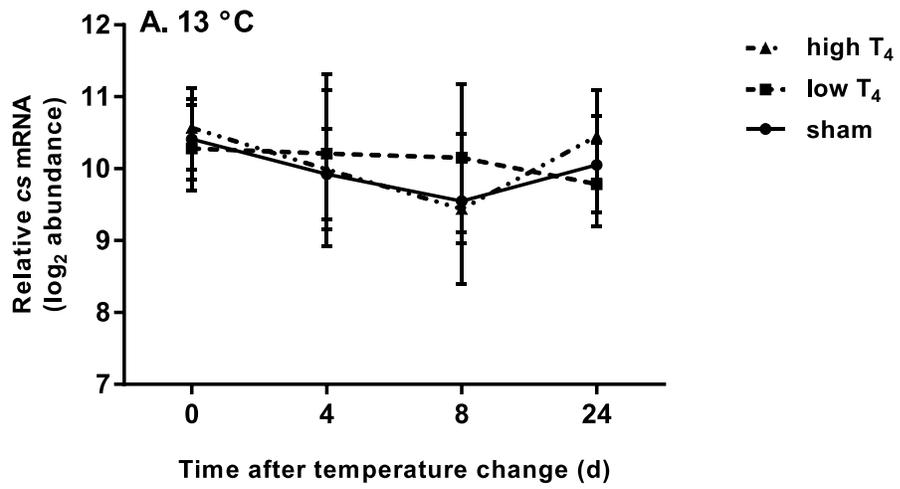


Figure 4. Abundance of *cs* mRNA transcripts (\log_2 abundance arbitrary units) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A), 17 (B) or 21 °C (C). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low T₄ (1 $\mu\text{g T}_4 \text{ g body weight}^{-1}$) or high T₄ (10 $\mu\text{g T}_4 \text{ g body weight}^{-1}$). Data was modeled within each temperature group using a Bayesian MCMC sampling scheme and the MCMC.qPCR package in R (Matz et al., 2013). Hormone treatment, time and treatment \times time were used as fixed factors. Plots represent posterior mean estimates from 7-10 fish \pm 95 % credible intervals. Letters above treatment groups indicate the hormone treatment at which 95 % credible intervals differed from sham-injected fish within each time point (L = low T₄, H = high T₄).

Table 6. Summary statistics for the effects of elevated temperature and T₄ exposure from 4 to 24 d on *cs*, *cox1* and *cox4* mRNA abundance in juvenile lake whitefish. Different lowercase letters indicate non-overlapping 95 % credible intervals between time points within a temperature. Bolded values indicate significant non-overlapping 95 % credible intervals relative to 13 °C controls within a time point.

Gene	Temperature	Treatment	Time			
			0 d	4 d	8 d	24 d
<i>cs</i>	13 °C	control	C/0	C/4	C/8	C/24
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24
	17 °C	control	C/0 ^a	C/4 ^{ab}	C/8 ^b	C/24 ^{ab}
		low T ₄	L/0 ^a	L/4 ^{ab}	L/8^{ab}	L/24 ^b
		high T ₄	H/0	H/4	H/8	H/24
	21 °C	control	C/0 ^a	C/4 ^b	C/8 ^{bc}	C/24 ^{ac}
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24
<i>cox1</i>	13 °C	control	C/0	C/4	C/8	C/24
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24
	17 °C	control	C/0	C/4	C/8	C/24
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24
	21 °C	control	C/0	C/4	C/8	C/24
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24
<i>cox4</i>	13 °C	control	C/0	C/4	C/8	C/24
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24
	17 °C	control	C/0 ^a	C/4 ^b	C/8 ^b	C/24 ^b
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24
	21 °C	control	C/0 ^a	C/4 ^b	C/8 ^b	C/24 ^{ab}
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0 ^a	H/4 ^{ab}	H/8 ^{ab}	H/24 ^b

direction of temperature on *cs* mRNA abundance in lake whitefish liver. Moderate recovery of mRNA levels was also observed on *day 4* in fish treated with low T₄, indicating TH may have dose-dependent effects on *cs* abundance, but 95 % credible intervals overlapped with sham-injected fish. Changes in *cs* mRNA abundance with TH treatment did not translate into effects on the activity level. Rather, CS activity in fish treated with either low or high T₄ closely reflected responses observed in sham-injected fish at all three temperatures (Figure 5A-C).

In contrast to the temperature-dependent effects of T₄ manipulation on *cs* mRNA, *cox1* and *cox4* transcripts in low and high T₄-treated fish did not differ from sham-injected controls at any temperature (Figures 6 and 7, Table 6). However, a modest decline in *cox4* mRNA abundance was observed over the 24 day period in 21 °C fish administered high T₄ (Figure 6C, Table 6), which was not observed in sham-injected controls. Like CS, TH manipulation had limited effects on COX activity (Figure 8A-C). While activity was mildly depressed by 1.39- and 1.27-fold on *day 0* in low and high T₄ treatment groups, respectively, no effects were observed throughout the period following temperature change. Most notably, the long-term depression in COX activity following exposure to 21 °C was not mitigated by elevated THs as suggested by effects on *cs* mRNA abundance (Figure 8C). Overall, these results demonstrate limited effects of elevated TH on long-term functional oxidative capacity in juvenile lake whitefish liver in response to elevated temperature exposure.

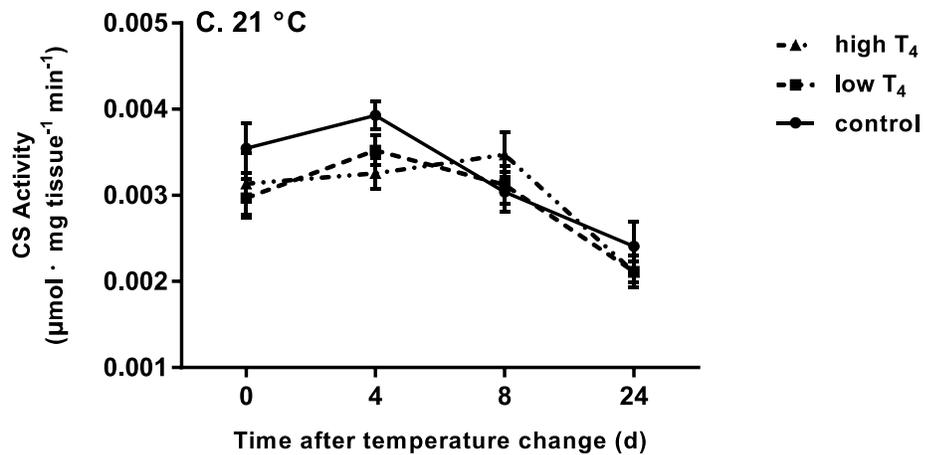
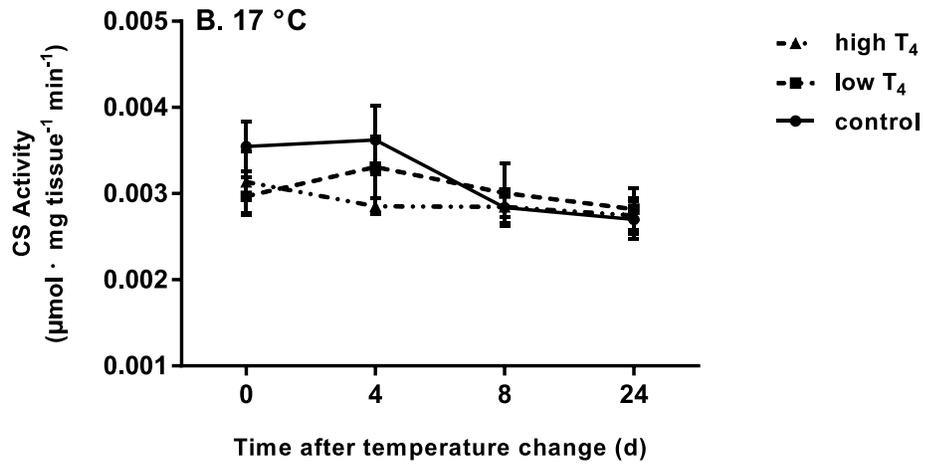
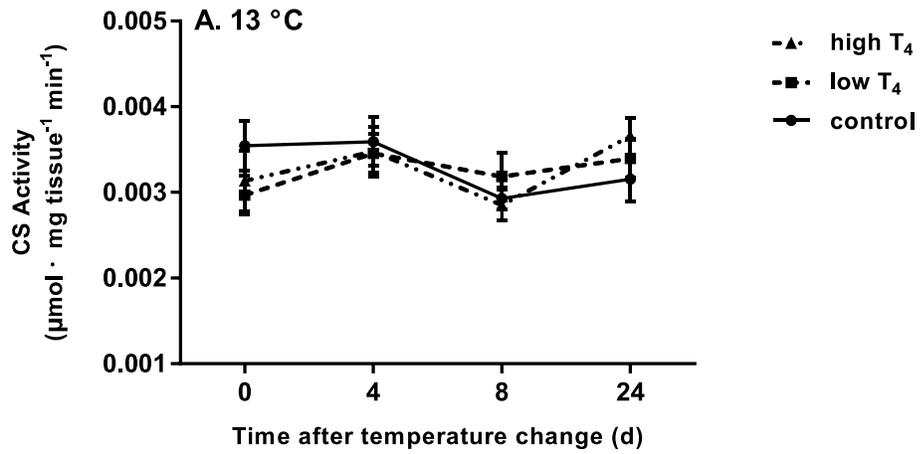


Figure 5. CS activity ($\mu\text{mol} \cdot \text{mg tissue}^{-1} \text{ min}^{-1}$) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A), 17 (B) or 21 °C (C). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low T₄ (1 $\mu\text{g T}_4$ g body weight⁻¹) or high T₄ (10 $\mu\text{g T}_4$ g body weight⁻¹). Enzyme activities were assayed at 25 °C from crude tissue homogenates using a temperature-controlled spectrophotometer. Data were analyzed using a two-way ANOVA, followed by Tukey post-hoc multiple comparisons. Significant pair-wise differences ($P < 0.05$) between sham fish and hormone treatments within a time point are indicated by L (low T₄) or H (high T₄) above the treatment group. Plots represent mean activity of 7-10 fish \pm SEM.

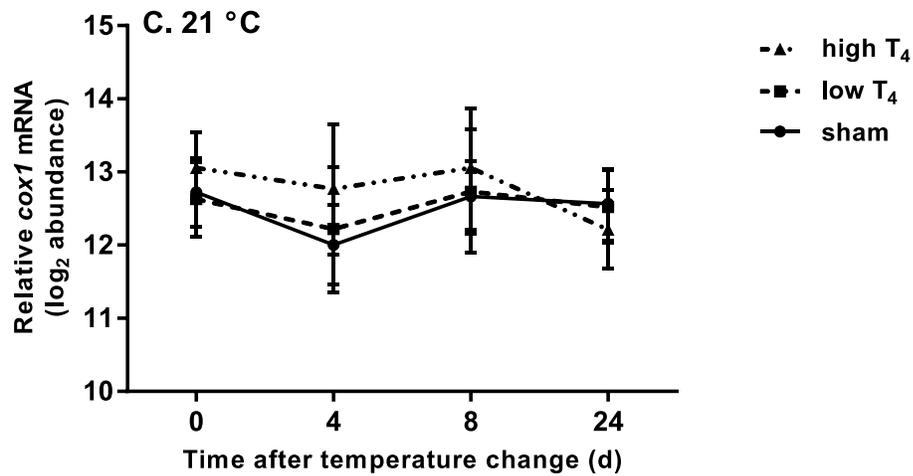
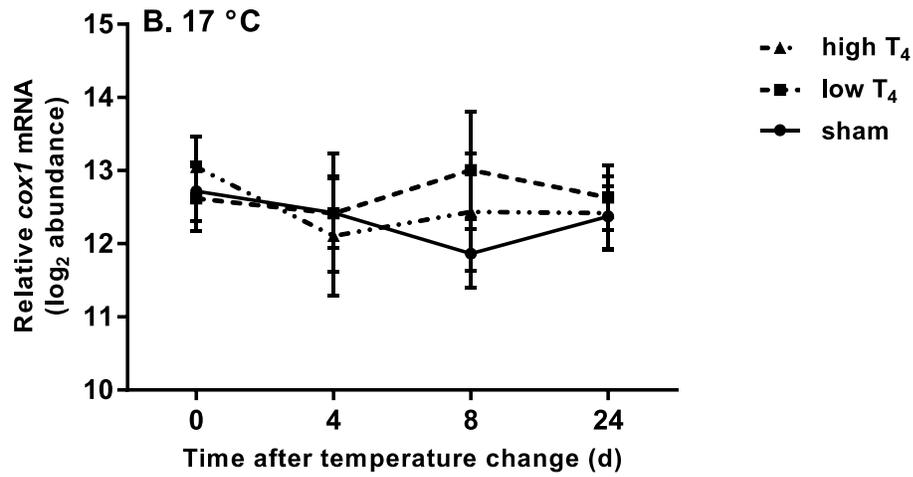
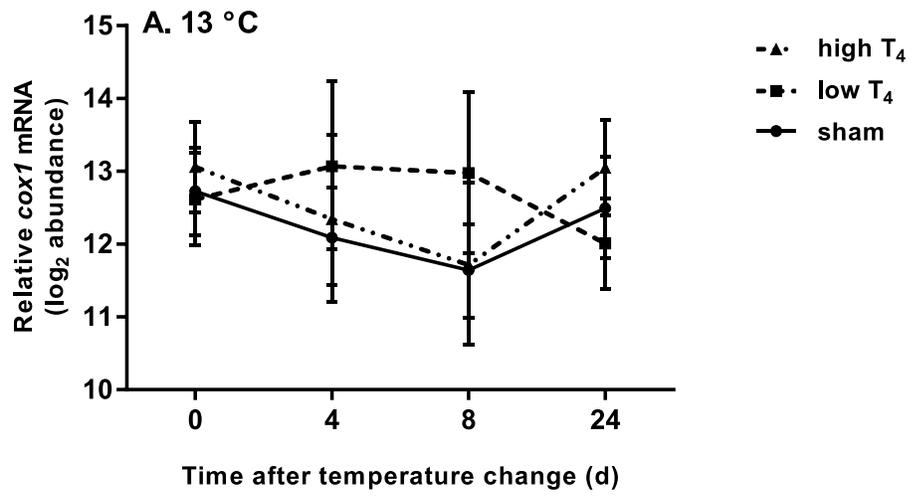


Figure 6. Abundance of *coxI* mRNA transcripts (\log_2 abundance arbitrary units) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A), 17 (B) or 21 °C (C). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low T₄ (1 $\mu\text{g T}_4 \text{ g body weight}^{-1}$) or high T₄ (10 $\mu\text{g T}_4 \text{ g body weight}^{-1}$). Data was modeled within each temperature group using a Bayesian MCMC sampling scheme and the MCMC.qPCR package in R (Matz et al., 2013). Hormone treatment, time and treatment \times time were used as fixed factors. Plots represent posterior mean estimates from 7-10 fish \pm 95 % credible intervals. Letters above treatment groups indicate the hormone treatment at which 95 % credible intervals differed from sham-injected fish within each time point (L = low T₄, H = high T₄).

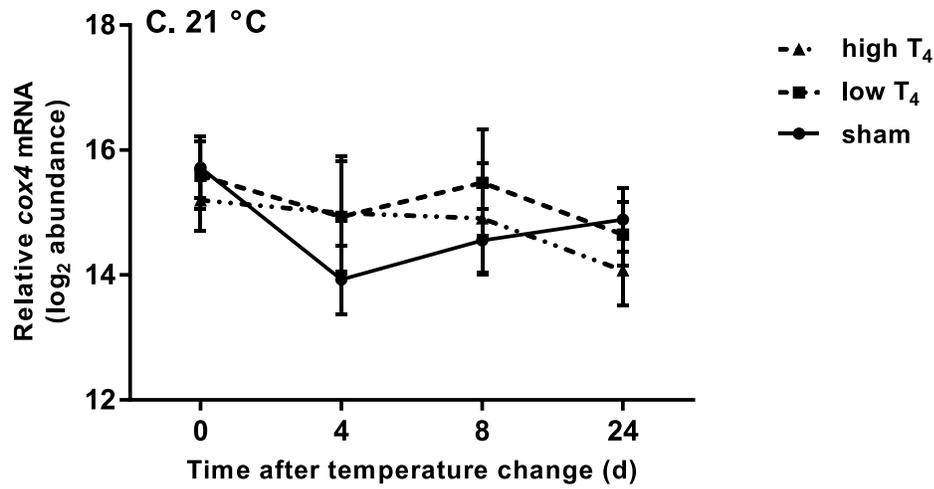
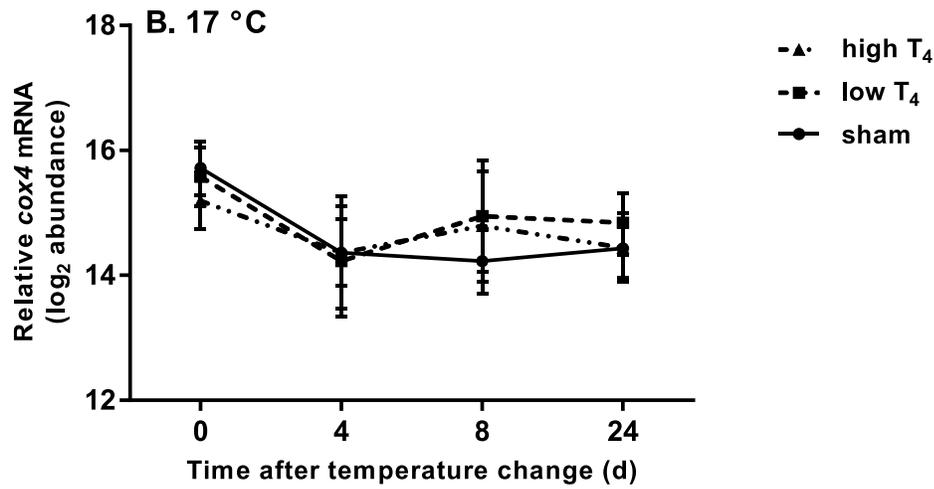
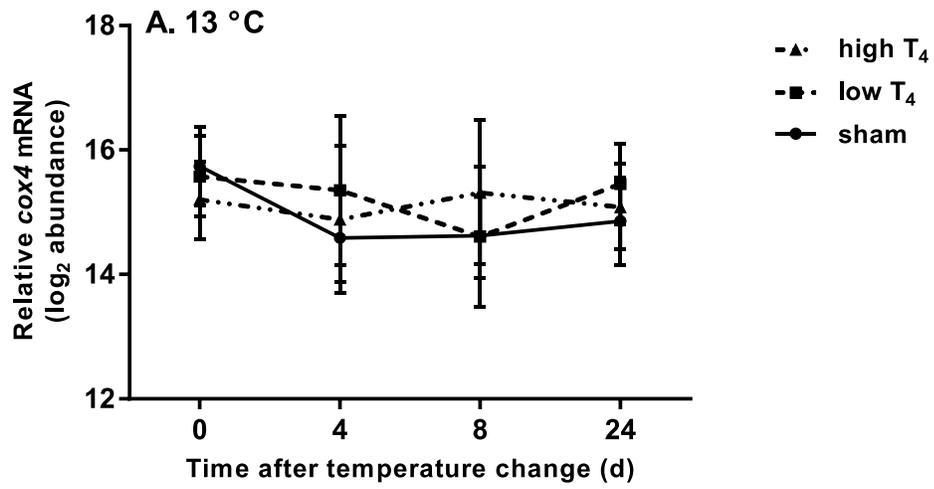


Figure 7. Abundance of *cox4* mRNA transcripts (\log_2 abundance arbitrary units) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A), 17 (B) or 21 °C (C). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low T₄ (1 $\mu\text{g T}_4$ g body weight⁻¹) or high T₄ (10 $\mu\text{g T}_4$ g body weight⁻¹). Data was modeled within each temperature group using a Bayesian MCMC sampling scheme and the MCMC.qPCR package in R (Matz et al., 2013). Hormone treatment, time and treatment \times time were used as fixed factors. Plots represent posterior mean estimates from 7-10 fish \pm 95 % credible intervals. Letters above treatment groups indicate the hormone treatment at which 95 % credible intervals differed from sham-injected fish within each time point (L = low T₄, H = high T₄).

Table 7. Two-way ANOVA statistics (F) on CS and COX activity in control and T₄-treated lake whitefish exposed to 13, 17 or 21 °C

	Treatment			Time			Treatment × Time		
	F	df_n, df_d	P	F	df_n, df_d	P	F	df_n, df_d	P
CS									
13 °C	0.044	2, 98	0.957	2.353	3, 98	0.077	0.923	6, 98	0.482
17 °C	0.984	2, 87	0.378	2.252	3, 87	0.088	0.702	6, 87	0.649
21 °C	1.751	2, 103	0.179	18.04	3, 103	< 0.001*	1.088	6, 103	0.375
COX									
13 °C	1.981	2, 99	0.143	2.988	3, 99	0.035*	1.584	6, 99	0.160
17 °C	1.912	2, 87	0.154	1.381	3, 87	0.254	1.394	6, 87	0.226
21 °C	2.905	2, 104	0.059	11.01	3, 104	< 0.001*	1.681	6, 104	0.133

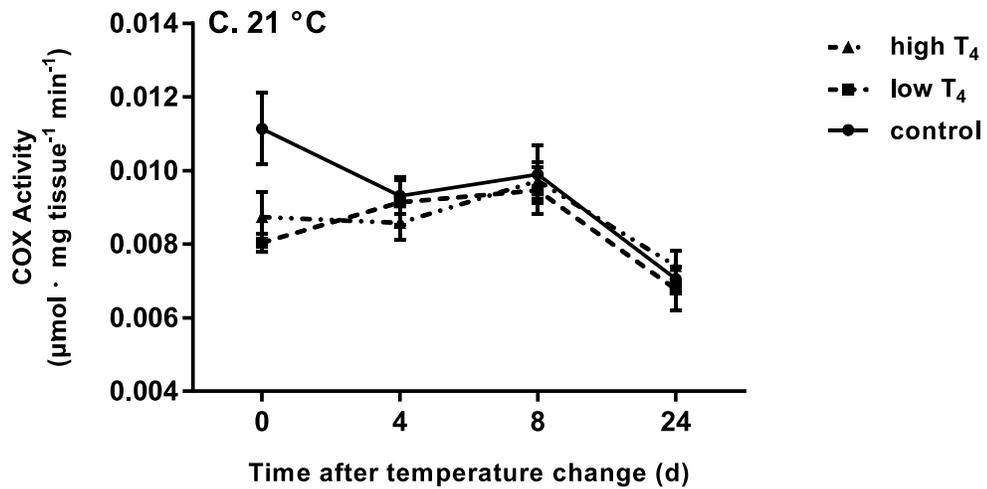
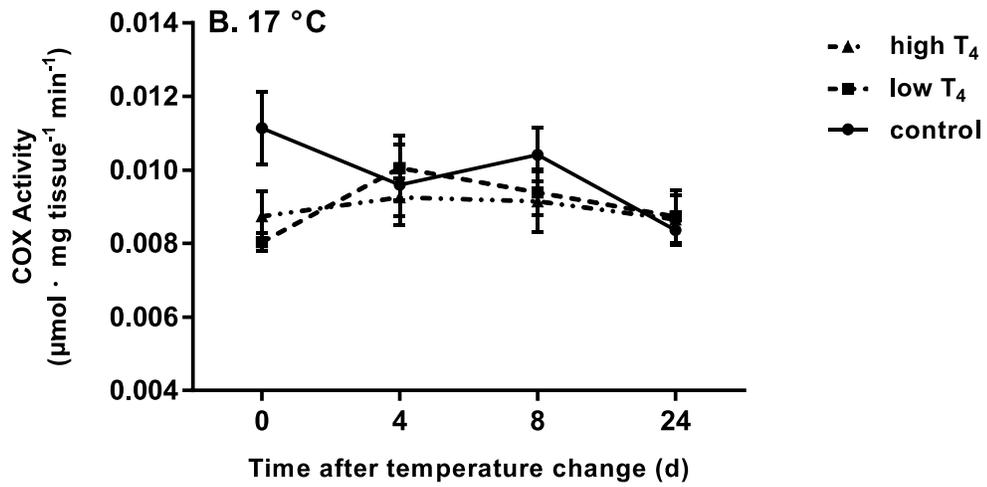
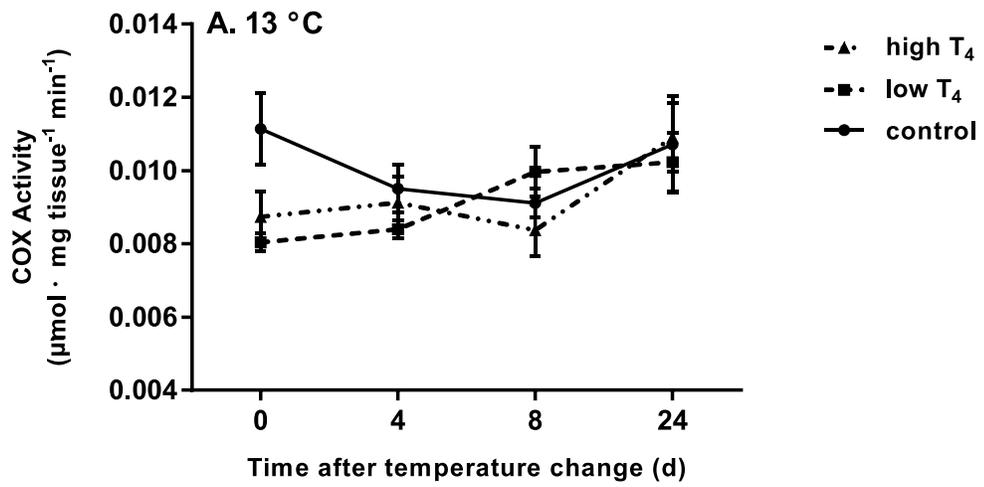


Figure 8. COX activity ($\mu\text{mol} \cdot \text{mg tissue}^{-1} \text{ min}^{-1}$) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A), 17 (B) or 21 °C (C). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low T₄ (1 $\mu\text{g T}_4 \text{ g body weight}^{-1}$) or high T₄ (10 $\mu\text{g T}_4 \text{ g body weight}^{-1}$). Enzyme activities were assayed at 25 °C from crude tissue homogenates using a temperature-controlled spectrophotometer. Data were analyzed using a two-way ANOVA, followed by Tukey post-hoc multiple comparisons. Significant pair-wise differences ($P < 0.05$) between sham fish and hormone treatments within a time point are indicated by L (low T₄) or H (high T₄) above the treatment group. Plots represent mean activity of 7-10 fish \pm SEM.

2.4. Discussion

2.4.1. Effects of elevated temperature on mRNA abundance and activity of oxidative enzymes

Metabolic remodeling in response to temperature change supports biochemical changes in enzyme and cellular function as well as changing metabolic demands. Due to its influence on metabolic function, long-term thermal acclimation has been extensively studied in fish and other ectotherms (Battersby and Moyes, 1998; Blier and Guderley, 1988; Duggan et al., 2011; Little et al., 2013; Lucassen et al., 2006; Sidell, 1980). However, the majority of these studies have focused on the physiological adjustments initiated by cold acclimation (Dean, 1989; Guderley, 2004; Johnston and Dunn, 1987, Hardewig et al., 1999; Lucassen et al., 2003; Lucassen, et al., 2006), with less emphasis placed on the long-term impacts of elevated heat stress on fish metabolism. From an environmental perspective, heat stress is an important physiological stressor, particularly for cool-water fishes that are already well-adapted to colder temperatures. Furthermore, increasing mean global water temperatures, such as those predicted by certain climate change models (IPCC, 2014), make the understanding of responses to elevated temperatures in fish species a priority. Here, I examine a time course of metabolic remodeling following exposure to elevated temperatures. I show that both mRNA abundance and activity of oxidative enzymes are influenced by elevated temperature exposure in the cool-water adapted species, lake whitefish, but at different points in the acclimation process.

Collectively, my data suggest that juvenile lake whitefish are capable of metabolic remodeling in response to elevated temperatures and that these effects are triggered at relatively low temperatures. Thermal preferences of lake whitefish decline towards adulthood (Holmes et al., 2002), but are typically between 12.7 and 16.8 °C for juveniles (Holmes et al., 2002, Wismer and Christie, 1987). Therefore, the temperatures chosen for this study represent the lower (13 °C), upper (17 °C) and extension (21 °C) of the predicted optimal physiological range for juveniles. However, they are still below the lethal limits of this species, which vary between 24 to 26 °C in laboratory-acclimated juveniles (Zak et al., unpublished, Table 1A) and have been recorded at 26.7 °C for certain wild lake whitefish populations (Edsall and Rottiers, 1976). While only minor (non-significant) metabolic adjustments were observed in response to 17 °C, exposure to 21 °C instigated clear, long-term effects of elevated temperature on CS and COX activities, suggesting the need for physiological compensation as acclimation temperature reaches 21 °C. Typically, cellular stress responses such as the heat shock response (HSR) are not observed until temperatures exceed 5 °C above the preferred temperature range of a species (Feder and Hoffmann 1999; Lindquist, 1986; Parsell and Lindquist, 1993) including in lake whitefish (Zak et al. unpublished). However, this temperature threshold does not necessarily apply to thermal acclimation responses as the tight relationship between temperature and catalytic rates promotes metabolic restructuring, even in the absence of a distinct stress response (Hofmann et al., 2010; Windisch et al 2011). Therefore, even at only 4 °C outside the preferred temperature range of lake whitefish (Holmes et al., 2002; Wismer and Christie, 1987) with enzymes that have relatively low Q_{10} values (Blier and Guderley, 1988), I observed significant metabolic restructuring to

elevated temperatures for both CS and COX. The presence of remodeling in response to temperature treatments used in the present study indicate that the temperatures used do indeed place some stress on lake whitefish physiology that require metabolic adjustments to maintain homeostasis.

Overall, the depression in CS and COX activities observed on *day 24* following exposure to 21 °C is in accordance with metabolic responses predicted by thermal enzymatic reaction rates, whereby, as catalytic rates increase with temperature, less enzyme is required to achieve an equivalent physiological rate of activity. The most likely interpretation of this result is that exposure to 21 °C does not significantly change overall metabolic demands in liver, allowing enzyme abundance to naturally fall as temperatures rise. However, this relationship with temperature is not always the case in teleosts. Windisch et al. (2011) observed rapid increases in liver COX activity following exposure to elevated temperatures in Antarctic eelpout (*Pachycara brachycephalum*), which stabilized at 90 % above controls within 7 days of temperature change. Similarly, warm acclimation has been shown to increase liver COX activity in goldfish (*Carassius auratus* L; Le Moine, 2008) as well as both CS and COX activity in juvenile lake whitefish red muscle (Zak et al., 2017). Combined, these results demonstrate the capacity for thermal plasticity of metabolic enzymes in fish, as well as the potential for differential responses related to extent of energy demand elicited by changes in temperature.

Both *cs* mRNA abundance and CS activity were reduced by exposure to 21 °C, but differed in the timing of depression. Whereas effects of 21 °C exposure on *cs* mRNA appeared within the first few days of temperature change and were only transiently

observed, CS activity did not decline until *day 24* post temperature change (Figs 1A and 2A) and are likely associated with a long-term establishment of a new CS activity steady state. A certain degree of lag between changes in expression and activity rates can be expected since transcriptional processes occur upstream of translation and functional enzyme production. However, given the degree of temporal difference in the effects of temperature between the observed effects on *cs* mRNA and CS activity in the present study (≈ 20 days), it is unclear if these two observations are physiologically linked. Rather, it is most likely that temperature causes an initial, short-term inhibition of *cs* transcription rates, leading to a decrease in *cs* mRNA, without significant impacts on overall CS abundance and activity. Previous studies in billfish (*Xiphias gladius* and *Tetrapturus audax*) and tuna (*Thunnus obesus* and *Thunnus albacares*) have suggested that the overall regulation of CS activity occurs at the translational level (Dalziel et al., 2005). This notion supported in other fish species which demonstrate a disparity between *cs* mRNA and long-term CS activity in response to temperature such as goldfish (*Carrasius auratus*; Le Moine et al., 2008) and the eelpout species, *Pachycara brachycephalum* and *Zoarces viviparus* (Lucassen et al., 2003; Windisch et al., 2011). Although similar translational control of CS activity may also be present in lake whitefish, this is likely not a universal mechanism among teleosts as tight correlations between *cs* mRNA and CS activity have been observed in cod (*Gadus morhua* L.; Lucassen et al., 2006), suggestive of transcriptional control of this enzyme.

Similar to the effects on CS, I observed inconsistent changes in mRNA abundance of COX subunits over time in relation to overall COX activity. COX is a very complex enzyme with both nuclear- and mitochondrial-encoded subunits. As such, assembly of the

functional COX enzyme requires transcriptional coordination between these two compartments. In mammals, the relationship between temperature and COX subunit transcript abundance and activity is predictable and highly-coordinated, with lower temperature leading to higher levels of COX subunit transcripts and a corresponding fold-increase in COX activity (Duggan et al., 2011). However, the present and other studies (Duggan et al., 2011, Windisch et al., 2011) suggest that this relationship is weak in fish. For instance, Duggan et al. (2011) noted that, despite increases in white muscle COX activity following cold acclimation in goldfish (*Carrasius auratus*), transcript abundance of *cox6* and *cox7* subunits were unchanged. Similar disparities between COX activity and mRNA subunit abundance have been observed in both zebrafish (*Danio rerio*) and northern redbelly dace (*Chrosomus eos*; Duggan et al., 2011) as well as Antarctic eelpout (*Pachycara brachycephalum*; Windisch et al., 2011).

The functional COX enzyme is composed of 13 distinct subunits, suggesting a high degree of coordination and complexity in the enzyme assembly process (Duggan et al., 2011; Nijtmans et al., 1998). This process is likely dependent on subunit availability, and could involve the presence of a rate-limiting subunit controlling overall enzyme assembly rate (Duggan et al., 2011; Nijtmans et al., 1998). Previous studies in both mammals (Nijtmans et al., 1998) and fish (Duggan et al., 2011) have identified *cox4* is a strong candidate as a rate-limiting subunit due to its early incorporation in the enzyme assembly process and its correlation with COX activity. However, the results of the current study show no evidence of this relationship on the mRNA level in juvenile lake whitefish. No change in *cox4* mRNA was observed at any time point following temperature change, despite the significant reduction in COX activity by *day 24*.

However, quantification of *cox4* mRNA abundance alone cannot rule out changes in steady state *cox4* subunit protein levels or the possibility of alternative subunits taking on the rate-limiting role in different fish species.

2.4.2. Effects of combined thyroid hormone manipulation and elevated temperature on mRNA abundance and activity of oxidative enzymes

Coconut oil implants are a popular and effective method for long-term administration of steroid (Holloway and Leatherland, 1996, Vijayan and Leatherland, 1989; Vijayan et al., 1994) and peptide hormones (Farbridge and Leatherland, 1988; Raine et al., 2010; Scott et al., 1999; Zak et al., 2017). While this method is well-established in the field of endocrinology, it can sometimes result in high or unpredictable hormone concentrations and difficulties in discerning physiological from supraphysiological (pharmacological) effects (Raine et al., 2010, Zak et al., 2017). In the present study, I administered low- ($1 \mu\text{g T}_4 \times \text{g body weight}^{-1}$) and high- T_4 ($10 \mu\text{g T}_4 \times \text{g body weight}^{-1}$) implants, both of which increased mean serum T_4 levels relative to sham-injected controls, but no more than 2.3-fold higher than observed physiological ranges for salmonids (Dickhoff et al., 1978). Therefore, the responses observed in this study are likely reflective of physiological, rather than supraphysiological, responses. However, differences in mean serum T_4 levels were observed among temperature groups, particularly in low- T_4 treated fish (Figure 3A). While coconut oil implant consistency (fluidity) at the various incubation temperatures may contribute to differential hormone absorption rates and thus levels among temperature groups, these effects could also be related to temperature-sensitive changes in T_4 release and/or uptake mechanisms.

Alternatively, T₄ conversion to other bioactive THs (i.e. T₃, T₂) via cellular deiodinases could be higher in fish exposed to 13 °C than those at elevated temperatures, thereby reducing circulating T₄ levels. This latter point is less likely given deiodinase activity in teleosts tends to decline as temperature decreases (Cyr et al., 1998, Eales and Fletcher, 1982).

Several instances of temperature and/or seasonal differences in serum T₄ levels have been reported in teleosts (Comeau et al., 2000; Eales and Fletcher, 1982; Johnston and Eales, 1995; Larsen et al., 2001). Plasma T₄ levels in rainbow trout (*Oncorhynchus mykiss*; Eales and Fletcher, 1982; Johnston and Eales, 1995), Atlantic cod (*Gadus morhua*; Comeau et al., 2000) and coho salmon (*Oncorhynchus kisutch*; Larsen et al., 2001) all increase with temperature. However, exceptions have also been observed in the literature. For instance, both T₃ and T₄ levels were elevated in cold-acclimated sea bream (*Sparus sarba*; O'Brien, 2010). While these results likely reflect differences in T₄ secretion rates from the thyroid follicles under different thermal conditions (Eales and Fletcher, 1982), they suggest a distinct relationship between temperature and overall TH physiology, at least among certain fish species.

While deiodination is unlikely to explain differences in serum T₄ concentrations among temperature groups in low-T₄ treated fish, it may contribute to the presence or absence of changes in oxidative enzyme mRNA abundance and activity following TH manipulation. Classical views of TH physiology propose T₃ as the most biologically active TH with T₄ primarily functioning as an inert prohormone (Hulbert, 2000). While recent evidence is challenging this view (Corderio et al., 2013), effects on oxidative

metabolism are still thought to be directed primarily through T₃- or T₂-mediated transcriptional pathways (Hulbert, 2000; Little et al., 2013, Peter and Oomenn, 1993) and, thus, are dependent on upstream deiodination processes. This, combined with increased deiodination activity at elevated temperatures (Cyr et al., 1998, Eales and Fletcher, 1982), suggests TH conversion may be a key player in TH action on oxidative metabolism following exposure to elevated temperatures. Unlike mammals, where T₄/T₃ ratios are typically above 20 and can even reach 50 (Hadley, 2000), T₄/T₃ ratios in fish and other ectotherms are typically below 5 (Burke and Leatherland, 1983; Eales and Fletcher, 1982; Zak et al., 2017). Although this ratio is known to fluctuate seasonally in fish (Burke and Leatherland, 1983, Eales and Fletcher, 1982; Levin and Bolotovskiy, 2015), likely due to a combination of both temperature and photoperiod (Burke and Leatherland, 1983, Eales and Fletcher, 1982), it remains very low. As such, T₃ levels are comparatively high in fish relative to mammals, and appear to have a more dynamic nature, corresponding with changes in environmental conditions. While this likely coincides with physiological adjustments initiated during reproductive cycles or other major life history events, such as migration, the full extent of the physiological implications of these changes have yet to be determined.

Previous studies in juvenile lake whitefish have demonstrated modest increases in liver CS activity with exogenous T₄ treatment, but only in fish exposed to elevated temperatures (Zak et al., 2017). The absence of TH-dependent effects on CS activity at colder temperatures suggests a distinct link between temperature and TH physiology in this species, and similar temperature-dependence of TH action has also been observed in other fish species (Gupta and Thapliyal, 1991). However, no effects of either low- or

high-T₄ treatment were observed on CS or COX activity in the present study, despite exposure to a higher acclimation temperature (Figures 5 and 8). Serum T₄ concentrations induced by Zak et al. (2017) were supraphysiological and were elevated approximately 165- to 175-fold over sham injected controls; in contrast, those in the present study were reflective of physiological levels observed in salmonids (Dickhoff et al., 1978). Therefore, the presence and absence of changes in CS activity between these two studies likely reflects responses induced by physiological versus supraphysiological serum T₄ levels. Despite the absence of changes in CS activity in response to physiologically-relevant T₄ concentrations, *cs* mRNA abundance was responsive to exogenous T₄ treatment at elevated temperatures. Low-T₄ treatment increased *cs* mRNA abundance in 17 °C fish at *day 8* and high-T₄ treatment elevated *cs* mRNA in 21 °C fish at *day 4*, relative to sham-injected controls (Figure 4B and C), demonstrating a certain level of cellular sensitivity to physiologically-relevant T₄ levels. Particularly in fish exposed to 21 °C, these results suggest either the stabilization of *cs* mRNA transcripts in the cell following thermal challenge or active suppression of typical metabolic responses to temperature through the promotion of *cs* transcription. The transient nature of these changes as well as the lack of enzyme-level effects suggest the observed elevation in *cs* mRNA abundance at *day 4* is unlikely to have significant long-term physiological impacts at either level of biological organization.

The presence of short-term effects of exogenous TH exposure on *cs* mRNA but neither of the two *cox* subunits demonstrates specificity of TH action on metabolism in juvenile lake whitefish. Currently, little information exists on the mechanisms controlling temperature-dependent sensitivities to TH in fish and other ectotherms (Gupta and

Thapliyal, 1991). Furthermore, the complexity of both metabolism and TH physiology facilitates numerous avenues for effects of temperature on TH action, ranging from the expression of TH transporters, TRs and accessory transcription factors to the metabolic state of tissues and overall energy demand (Gupta and Thapliyal, 1991) or a combination of these factors. Modifications in certain elements of these pathways have been observed in various teleost species. For instance, PGC-1 α , which is thought to be responsible for coordinating several aspects of oxidative metabolism and mitochondrial biogenesis in mammals (Bremer et al., 2016; Coffi et al., 2013; Weitzel and Iwen, 2011) has incurred multiple mutations leading to amino acid substitutions in the putative NRF1-binding domain in goldfish (LeMoine et al., 2010). High incidences of mutations have been observed in one of its target genes, PPAR γ in several different fish species (Batista-Pinto et al., 2005). Therefore, it is unclear whether the integrity of PGC-1 α -mediated cellular pathways influencing oxidative metabolism have been conserved in fish. Certain studies have disputed the involvement of this pathway in zebrafish muscle (Bremer et al., 2015; Bremer and Moyes, 2011; Gao and Moyes, 2016), but have possibly identified an alternative coordinator, PGC-1 β (Bremer et al., 2012). Other studies in the same species have lent support to the conservation of PGC-1 α -mediated pathways (Little et al., 2013). Nevertheless, the suite of coregulatory proteins and transcriptions either directly or indirectly interacting with TR have the potential to elicit a diverse range of effects on target genes and could possibly lead to the differential effects observed on the abundance of oxidative transcripts following the treatment with exogenous T₄.

3. CHAPTER TWO: EFFECTS OF TEMPERATURE AND THYROID HORMONE ON LIPID METABOLISM IN JUVENILE LAKE WHITEFISH

3.1. Introduction

Variable temperatures place demands on physiological processes. For fish and other ectotherms, body temperature typically reflects that of ambient conditions. Therefore, environmental temperature fluctuations can have a profound impact on temperature-sensitive physiological processes, such as metabolism. Despite the tight relationship between environmental and body temperatures observed in ectothermic species, thermal sensitivities of enzyme catalytic rates remain comparable to those observed in mammals (Hochachka and Somero, 2002; Reyes et al., 2008). As a result, fish and other ectotherms must adjust physiological processes to cope with temperature stress experienced in their environment.

Metabolic remodeling following long-term exposure to high or low temperatures has been observed in several teleost species (Johnston and Dunn, 1987), but predominantly in those that inhabit temperate regions (Battersby and Moyes, 1998; Dhillon and Schulte, 2011; Johnston and Dunn, 1987). These studies typically demonstrate an increase in mitochondrial density and/or function following cold acclimation. For instance, cold acclimation from 18 to 4 °C in rainbow trout (*Oncorhynchus mykiss*) has been shown to increase red and white muscle citrate synthase (CS) activity by 40 and 70 %, respectively (Battersby and Moyes, 1998). Similarly, white muscle CS activity in northern, temperate populations of Atlantic killifish (*Fundulus heteroclitus*) has also been shown to increase nearly 70 % following acclimation from 25

to 5 °C (Dhillon and Schulte, 2011). The majority of studies examining phenotypic plasticity under chronic thermal stress conditions have focused on mitochondrial-based (oxidative) markers (Battersby and Moyes, 1998; Blier and Guderley, 1988; Bremer et al., 2012; Bremer and Moyes, 2011; Dhillon and Schulte, 2011; Duggan et al., 2011; Guderley, 2004; Hardewig et al., 1999; Little et al., 2013; McClelland et al., 2006; O'Brien, 2011; Windisch et al., 2011), due to interest in the effects of temperature on electron transport chain (ETC) capacity and ATP regeneration. However, secondary pathways of intermediary metabolism, such as carbohydrate and lipid pathways, play a pivotal role in overall energy consumption by supplying substrates for subsequent oxidative processes. Furthermore, studies in fish have demonstrated significant metabolic shifts between the priority of lipid and carbohydrate pathways following temperature change (Johnston and Dunn, 1987; Moon et al., 1985; Tocher et al., 2004; Windisch et al., 2011), suggesting temperature exerts significant control over intermediary metabolic pathways in addition to the oxidative metabolism core.

The high energy/mass ratio of lipid molecules makes them an ideal fuel source during energy-intensive events such as development, somatic growth, migration and reproduction (Sheridan, 1994; Tarr, 1972). The balance between fat accumulation and depletion is coordinated through fatty acid synthesis (lipogenesis) and β -oxidation (lipolysis), which occur through independent metabolic pathways (Houten and Wanders, 2010; Leaver et al., 2008; Poirier et al., 2006). β -oxidation occurs in both the mitochondria and peroxisomes, facilitating the breakdown of a wide array of fatty acids (Houten and Wanders, 2010). Mitochondrial and peroxisomal pathways are rate-limited by the enzymes carnitine palmitoyltransferase (CPT1) and acyl-coA oxidase (ACOX),

respectively (Leaver et al., 2008). Lipogenesis is limited by the production of malonyl-coA, which is catalyzed by acetyl-coA carboxylase (ACC; Brownsey et al., 2006; Leaver et al., 2008). Although regulation of lipid metabolism occurs to some extent through allosteric regulation (Brownsey et al., 2006) and sensing of lipid stores (Mullur et al., 2014), there is also evidence that lipid pathways are under both temperature (Bermejo-Nogales et al., 2014; Rodnick and Sidell, 1994) and endocrine (Polakof et al., 2010; Vijayan and Leatherland, 1989) influence in fish, including via thyroid hormones (Chen et al., 2015; Kao et al., 1999).

The metabolic effects of thyroid hormones (THs) are well established in mammals (Cioffi et al., 2013; Hulbert, 2000; Weitzel and Iwen, 2011) and becoming increasingly recognized in fish (Gupta and Thapliyal, 1991; Kao et al., 1999; Little et al., 2013; Peter and Oommen, 1993). However, these responses appear complex, particularly with respect to lipid pathways. Increases in blood triglycerides, indicative of lipid mobilization and oxidation, have been observed following TH treatment in gilthead seabream (*Sparus aurata* L.; Vargas-Chacoff, 2016) and the common carp (*Cyprinus carpio*; Plisetskaya et al., 1983). In contrast, ACC activity also increases following TH treatment in certain fish species (Kao et al., 1999), refuting exclusive stimulation of catabolic pathways via THs. Unlike the effects of THs on oxidative metabolism, which are thought to be mediated primarily through 3',5,3-triiodothyronine (T₃) and nuclear-based TH receptors (TR; Cioffi et al., 2013; Hulbert, 2000; Weitzel and Iwen, 2011), both thyroxine (T₄) and 5,3-diiodothyronine (T₂) have been implicated in metabolic remodeling in fish (Varghese and Oommen, 2000), potentially contributing to the complexity of effects on lipid metabolism. Furthermore, many of the effects of TH on

metabolism are temperature-dependent (Gupta and Thapliyal, 1991), suggesting the potential for unique physiological associations between temperature and TH physiology in fish that routinely experience thermal variability in their environments.

Lake whitefish (*Coregonus clupeaformis*) is a member of the salmonid family and is prevalent in cool, freshwater systems across North America (Bernachez and Dodson, 1991; Holmes et al., 2002). Juvenile lake whitefish are exposed to a wide array of temperatures over an annual cycle due to their geographic range, which spans across temperate regions. However, they still maintain a relatively cool and narrow optimal thermal range, which lies between 12.7 and 16.8 °C (Holmes et al., 2002; Wismer and Christie, 1987). Some lake whitefish populations inhabit large bodies of water such as the Laurentian Great Lakes (Bernachez and Dodson, 1991; Holmes et al., 2002) among many others (Bernachez and Dodson, 1991), where they are partially protected from long-term exposure to adverse temperatures by the thermal stratification present in these deep lakes. However, certain lake whitefish populations are also known to inhabit shallow, isolated water systems where temperatures can approach the lethal limits of their temperature range (Smith, 1978; Dr. Rebecca North, 2017, personal communication). As such, they require effective mechanisms to minimize the negative impacts of chronic exposure to elevated temperatures experienced in their environment. This, combined with the fact that they are known to be more sensitive to thermal stress than other species that occupy a similar geographic range such as northern pike (*Esox lucius*) and coho salmon (*Oncorhynchus kisutch*; Wismer and Christie, 1987), makes lake whitefish an ideal candidate to evaluate the effects of chronic thermal stress and TH on metabolic plasticity from both a biochemical and ecological perspective.

Overall, the impacts of elevated temperature associated with climate change are predicted to be most substantial for stenothermal species that have adapted to thermally-stable waters (Gunderson and Stillman, 2015), largely due to limited internal mechanisms to cope with thermal fluctuations (Hofmann et al., 2000; Windisch et al., 2011). However, despite the plasticity in metabolic responses predicted to be present in temperate fishes that frequently encounter temperature fluctuations, these species may still be at risk of elevated temperature stress, particularly when it is combined with naturally occurring thermal fluctuations that can already approach lethal temperature limits. Currently, the adaptive potential for metabolic remodeling in response to chronic temperature change in juvenile lake whitefish is unknown, as are the mechanisms responsible for initiating and mediating these responses. The aims of the current study were to (1) examine alterations in lipid metabolism pathways at the transcriptional level to detect potential metabolic shifts in response to temperature exposure and (2) determine if THs may be involved in regulating these adjustments. To this end, mRNA abundance of key, rate-limiting enzymes associated with lipid biosynthesis and breakdown pathways were quantified in juvenile lake whitefish exposed to temperatures both at and above thermal optimum and in the presence or absence of exogenous T₄ treatment.

3.2. Materials and Methods

3.2.1. Animal husbandry, experimental procedures and serum thyroxine (T₄) quantification

Liver samples obtained the experiment described in Chapter 1 (Section 2.2.2.) were used for calculation of the HSI and detection of changes in transcript abundance of

enzymes associated with lipid metabolism. Therefore, animal collection, rearing and husbandry procedures as well as experimental design are as described in Sections 2.2.1. and 2.2.2. Serum T₄ levels were the same as those taken in Chapter 1 using quantification and analysis methods described in Section 2.2.3. and 2.2.6.1.

3.2.2. RNA extraction, cDNA preparation and real-time quantitative-PCR (RT-qPCR) of metabolic enzyme mRNA transcripts

Isolated RNA samples generated for the experiment described in Chapter 1 (Section 2.2.2.) were used for quantification of transcript abundance of enzymes associated with lipid metabolism. Therefore, RNA extraction procedures and quality checks are as described in Section 2.2.4. Partial lake whitefish cDNA sequences for target genes *acc α* , *acc β* , *cpt1 α* , *cpt1 β* , *acox1* and *acox3* were amplified, sub-cloned and sequenced using PCR-based sequencing methods. Degenerate primers for the amplification process were designed using BlockMaker and CODEHOP designer as previously described (Chapter 1). Predicted amino acid sequences for each cloned cDNA fragment were generated using EMBOSS Sixpack (EMBL-EBI) and compared to sequences available on the NCBI database for several teleost species to confirm clone specificity (Table 8). Gene-specific primers were designed as previously described using cloned partial cDNA sequences for *acc α* , *acc β* , *acox1*, *acox3*, *cpt1 α* and *cpt1 β* . All primers used for RT-qPCR were validated to meet Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009) prior to use on experimental samples. Appropriate cDNA and primer concentrations were determined empirically by constructing a standard curve for each target sequence using

serially diluted liver cDNA from a reference animal. Standard curves were also used to ensure amplification efficiency of each primer pair ranged between 90-110 %. mRNA transcript abundance of target and reference genes was quantified in triplicate using 2x SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Mississauga, ON, Canada) in a final reaction volume of 20 µl. All RT-qPCR quantifications were performed using a CFX Connect Real-Time detection system (BioRad) using CFX Manager 3.1 software (BioRad) under the following reaction conditions: 1 cycle of 95 °C for 2 min and 40 cycles of 95 °C for 5 s followed by a 30 s extension phase at 56, 58, 60 or 62 °C (Table 9). The absence of genomic DNA contamination was confirmed by melt curve analysis. Inter-run variation between RT-qPCR runs was monitored using an inter-run calibrator (IRC) consisting of a cDNA pool from several treatment groups.

Cross-amplification checks were performed between isoforms of the same gene to ensure that primers were targeting the gene of interest. This was done by performing RT-qPCR reactions using a serial dilution series of plasmid preps (E.N.Z.A. Plasmid DNA Kit, Omega Bio-tek, Norcross, GA) generated for each gene during the gene cloning. For each serial dilution of the plasmid prep, reactions were run as described above using primer pairs for both the target and non-target isoform to compare cDNA amplification

Table 8. Percent amino acid identity (id) and similarity (si) of predicted amino acid sequences of cloned lake whitefish lipid enzyme cDNA fragments with those of select teleost species. Accession numbers of NCBI sequences used for each comparison are provided below id/si percentages.

Target	<i>Salmo salar</i> (id/si)	<i>Oncorhynchus mykiss</i> (id/si)	<i>Esox lucius</i> (id/si)	<i>Danio rerio</i> (id/si)	<i>Oreochromis niloticus</i> (id/si)
<i>accα</i>	97.6/99.0 (XM_014138096.1)	93.3/96.7 (CDQ67881.1)	96.7/99.0 (XM_010870361.2)	95.2/98.1 (NM_001271308.1)	93.3/98.1 (XM_013274550.1)
<i>accβ</i>	97.2/100 (XM_014160782.1)	97.2/99.7 (CDQ68380.1)	95.4/99.4 (XM_010876742.1)	93.3/99.6 (XM_009301375.1)	90.7/97.2 (XM_003451659.3)
<i>acox1</i>	97.1/99.5 (XP_013986886.1)	n/a	89.8/96.6 (XM_010889413.2)	84.3/97.4 (NM_001005933.2)	84.0/97.9 (NM_001290199.1)
<i>acox3</i>	92.3/95.4 (XM_014207108.1)	n/a	83.8/94.6 (XM_010899166.1)	83.1/94.6 (NM_213147.1)	84.6/91.5 (XM_013266255.1)
<i>cpt1α</i>	n/a	97.2/98.6 (GU592679.1)	n/a	70.1/92.2 (XM_005166473.3)	n/a
<i>cpt1β</i>	96.2/98.7 (XM_014124962.1)	96.4/99.2‡ (XM_020456624.1)	90.5/97.3 (XM_010890400.2)	84.0/95.2 (NM_001328192.1)	n/a

n/a = insufficient sequence information available on NCBI database for comparison

‡ Comparison to coho salmon (*Oncorhynchus kisutch*) rather than rainbow trout (*Oncorhynchus mykiss*)

Table 9. RT-qPCR primer sequences for target lake whitefish transcripts

Target	Sequence (5'–3')	Amplicon (bp)	cDNA (μl)	[Final Primer] (nM)	Annealing Temp (°C)	Primer Efficiency (%)
<i>accα</i>	F: GTC GTT CTC GTC TAA CCT GA R: CCA TAG AGC ACA GGG TTC CC	228	0.5	250 250	58	108.0
<i>accβ</i>	F: AGT GTG GGA CAG TTT GAG AT R: TCG TAG AGG CTG GAG CAG GA	206	0.5	375 375	58	97.4
<i>acox1</i>	F: ACA AAC TAC GAG CTG CCA AG R: ATC CTC CTG ACT GAC CCT CT	83	0.5	250 250	62	103.5
<i>acox3</i>	F: GCT CTC GTC CTT CAT CAT CAA A R: GCG TCC GAT CCC ATA CCT G	109	0.5	250 250	60	102.2
<i>cpt1α</i>	F: AAG CGG CAA GAA CAA GCA GT R: AGC CTC GTA TTT CTG TTC TGA	88	0.5	250 250	56	102.3
<i>cpt1β</i>	F: GCA AGT TCT CCA GCC CTG AG R: ATA GCG CAC GAA TGT CCA GC	81	0.5	250 250	58	105.6

No cross-amplification was detected between primers and non-target isoforms (Table A2).

3.2.3. Data handling and statistical analysis

3.2.3.1. Hepatosomatic index

Hepatosomatic index (HSI) was calculated for each individual using the formula,

$$HSI = \frac{\text{liver weight}}{\text{body weight}} \times 100$$

where liver weight and body weight were in common units. Significant effects of temperature on HSI were calculated using a two-way ANOVA (GraphPad Prism 6) to detect effects of temperature over time followed by Bonferroni's multiple comparison tests to detect pair-wise differences in enzyme activity among temperature groups. Similarly, significant effects of TH treatment on HSI were determined within each temperature group using two-way ANOVA to detect effects of TH treatment over time, followed by Bonferroni's multiple comparison tests to detect pair-wise difference in enzyme activity among TH treatment groups. All results were considered significant when $P \leq 0.05$.

3.2.3.2. RT-qPCR mRNA transcript abundance

Statistical analysis of liver *acc α* , *acc β* , *cpt1 α* , *cpt1 β* , *acox1* and *acox3* mRNA abundance was completed using the MCMC.qPCR package (Matz 2013) in R Studio (R version 3.3.1; R Studio version 1.0.136; R core team, R Foundation for Statistical

Computing, Vienna, Austria). As described in Section 2.2.6.3. above, this package predicts posterior mean estimates of mRNA transcript levels within a Bayesian framework using a Poisson-lognormal generalized linear mixed model and a Markov Chain Monte Carlo (MCMC) sampling scheme (Matz et al., 2013). Random effects representing variation among technical replicates due to uneven template loading are also incorporated into the model, acting to normalize the data without the use of reference genes. Raw quantification cycle (Cq) values for target genes were exported from CFX Manager 3.1 software (BioRad) and converted into molecular counts with the `cq2counts()` function using a Cq1 value of 37. The Cq1 value represents the number of qPCR cycles required to detect a single target molecule. Previous tests in our laboratory have shown no difference in molecule count approximation or model fit using either a Cq1 value of 37, as recommended by Matz et al. (2013), or empirically determined values for single-molecule amplification (Stefanovic et al. 2016). All models were run in R with the `mcmc.qpcr()` function through 45000 iterations using a thinning value of 20 and a burn-in value of 5000. Impacts of temperature were modeled using temperature, time and temperature \times time as fixed factors. Impacts of TH manipulation were modeled separately for each temperature using hormone treatment (sham, low T₄ or high T₄), time and treatment \times time as fixed factors. Similar to statistical models run for oxidative genes, models were run without priors since use of the reference genes *efl α* and *β -actin* as priors did not alter model fit or data interpretation. Posterior mean estimates of target transcript levels are plotted as log₂ mRNA abundance and error bars represent 95 % credible intervals surrounding the posterior mean estimate. Estimates were considered significant when 95 % credible intervals did not overlap.

3.3. Results

3.3.1. Time-course of effects of elevated temperatures alone on the hepatosomatic index and mRNA abundance of enzymes associated with lipid metabolism in juvenile lake whitefish liver

A time course of temperature-induced effects on hepatic lipid metabolism was examined in juvenile lake whitefish by quantifying HSI and mRNA abundance of key enzymes involved in lipogenesis and β -oxidation at 0, 4, 8 and 24 days following exposure to 13 (control), 17 or 21 °C. The HSI, which provides an indication of hepatic energy reserves, was significantly affected over time in fish exposed to elevated temperatures (Figure 9, Table 10). Relative to control fish maintained at 13 °C, the HSI declined modestly in fish exposed to 17 or 21 °C on *day 4* and *day 8* and exhibited 1.24- and 1.39-fold increase above 13 °C controls by *day 24* in 17 and 21 °C temperature groups, respectively. However, the increase in HSI between *days 4* and *24* was only significant for fish exposed to 21 °C. Overall, these results show that elevated temperatures promote long-term increases in HSI in juvenile lake whitefish.

Regulation of fatty acid biosynthesis in response to temperature change was estimated by mRNA abundance of the rate-limiting lipogenic enzyme, *acc*. Two *acc* isoforms (i.e. α and β) were quantified since they occupy different functional roles in the regulation of lipid metabolism. While mRNA abundances of both *acc α* and *acc β* were sensitive to temperature, they were driven in opposite directions on different time scales following elevated temperature exposure. On *day 4* following the onset of 21 °C, mean

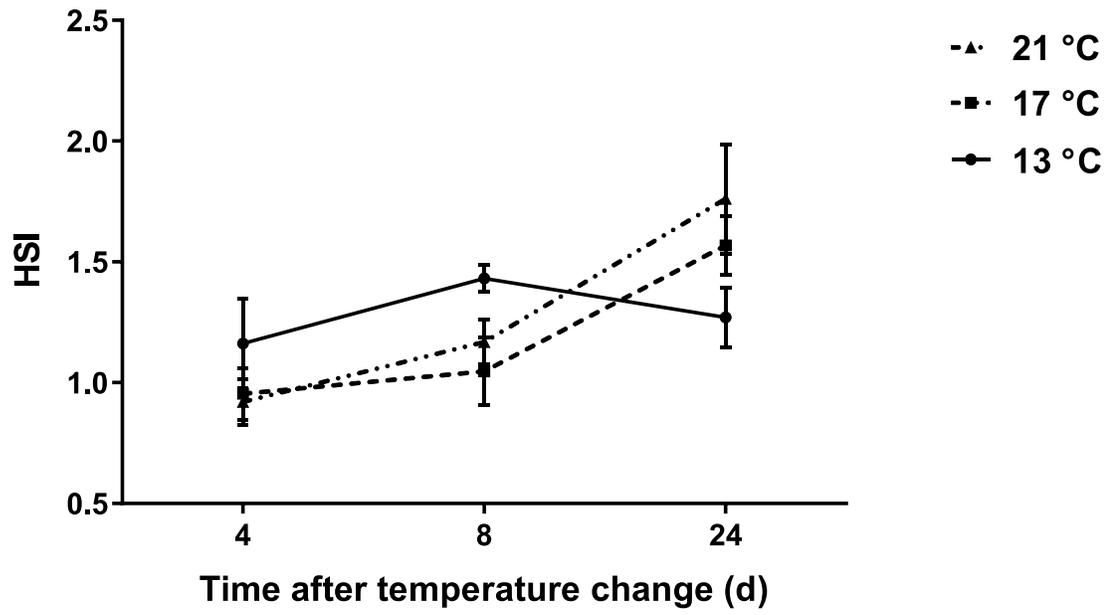


Figure 9. HSI [(liver weight • body weight⁻¹) × 100] of juvenile lake whitefish exposed to 13 (control), 17 or 21 °C for 4, 8 or 24 d. Data was analyzed using a two-way ANOVA to detect effects of temperature, time and their interaction on HSI. Plots represent mean HSI of 7-10 fish ± SEM. No significant pair-wise differences (P < 0.05) were observed within each time point.

Table 10. Two-way ANOVA statistics on HSI in lake whitefish exposed to 13, 17 or 21 °C.

	Temperature			Time			Temperature × Time		
	F	df _n , df _d	P	F	df _n , df _d	P	F	df _n , df _d	P
HSI	0.473	2, 70	0.625	10.57	2, 70	< 0.001*	2.768	4, 70	0.034*

liver *acc α* mRNA abundance was 4.55-fold lower than 13 °C controls, but recovered to control levels by *day 8* (Figure 10A, Table 11). In contrast, mean *acc β* mRNA levels were elevated 3.60-fold in fish exposed to 21 °C, but not until *day 8* post-temperature-change (Figure 10B). Furthermore, they remained elevated on *day 24*, demonstrating delayed, but long-term effects of elevated temperature on liver *acc β* mRNA abundance. Exposure to the intermediate temperature of 17 °C had no significant effects on either *acc α* or *acc β* at any time point. mRNA abundance of rate-limiting enzymes involved in mitochondrial (*cpt1 α* , *cpt1 β*) and peroxisomal (*acox1*, *acox3*) β -oxidation were also quantified to investigate potential transcriptional regulation of lipid catabolism pathways in response to elevated temperature stress. *cpt1 α* mRNA abundance fluctuated in 13 °C control fish over the course the experimental period, but was not affected by temperature at any time point (Figure 11A). Similarly, *cpt1 β* mRNA exhibited time-dependent changes in abundance, which declined by either *day 8* or *24* in all three temperature groups (Figure 11B). With respect to peroxisomal β -oxidation enzymes, *acox1* displayed constant expression in response to temperature change (Figure 11C), but the expression of *acox3* was elevated 2.07-fold following the onset of 21 °C on *day 4*, before returning to control levels by *day 8* (Figure 11D). Overall, these results show limited effects of elevated temperature on the expression and/or stability of rate-limiting enzymes in β -oxidation in both the mitochondrial and peroxisomal pathways in juvenile lake whitefish.

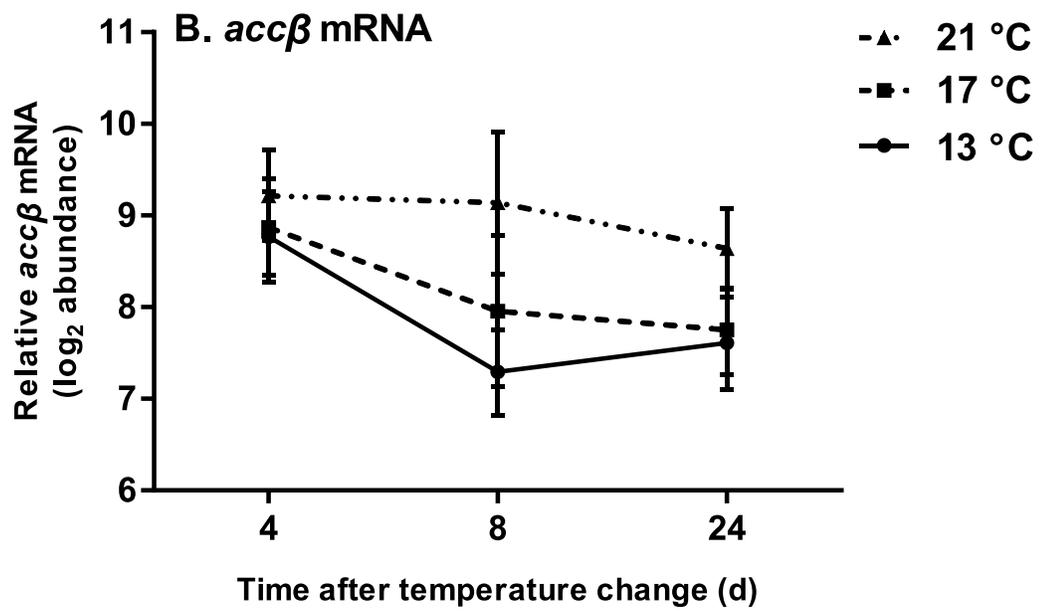
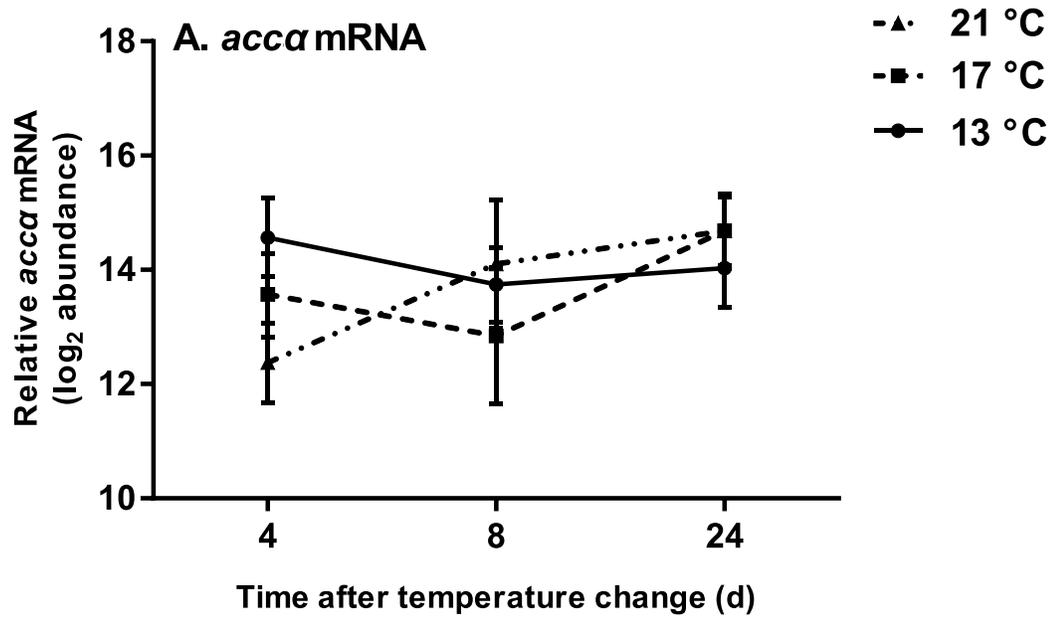


Figure 10. Effects of elevated temperature exposure on *acc α* (A) and *acc β* (B) mRNA abundance (\log_2 abundance arbitrary units) in juvenile lake whitefish. mRNA abundance of oxidative enzyme was quantified in liver 0, 4, 8 or 24 d following exposure to 13, 17 or 21 °C. Data was modeled using a Bayesian MCMC sampling scheme and the MCMC.qPCR package in R (Matz et al. 2013) with temperature, time and temperature \times time as fixed factors. Plots represent posterior mean estimates from 7-10 fish \pm 95 % credible intervals. Numbers above treatment groups indicate the temperature at which 95 % credible intervals differed from fish maintained at 13 °C within each time point.

Table 11. Summary statistics for the effects of elevated temperature exposure from 4 to 24 d on *acc α* , *acc β* , *cpt1 α* , *cpt1 β* , *acox1* and *acox3* mRNA abundance in juvenile lake whitefish. Different lowercase letters indicate non-overlapping 95 % credible intervals between time points within a temperature. Bolded values indicate significant non-overlapping 95 % credible intervals relative to 13 °C controls within a time point.

Gene		Time		
		4 d	8 d	24 d
<i>accα</i>	13 °C	13/4	13/8	13/24
	17 °C	17/4	17/8	17/24
	21 °C	21/4^a	21/8 ^{ab}	21/24 ^b
<i>accβ</i>	13 °C	13/4 ^a	13/8 ^b	13/24 ^b
	17 °C	17/4 ^a	17/8 ^{ab}	17/24 ^b
	21 °C	21/4	21/8	21/24
<i>cpt1α</i>	13 °C	13/4 ^a	13/8 ^b	13/24 ^{ab}
	17 °C	17/4	17/8	17/24
	21 °C	21/4	21/8	21/24
<i>cpt1β</i>	13 °C	13/4 ^a	13/8 ^b	13/24 ^b
	17 °C	17/4 ^a	17/8 ^{ab}	17/24 ^b
	21 °C	21/4 ^a	21/8 ^b	21/24 ^b
<i>acox1</i>	13 °C	13/4	13/8	13/24
	17 °C	17/4	17/8	17/24
	21 °C	21/4	21/8	21/24
<i>acox3</i>	13 °C	13/4 ^a	13/8 ^b	13/24 ^a
	17 °C	17/4	17/8	17/24
	21 °C	21/4 ^a	21/8 ^{ab}	21/24^b

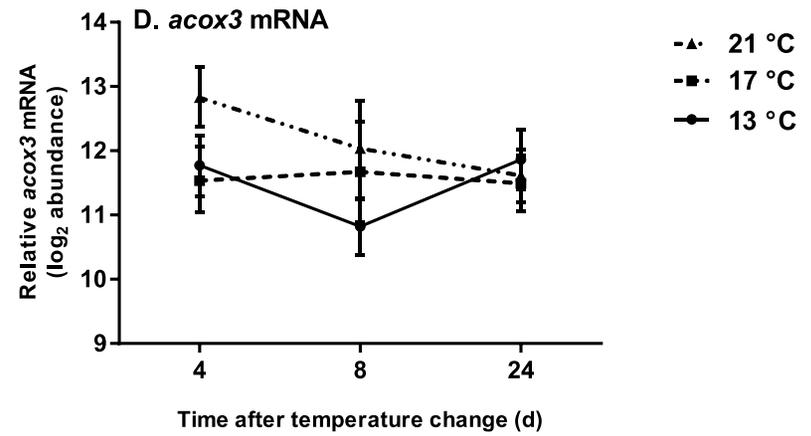
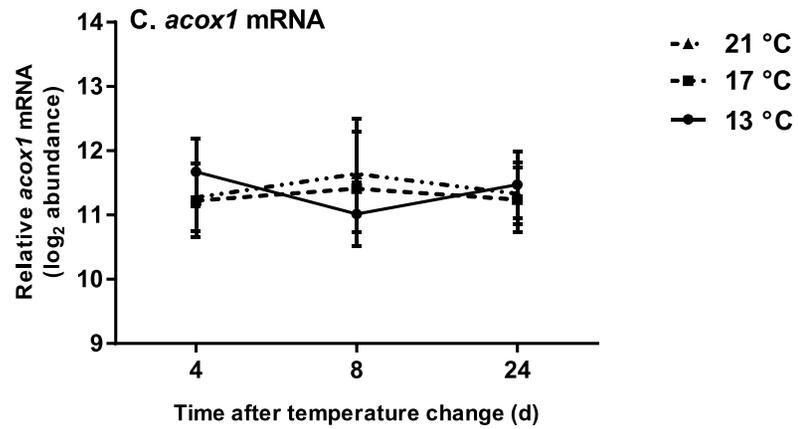
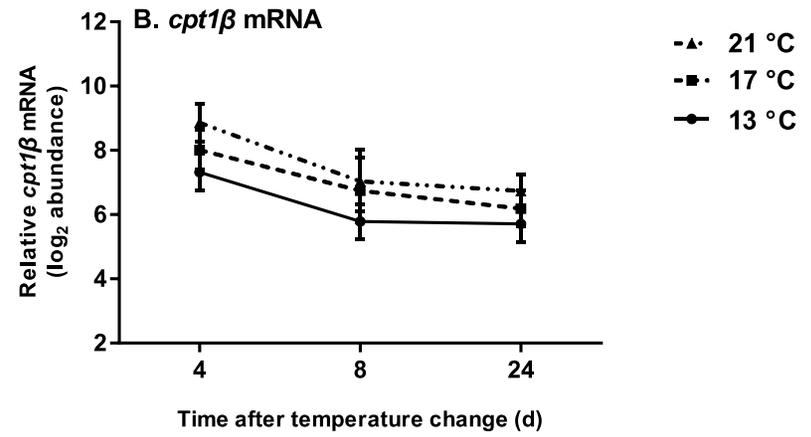
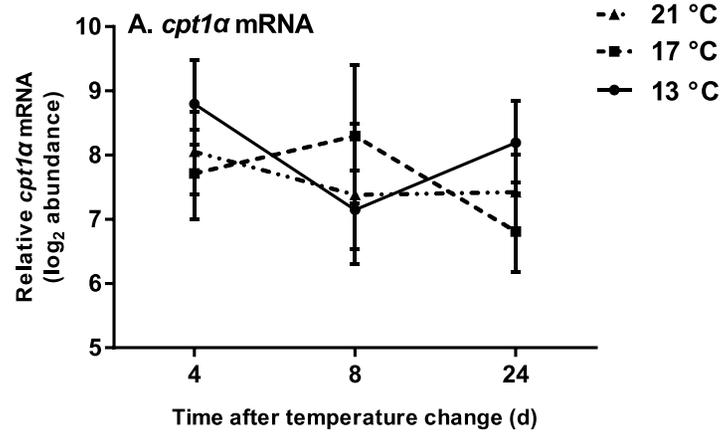


Figure 11. Effects of elevated temperature exposure on *cpt1 α* (A), *cpt1 β* (B), *acox1* (C) and *acox3* (D) mRNA abundance (log₂ abundance arbitrary units) in juvenile lake whitefish. mRNA abundance of oxidative enzyme was quantified in liver 0, 4, 8 or 24 d following exposure to 13, 17 or 21 °C. Data was modeled using a Bayesian MCMC sampling scheme and the MCMC.qPCR package in R (Matz et al. 2013) with temperature, time and temperature \times time as fixed factors. Plots represent posterior mean estimates from 7-10 fish \pm 95 % credible intervals. Numbers above treatment groups indicate the temperature at which 95 % credible intervals differed from fish maintained at 13 °C within each time point.

3.3.2. Combined effects of elevated temperature and TH manipulation on the hepatosomatic index and mRNA abundance of enzymes associated with lipid metabolism in juvenile lake whitefish liver

Circulating T₄ levels in juvenile lake whitefish were altered using coconut oil implants containing either low (1 µg T₄ × g body weight⁻¹) or high (1 µg T₄ × g body weight⁻¹) T₄ to examine the role of THs in the short- and long-term metabolic responses to elevated temperature stress. As described previously (Section 2.4.2.), T₄ implants were administered 7 days prior to the onset of temperature change and serum T₄ levels were quantified to confirm manipulation the TH status. While circulating serum T₄ levels were stable in sham-injected fish among 13, 17 and 21 °C temperature groups (Table 5), levels in low- and high-T₄ treated fish exhibited variability with respect to both temperature and time. Serum T₄ levels were significantly elevated relative to sham-injected controls at all three temperatures in high-T₄ treated fish and higher than sham levels in low-T₄ treated fish. However, elevations in T₄ levels among the low-T₄ fish were much more pronounced in 17 and 21 °C temperature groups on *day 4* and *day 8* post temperature change and absent in 13 °C groups at all time points as well as 17 and 21 °C groups on *day 24*. These altered thyroid states had modest, temperature-dependent effects on HSI. At 13 °C, the HSI in low T₄-treated fish dropped modestly on *day 4* and rose significantly from this low point by *day 24* (Figure 12A; Table 12), mimicking the effects of elevated temperature alone on HSI. However, this trend was not observed in either sham or high T₄ fish, where HSI stayed constant throughout the experimental period. Similarly, in elevated temperature groups, HSI closely modeled those observed in sham-injected fish

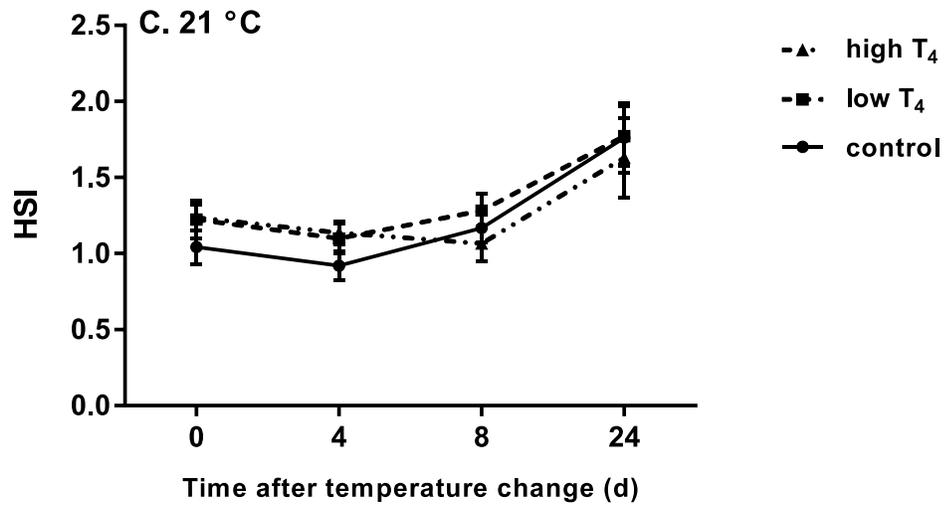
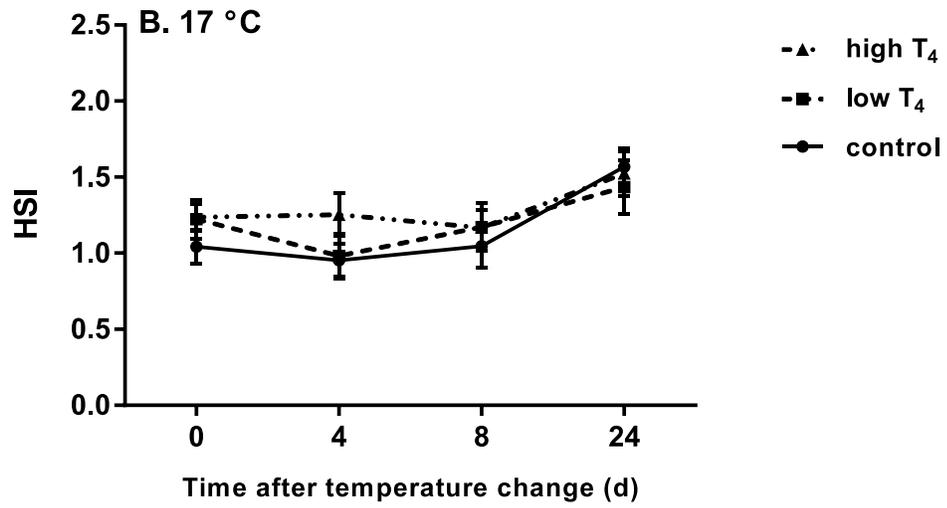
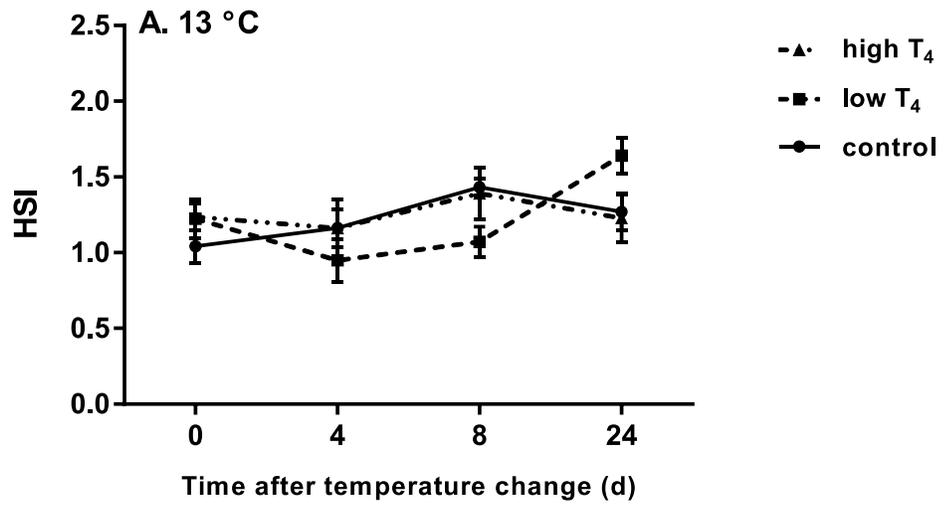


Figure 12. HSI [dimensionless; (liver weight • body weight⁻¹) × 100] of juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A), 17 (B) or 21 °C (C). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low T₄ (1 µg T₄ g body weight⁻¹) or high T₄ (10 µg T₄ g body weight⁻¹). Data was analyzed within each temperature using a two-way ANOVA to detect effects of thyroid hormone treatment, time and their interaction on HSI. Plots represent mean HSI of 7-10 fish ± SEM. No significant pair-wise differences (P < 0.05) were observed within each time point.

Table 12. Two-way ANOVA statistics on HSI in control and T₄-treated lake whitefish exposed to 13, 17 or 21 °C

	Treatment			Time			Treatment × Time		
	F	df_n, df_d	P	F	df_n, df_d	P	F	df_n, df_d	P
HSI									
13 °C	0.074	2, 99	0.929	3.041	3, 99	0.033*	2.350	6, 99	0.037*
17 °C	1.149	2, 87	0.322	6.546	3, 87	< 0.001*	0.511	6, 87	0.799
21 °C	0.755	2, 105	0.472	13.49	3, 105	< 0.001*	0.467	6, 105	0.831

throughout the experimental period (Figure 12B and C), suggesting limited effects of elevated T₄ levels on HSI, even at elevated temperatures.

Expression levels of several enzymes associated with lipid metabolism were altered by TH, both in the presence and absence of elevated temperature stress. *acc α* mRNA abundance was variable in sham-injected fish among time points, but short-term effects of hyperthyroidism were still observed in fish maintained at 13 °C. Here, *acc α* mRNA abundance in high T₄-treated fish was elevated 4.88-fold above shams on *day 0* (Figure 13A). However, no effects of TH treatments were observed at subsequent time points. Both low and high T₄ treatment increased *acc α* mRNA abundance on *day 8* in fish exposed to 17 °C (Figure 13B) and low T₄ treatment increased levels at 4 d post temperature change at 21 °C (Figure 13C). Likewise, effects of T₄ treatment on *acc β* were temperature-dependent. While no effects of elevated T₄ were observed at either of the two lower temperatures, *acc β* mRNA abundance was reduced in low T₄-treated fish at both *day 4* and *day 8* (Figure 14A-C). These results demonstrate temperature-dependent effects of TH action on the transcription and/or mRNA stability of lipogenic enzymes.

Of the two *cpt1* isoforms, *cpt1 β* was more strongly affected by T₄ treatment than *cpt1 α* . *cpt1 α* mRNA abundance was stable in response to T₄ treatment at all temperatures except for high T₄-treated fish exposed to 21 °C on *day 8* where *cpt1 α* mRNA abundance remained elevated 7.37-fold above shams (Figure 15C). In contrast, *cpt1 β* exhibited short-term effects of TH treatment which were amplified at elevated temperatures. At 13 °C, mean *cpt1 β* mRNA levels were 6.73-fold lower with high T₄-treatment on *day 4* post-temperature change (Figure 16A), but this difference increased to 18.15-fold at 17 °C and

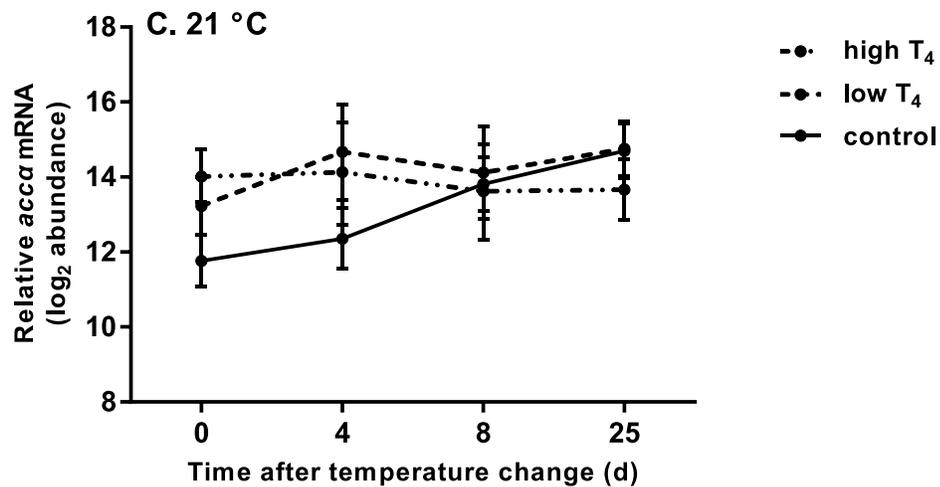
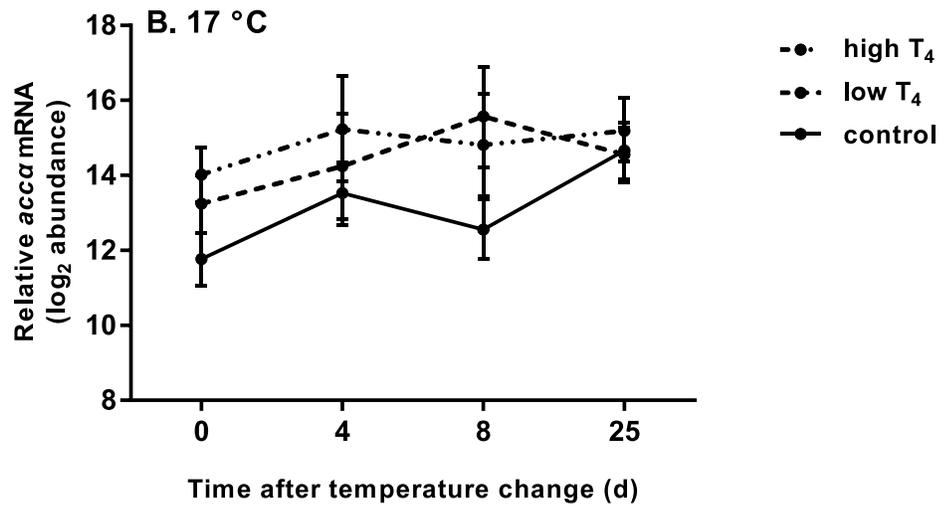
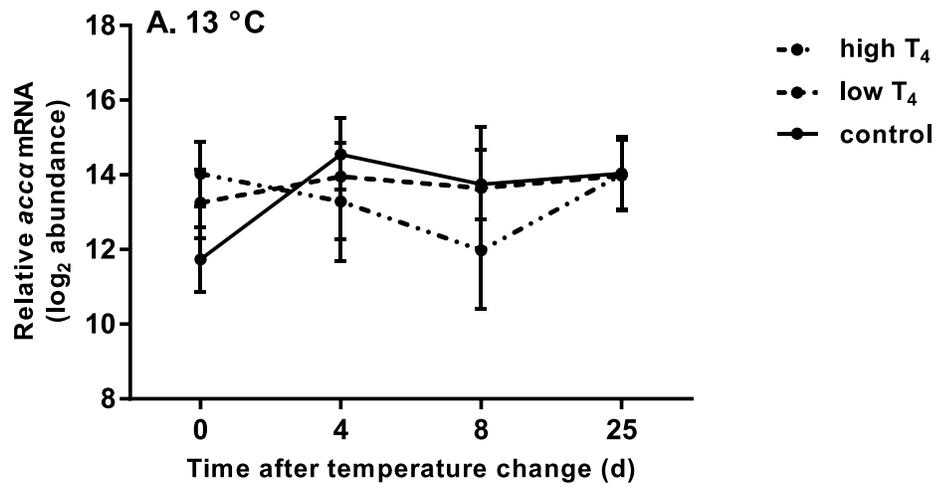


Figure 13. Abundance of *acc α* mRNA transcripts (\log_2 abundance arbitrary units) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A), 17 (B) or 21 °C (C). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low T₄ (1 $\mu\text{g T}_4$ g body weight⁻¹) or high T₄ (10 $\mu\text{g T}_4$ g body weight⁻¹). Data was modeled within each temperature group using a Bayesian MCMC sampling scheme and the MCMC.qPCR package in R (Matz et al., 2013). Hormone treatment, time and treatment \times time were used as fixed factors. Plots represent posterior mean estimates from 7-10 fish \pm 95 % credible intervals. Letters above treatment groups indicate the hormone treatment at which 95 % credible intervals differed from sham-injected fish within each time point (L = low T₄, H = high T₄).

Table 13. Summary statistics for the effects of elevated temperature and T₄ exposure from 4 to 24 d on *accα*, *accβ*, *cpt1α*, *cpt1β*, *acox1* and *acox3* mRNA abundance in juvenile lake whitefish. Different lowercase letters indicate non-overlapping 95 % credible intervals between time points within a temperature. Bolded values indicate significant non-overlapping 95 % credible intervals relative to 13 °C controls within a time point.

Gene	Temperature	Treatment	Time			
			0 d	4 d	8 d	24 d
<i>accα</i>	13 °C	control	C/0 ^a	C/4 ^b	C/8 ^b	C/24 ^b
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24
	17 °C	control	C/0 ^a	C/4 ^b	C/8 ^{ab}	C/24 ^b
		low T ₄	L/0 ^a	L/4 ^{ab}	L/8^b	L/24 ^{ab}
		high T ₄	H/0	H/4	H/8	H/24
	21 °C	control	C/0 ^a	C/4 ^{ab}	C/8 ^{bc}	C/24 ^c
		low T ₄	L/0 ^a	L/4^{ab}	L/8 ^{ab}	L/24 ^b
		high T ₄	H/0	H/4	H/8	H/24
<i>accβ</i>	13 °C	control	C/0 ^a	C/4 ^b	C/8 ^a	C/24 ^{ab}
		low T ₄	L/0 ^{ab}	L/4 ^{ab}	L/8 ^a	L/24 ^b
		high T ₄	H/0	H/4	H/8	H/24
	17 °C	control	C/0 ^a	C/4 ^b	C/8 ^{ab}	C/24 ^{ab}
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24
	21 °C	control	C/0 ^a	C/4 ^b	C/8 ^b	C/24 ^b
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24
<i>cpt1α</i>	13 °C	control	C/0 ^a	C/4 ^a	C/8 ^b	C/24 ^{ab}
		low T ₄	L/0 ^a	L/4 ^{ab}	L/8 ^{ab}	L/24 ^b
		high T ₄	H/0	H/4	H/8	H/24
	17 °C	control	C/0 ^a	C/4 ^b	C/8 ^b	C/24 ^b
		low T ₄	L/0 ^a	L/4 ^b	L/8 ^{ab}	L/24 ^b
		high T ₄	H/0 ^a	H/4 ^b	H/8 ^b	H/24 ^b
	21 °C	control	C/0 ^a	C/4 ^b	C/8 ^c	C/24 ^{bc}
		low T ₄	L/0 ^a	L/4 ^b	L/8 ^{ab}	L/24 ^b
		high T ₄	H/0 ^a	H/4 ^{ab}	H/8^a	H/24 ^b

Table 13. Continued

Gene	Temperature	Treatment	Time			
			0 d	4 d	8 d	24 d
<i>cpt1β</i>	13 °C	control	C/0 ^{ab}	C/4 ^a	C/8 ^b	C/24 ^b
		low T ₄	L/0 ^a	L/4 ^{ab}	L/8 ^{ab}	L/24 ^b
		high T ₄	H/0 ^a	H/4^{ab}	H/8 ^b	H/24 ^{ab}
	17 °C	control	C/0 ^a	C/4 ^b	C/8 ^{ab}	C/24 ^a
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0 ^a	H/4^b	H/8^{ab}	H/24 ^{ab}
	21 °C	control	C/0 ^a	C/4 ^b	C/8 ^a	C/24 ^a
		low T ₄	L/0 ^a	L/4^b	L/8 ^{ab}	L/24 ^a
		high T ₄	H/0 ^a	H/4^b	H/8 ^{ab}	H/24^{ab}
<i>acox1</i>	13 °C	control	C/0	C/4	C/8	C/24
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24
	17 °C	control	C/0	C/4	C/8	C/24
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24
	21 °C	control	C/0	C/4	C/8	C/24
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24
<i>acox3</i>	13 °C	control	C/0	C/4	C/8	C/24
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24
	17 °C	control	C/0 ^a	C/4 ^{ab}	C/8 ^b	C/24 ^{ab}
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24
	21 °C	control	C/0 ^{ab}	C/4 ^a	C/8 ^b	C/24 ^{ab}
		low T ₄	L/0	L/4	L/8	L/24
		high T ₄	H/0	H/4	H/8	H/24

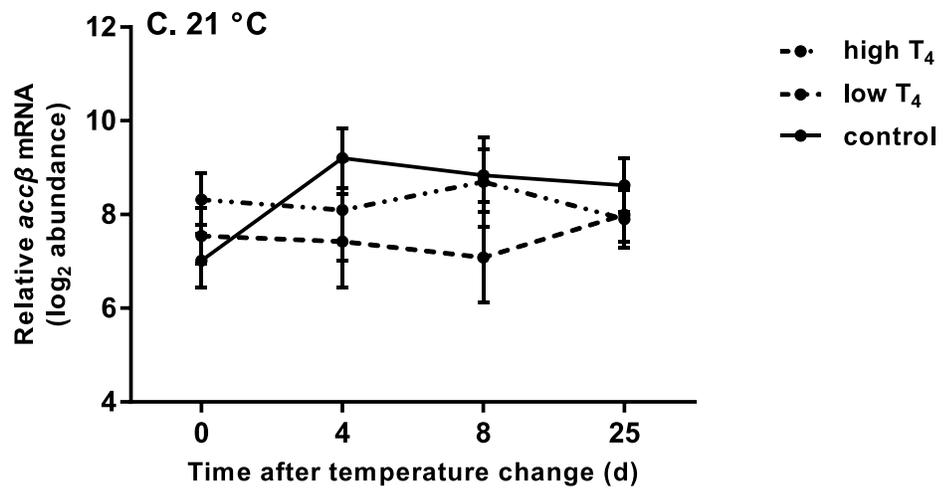
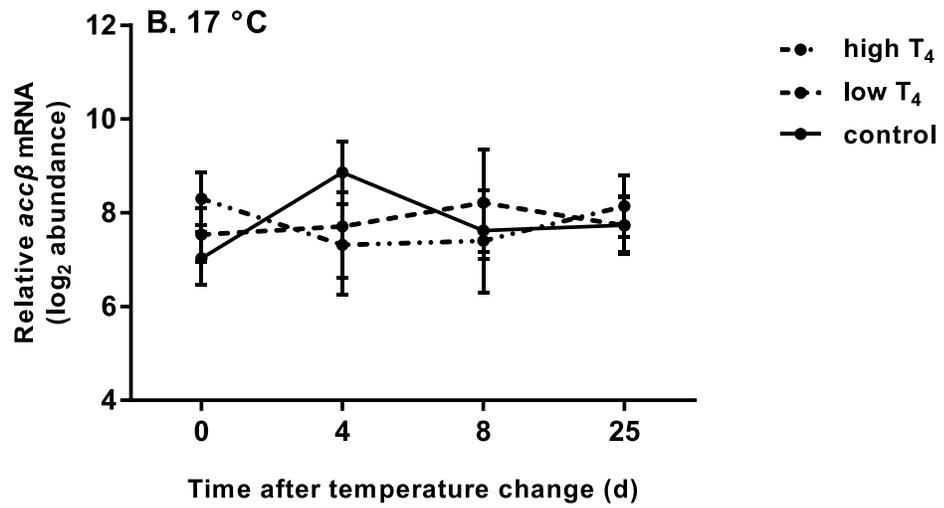
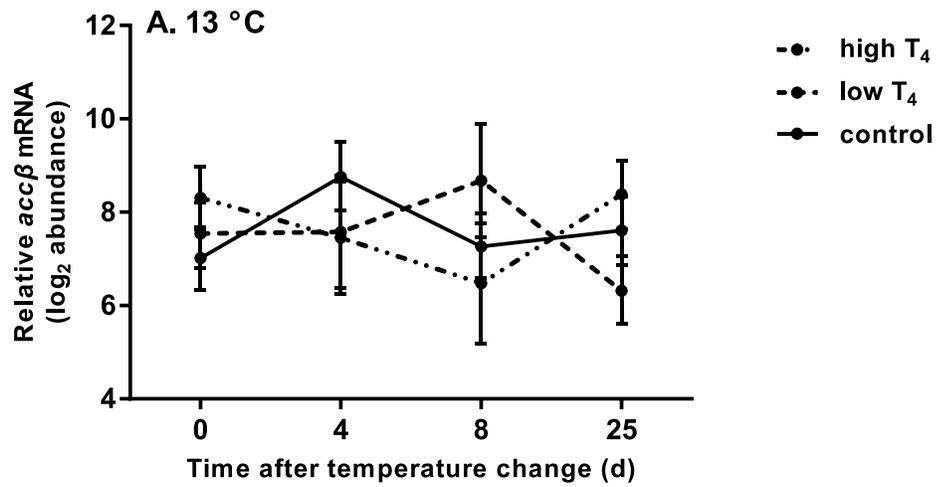


Figure 14. Abundance of *accβ* mRNA transcripts (log₂ abundance arbitrary units) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A), 17 (B) or 21 °C (C). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low T₄ (1 μg T₄ g body weight⁻¹) or high T₄ (10 μg T₄ g body weight⁻¹). Data was modeled within each temperature group using a Bayesian MCMC sampling scheme and the MCMC.qPCR package in R (Matz et al., 2013). Hormone treatment, time and treatment × time were used as fixed factors. Plots represent posterior mean estimates from 7-10 fish ± 95 % credible intervals. Letters above treatment groups indicate the hormone treatment at which 95 % credible intervals differed from sham-injected fish within each time point (L = low T₄, H = high T₄).

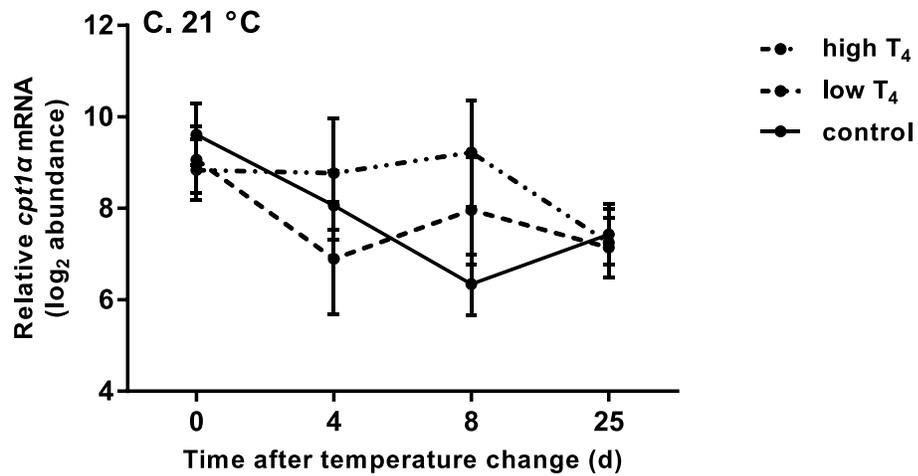
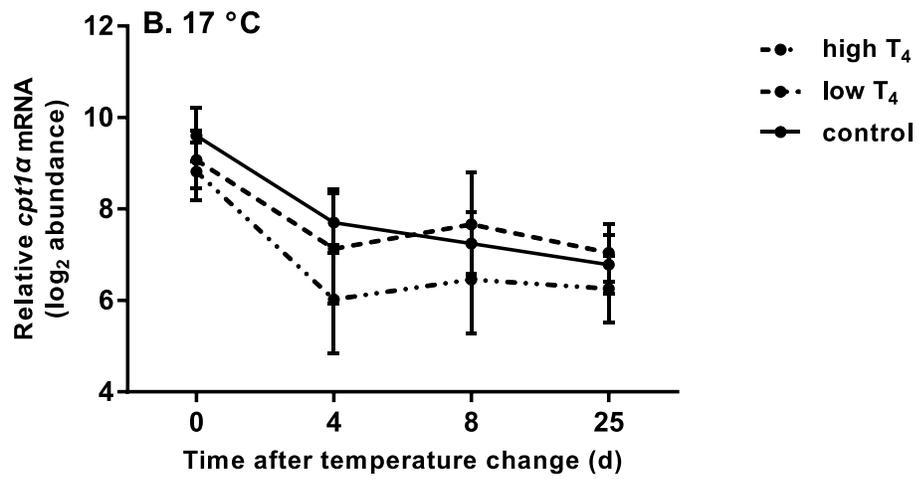
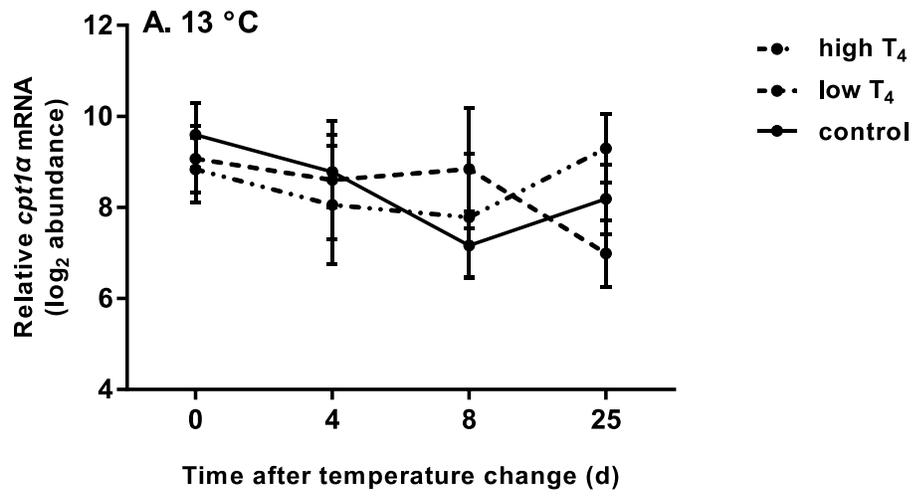


Figure 15. Abundance of *cpt1 α* mRNA transcripts (\log_2 abundance arbitrary units) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A), 17 (B) or 21 °C (C). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low T₄ (1 $\mu\text{g T}_4$ g body weight⁻¹) or high T₄ (10 $\mu\text{g T}_4$ g body weight⁻¹). Data was modeled within each temperature group using a Bayesian MCMC sampling scheme and the MCMC.qPCR package in R (Matz et al., 2013). Hormone treatment, time and treatment \times time were used as fixed factors. Plots represent posterior mean estimates from 7-10 fish \pm 95 % credible intervals. Letters above treatment groups indicate the hormone treatment at which 95 % credible intervals differed from sham-injected fish within each time point (L = low T₄, H = high T₄).

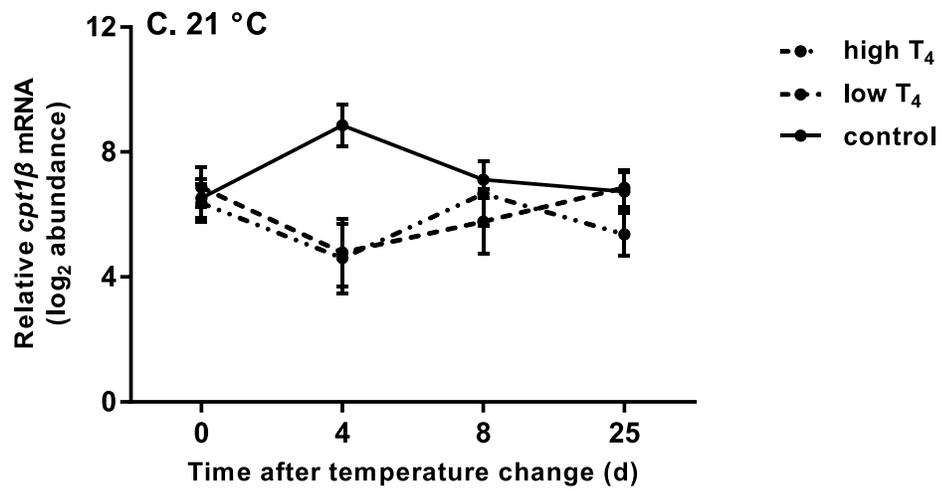
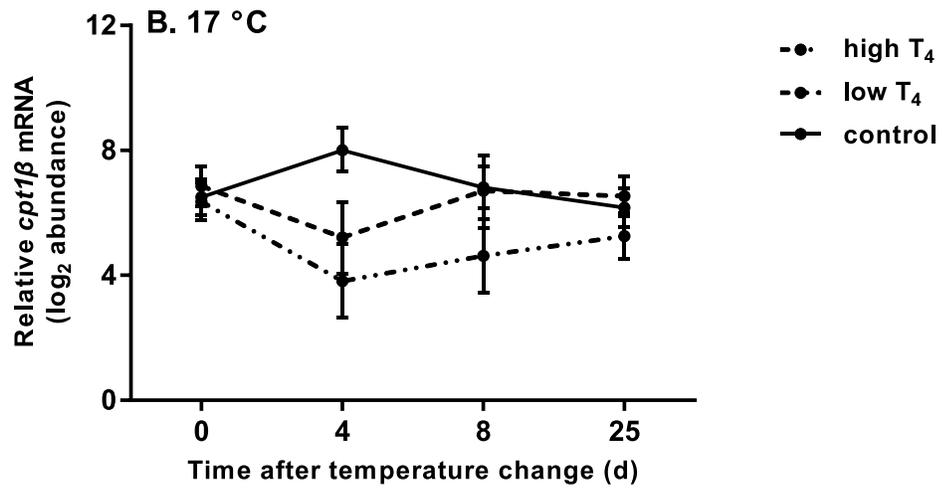
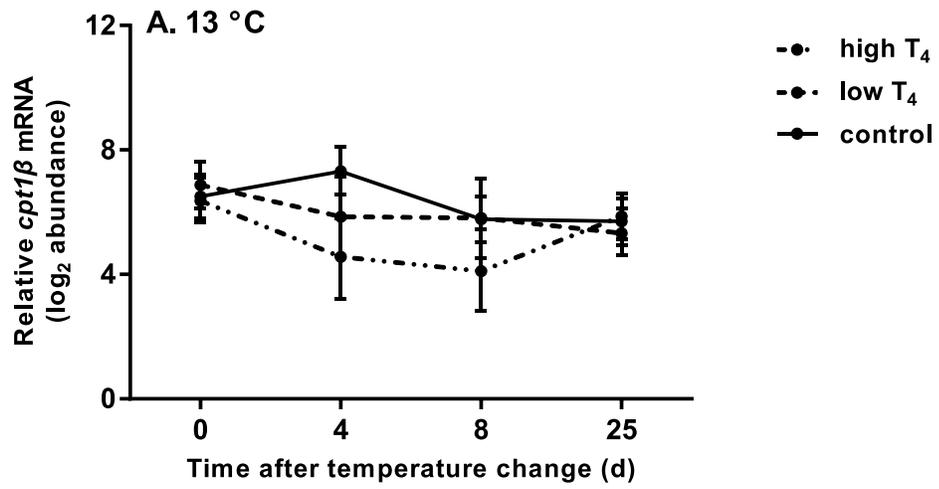


Figure 16. Abundance of *cpt1 β* mRNA transcripts (\log_2 abundance arbitrary units) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A), 17 (B) or 21 °C (C). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low T₄ (1 μ g T₄ g body weight⁻¹) or high T₄ (10 μ g T₄ g body weight⁻¹). Data was modeled within each temperature group using a Bayesian MCMC sampling scheme and the MCMC.qPCR package in R (Matz et al., 2013). Hormone treatment, time and treatment \times time were used as fixed factors. Plots represent posterior mean estimates from 7-10 fish \pm 95 % credible intervals. Letters above treatment groups indicate the hormone treatment at which 95 % credible intervals differed from sham-injected fish within each time point (L = low T₄, H = high T₄).

19.00-fold at 21 °C (Figure 16B and C). Similarly, mean *cpt1β* mRNA levels in low T₄-treated fish on *day 4* were also reduced relative to sham-injected fish by 6.93- and 16.78-fold at 17 and 21 °C by, respectively, showing that *cpt1β* mRNA levels experience strong and consistent short-term down-regulation by elevated THs, which is intensified at higher temperatures. While the effects of TH on *cpt1β* in 13 °C fish were limited to *day 4*, longer-term effects of high T₄ treatment were observed at elevated temperatures. At 17 °C, mean *cpt1β* mRNA levels remained depressed on *day 8* post-temperature change, but recovered to sham levels by *day 24*. In contrast, mean *cpt1β* mRNA abundance in high T₄-treated fish was not lower on *day 8*, but once again fell below sham levels at *day 24*.

Unlike the pronounced effects of TH on mRNA levels of mitochondrial β-oxidation enzymes, peroxisomal β-oxidation enzymes were largely unaffected by elevated THs. *acox1* mRNA abundance was not altered by either low or high T₄ treatment at any temperature (Figure 17A-C). Similarly, no effects of T₄ treatment were observed on *acox3* mRNA abundance at 13 °C (Figure 18A). However, *acox3* mRNA abundance was modestly affected by T₄-treatment on *day 8* post temperature change at 17 and 21 °C groups (Figure 18B and C). In both cases, elevated T₄ increased *acox3* mRNA abundance between 2.58- and 3.32-fold. Despite the relatively modest effects observed on *acox3*, these results still demonstrate temperature-dependent effects of TH action on mRNA abundance of peroxisomal β-oxidation enzymes in juvenile lake whitefish.

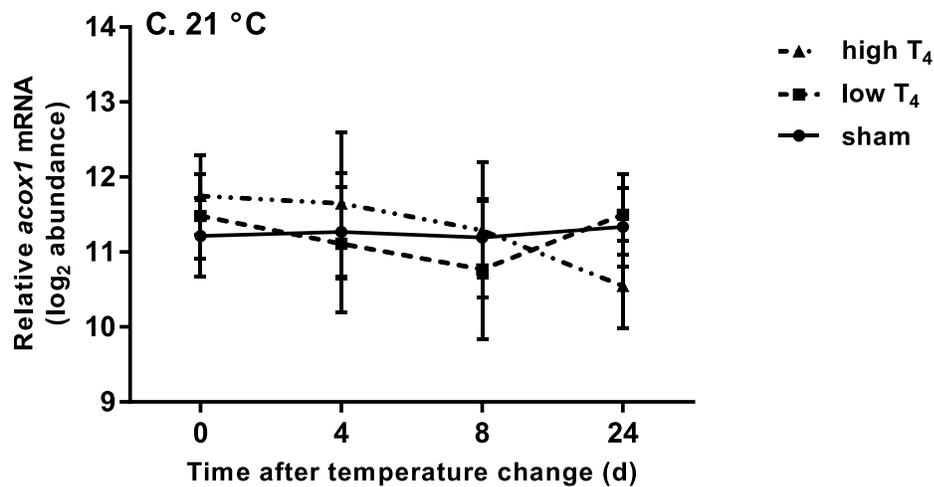
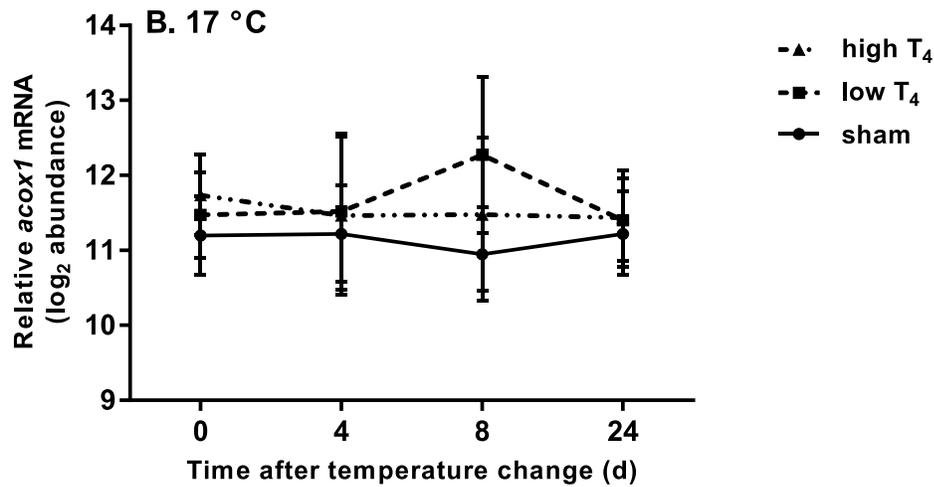
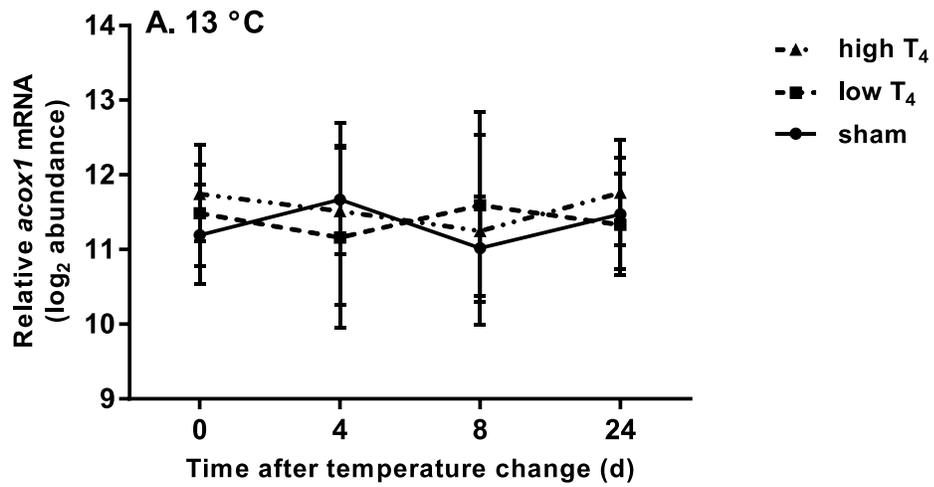


Figure 17. Abundance of *acox1* mRNA transcripts (\log_2 abundance arbitrary units) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A), 17 (B) or 21 °C (C). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low T₄ (1 $\mu\text{g T}_4$ g body weight⁻¹) or high T₄ (10 $\mu\text{g T}_4$ g body weight⁻¹). Data was modeled within each temperature group using a Bayesian MCMC sampling scheme and the MCMC.qPCR package in R (Matz et al., 2013). Hormone treatment, time and treatment \times time were used as fixed factors. Plots represent posterior mean estimates from 7-10 fish \pm 95 % credible intervals. Letters above treatment groups indicate the hormone treatment at which 95 % credible intervals differed from sham-injected fish within each time point (L = low T₄, H = high T₄).

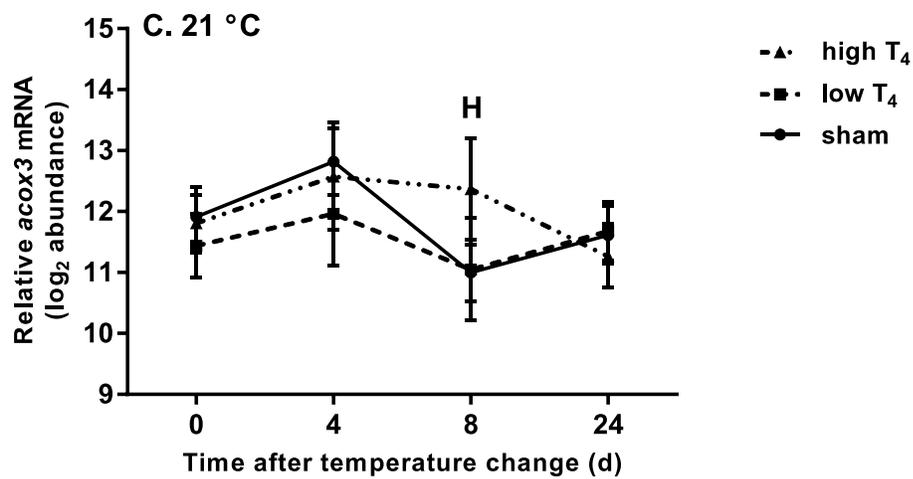
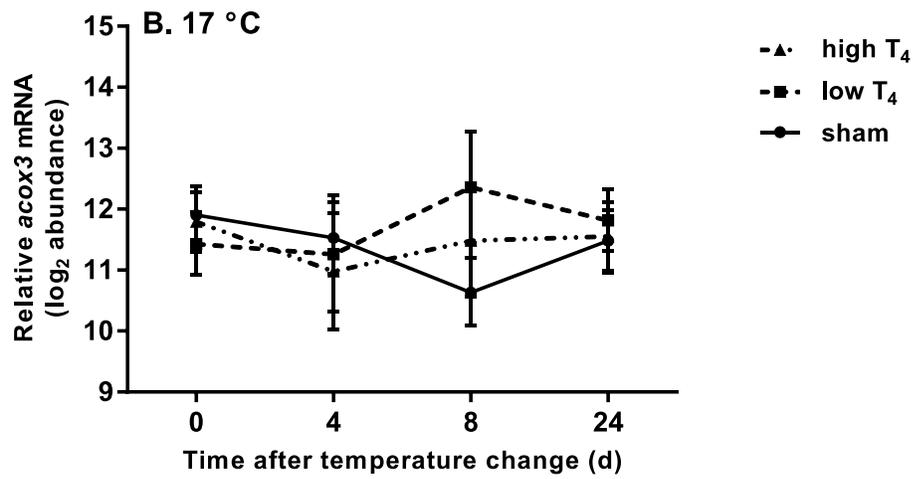
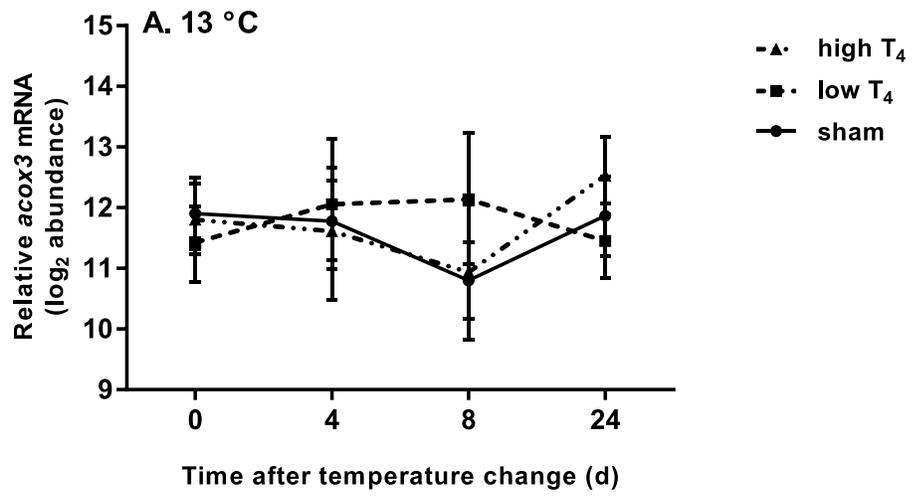


Figure 18. Abundance of *acox3* mRNA transcripts (\log_2 abundance arbitrary units) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A), 17 (B) or 21 °C (C). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low T₄ (1 $\mu\text{g T}_4$ g body weight⁻¹) or high T₄ (10 $\mu\text{g T}_4$ g body weight⁻¹). Data was modeled within each temperature group using a Bayesian MCMC sampling scheme and the MCMC.qPCR package in R (Matz et al., 2013). Hormone treatment, time and treatment \times time were used as fixed factors. Plots represent posterior mean estimates from 7-10 fish \pm 95 % credible intervals. Letters above treatment groups indicate the hormone treatment at which 95 % credible intervals differed from sham-injected fish within each time point (L = low T₄, H = high T₄).

3.4. Discussion

3.4.1. *Effects of temperature on the hepatosomatic index and mRNA abundance of enzymes associated with lipid metabolism*

The liver plays a vital role in several diverse aspects of physiology, including bile production (Campbell and Reece, 2005), energy storage (Sheridan, 1994; Tarr, 1972) and metabolism (Mullur et al., 2013; Sheridan, 1994; Tarr, 1972). I observed an increase in the hepatosomatic index (HSI) of juvenile lake whitefish on *day 24* following exposure to 21 °C (Figure 9). Changes in the HSI, which reflect the relative size of the liver in relation to overall body mass, are indicative of changes in hepatic energy stores, primarily those associated with glycogen (carbohydrate) and triglyceride (lipid) reserves (Johnston and Dunn, 1987). Overall, glycogen contributes a large percentage of liver dry weight in fish and is an important variable in dictating liver size and weight. Furthermore, the volume of glycogen stores can be quite variable. Changes in diet alone have been shown to cause shifts in glycogen contributions to overall liver dry weight between 1.5 and 4 % in blunt snout bream (*Megalobrama amblycephala*; Wang et al., 2017). Comparatively, lipid stores contribute an even larger percentage of overall liver mass in fish, which can range from 5.5 up to 40 % (Johnston and Dunn, 1987; Rodríguez et al., 2004; Wang et al., 2017), depending on both nutritional and environmental factors (i.e. temperature). The increase in the HSI following long-term exposure to elevated temperatures is in accordance with observations in coho salmon (*Oncorhynchus kisutch*; Larsen et al., 2001) and Atlantic salmon (*Salmo salar*; Kullgren et al., 2013) which also noted an increase in HSI in warm-acclimated fish relative to their cold-acclimated

counterparts. However, different patterns in the utilization of hepatic energy reserves following temperature change have been observed among fish species. For instance, Corey et al. (2017) noted decreases in liver glycogen levels following the implementation of elevated thermal cycling regimes in juvenile Atlantic salmon as well as on overall HSI (Kullgren et al., 2013). Differential responses to elevated temperature exposure in closely related fish species indicates that control of the HSI in fish may represent a delicate balance of energy demand and the mobilization of energy reserves that may be influenced by a complex array of factors and dependent on the nature of the temperature change (i.e. fluctuation vs constant temperatures). Overall, the results of the present study suggest there is a significant deposition of hepatic glycogen and/or lipid following exposure to 21 °C and, therefore, limited mobilization of hepatic energy stores and overall metabolic demand.

With respect to the transcript abundance of rate-limiting enzymes in lipid metabolism pathways, effects of elevated temperature exposure were relatively mild, both for lipid biosynthesis and β -oxidation pathways. The functional divergence in the two *acc* isoforms I quantified means that they provide information on both the effects of temperature on lipid biosynthesis as well as inhibition of mitochondrial β -oxidation. Exposure to elevated temperature had clear differences on the transcript abundance of these two isoforms, leading to opposing interpretations of the effects of temperature on this aspect of metabolism. The transient downregulation of *acc α* at *day 4* following exposure to 21 °C suggests some level of negative impact on *acc α* transcription and/or stability following temperature change. However, given the short-term nature of this depression, I predict it has limited long-term impacts on the production of functional

ACC α enzyme and the overall lipid biosynthetic pathway. More likely to be of biological significance are the increases in *acc β* mRNA abundance observed in response to 21 °C exposure (Figure 10B). Although these effects did not occur until *day 8*, *acc β* mRNA abundance remained elevated at *day 24*, suggesting the establishment of a new steady state mRNA level in response to elevated temperature. A corresponding change on *acc β* -derived ACC enzyme abundance could lead to excess malonyl-coA production and inhibition of mitochondrial β -oxidation via allosteric inhibition of CPT1 activity. Furthermore, the inhibition of β -oxidation suggested by the effects of elevated temperature on *acc β* abundance correlate with the long-term increase in HSI observed at elevated temperatures.

Overall, temperature had surprisingly few effects on transcript abundance of enzymes associated with both mitochondrial and peroxisomal β -oxidation. *cpt1 α* , *cpt1 β* and *acox1* were all unaffected over the experimental period, despite a temperature difference of 8 °C between the highest and lowest treatments. Exposure to 21 °C did increase *acox3* mRNA abundance, but these effects were transient and were absent at both *day 8* and *day 24*. The role of the peroxisomal β -oxidation enzymes in cell physiology is broader than their mitochondrial counterparts. In mammals, *acox3* is thought to be responsible not only for the breakdown of very-long chained, branched fatty acids, but also involved in the production of bile acids (Ferdinandusse and Houter, 2006) which aid in the emulsification of fats in the diet (Campbell and Reece, 2005). It is unclear if *acox3* has a similar role in bile acid production in fish. However, given the transient nature of this effect on *acox3* mRNA transcript abundance, it is unlikely to

dictate significant long-term impacts on downstream events related to either bile production or peroxisomal β -oxidation.

Clear functional differences exist between the gene isoforms for *acc* and *acox*, and in both cases, the two isoforms appear to be regulated independently in response to temperature change. My results show that *acc α* and *acc β* mRNA abundancies are driven in opposite directions following elevated temperature exposure. Furthermore, these changes occur on different timescales, demonstrating pronounced regulatory differences in gene expression. Transcriptional regulation of *acc* isoforms in mammals is thought to primarily occur through the peroxisome-proliferator-activated receptor (PPAR) transcription factor family, along with several other accessory transcription factors such as liver X receptor (LXR) and retinoid X receptors (RXR; Brownsey et al. 2006). Similarly, *acox1* and *acox3* exhibited differential expression patterns at *day 4* and are known to be heavily influenced by the PPAR family of transcriptional regulators (Madureira et al., 2016). Despite the commonalities between transcription factors among these genes, the presence or absence of several accessory proteins may facilitate complex responses to temperature and/or other environmental stimuli accounting, in part, for the regulation of their expression.

3.4.2. Effects of combined thyroid hormone manipulation and elevated temperature on the hepatosomatic index and mRNA abundance of enzymes associated with lipid metabolism

Overall, serum T₄ levels following administration of coconut oil implants were significantly elevated above control levels in juvenile lake whitefish, but remained close

to physiological serum TH levels observed in other salmonid species (Dickhoff et al., 1978). Furthermore, low- ($1 \mu\text{g T}_4 \times \text{g body weight}^{-1}$) and high- T_4 ($10 \mu\text{g T}_4 \times \text{g body weight}^{-1}$) treatments differentially increased serum T_4 levels in juvenile lake whitefish, allowing for examination of both mild and moderate serum T_4 elevations on lipid metabolism markers. A more detailed discussion on the variability in serum T_4 levels observed among temperature groups and overall implications for juvenile lake whitefish is given in Chapter 1 (Section 2.4.2.).

Several instances of temperature-dependent modifications in TH abundance and action on oxidative metabolism have been reported in the literature for fish (Kao et al., 1999; Gupta and Thapliyal, 1991; Zak et al., 2017) and other ectothermic species (Gupta and Thapliyal, 1991). An interaction between temperature and exogenous T_4 treatment on the transcript abundance of enzymes associated with lipid metabolism were also observed in the current study, suggesting these effects extend beyond those on oxidative enzymes to influence several aspects of metabolism in juvenile lake whitefish. Of the six transcripts I examined, the largest effect of exogenous T_4 exposure, as well as the clearest depiction of a temperature-TH interaction on transcript abundance, was observed on the mitochondrial β -oxidation enzyme, *cpt1 β* . Here, high- T_4 treatment depressed *cpt1 β* transcript abundance at *day 4* in fish maintained at 13 °C and effects of high- T_4 treatment at this time point were highly exaggerated and accompanied by effects of low- T_4 treatment at elevated temperatures (Figure 16). These results depict clear, short-term effects of THs on the transcript abundance of *cpt1 β* in juvenile lake whitefish, particularly given CPT1 activity is thought to be largely regulated at the transcriptional level in fish (Leaver et al., 2008). However, the biological interpretation of these results

is inconsistent with expected effects of TH on metabolic processes. Under most circumstances, mammalian studies suggest a pronounced catabolic effect of THs on both lipid (Mullur et al., 2013; Pucci et al., 2000) and oxidative (Barker and Kiltgaard, 1952; Cioffi et al., 2013; Etkin et al., 1940; Hulbert, 2000; Pucci et al. 2000) metabolism. The majority of reports on the effects of TH on cellular metabolism in ectotherms suggest the effects of TH are also calorogenic in nature (Chen et al., 2015; Gupta and Thapliyal, 1991). Therefore, the depression of *cpt1 β* observed in the present study following TH exposure, suggesting a reduction in the expression of the rate-limiting enzyme for mitochondrial β -oxidation, is contradictory to the assumed catabolism-promoting effects of THs. However, a decline in triacylglycerol lipase (TGL) activity, suggesting a depression in lipid breakdown, has been observed in hypothyroid sea lamprey (*Petromyzon marinus*) following exogenous T₄ treatment (Kao et al., 1999). Therefore, TH stimulation of oxidative metabolism is not universal in all fish species.

Effects of exogenous T₄ exposure on transcript abundance of *acc α* , *acc β* , *cpt1 α* and *acox3* were temperature-dependent, as effects were largely absent at lower temperatures, but became increasingly evident as temperatures increased. Notwithstanding *cpt1 β* and the mild effects on *acc α* and *acc β* on *day 0*, temperature had no effect on transcript abundance of enzymes associated with lipid metabolism in juvenile lake whitefish maintained at 13 °C. However, in fish exposed to 17 °C, mild transient effects of T₄ treatment were observed on both *acc α* and *acox3* abundance at *day 8* and at 21 °C, effects were observed on all gene transcripts except *acox1* at various points in the acclimation process (Figures 13-15, 17, 18). The limited effects of exogenous T₄ treatment at low temperatures suggests temperature plays an important role

in the transcriptional responses initiated via THs with respect to lipid metabolism genes in juvenile lake whitefish, which is supported by temperature-dependent effects of THs observed in other ectothermic species (Gupta and Thapliyal, 1991). Biologically, the effects associated with exogenous T₄ exposure at higher temperatures in lake whitefish on *accα*, *accβ*, *cpt1α* and *acox3* transcript abundance were mild and unlikely to produce long-term physiological effects on lipid metabolism pathways. Nevertheless, these results demonstrate regulatory potential on enzymes associated with lipid metabolism by physiologically-relevant serum TH levels. It is possible that effects of THs on transcription of lipid enzymes could become more pronounced as temperatures continue to increase, but, currently, it is unclear how metabolic responses to elevated TH status would be altered as temperatures approach the lethal limit for juvenile lake whitefish. Temperature-dependent changes in metabolism and their potential relationship to both TH levels (Comeau et al., 2000; Eales and Fletcher, 1982; Johnston and Eales, 1995; Larsen et al., 2001; Levin and Bolotovskiy, 2015; O'Brien, 2010) and TH action (Gupta and Thapliyal, 1991) could become increasingly relevant due to rising global temperatures associated with climate change. Information on the relationship between temperature and TH action is likely to shed light on the regulation of adaptive responses in lake whitefish and other cool-water fish species subjected to temperatures approaching lethal limits.

Similar to the effects of temperature alone on transcription of metabolic enzymes, I observed differential regulation of gene isoforms following exogenous T₄ exposure for all three sets of genes studied. With the exception of *cpt1*, the isoforms of each gene I examined in the current study are known to occupy distinct functional niches that make

them unique from their partner isoform. Therefore, differential regulation in response to combined temperature and exogenous T₄ treatment can be expected as they maintain different function roles in lipid pathways. The most pronounced differences among regulation patterns were observed for *accα* and *accβ*, which were driven in opposite directions by exogenous T₄ treatment in fish exposed to 21 °C. In both cases, the effects of TH exposure were opposite to those experienced by temperature change alone. Given the differential effects of TH on gene isoforms as well as the observed relationship in opposition to temperature responses, it is possible that the primary effects of THs during thermal acclimation events is associated with stabilizing mRNA expression of metabolic genes rather than specifically driving towards an increase in cellular oxidative processes. A similar relationship with temperature and oxidative enzyme activity has previously been observed in juvenile lake whitefish in the presence of supraphysiological serum T₄ levels (Zak et al., 2017). Repeated observations demonstrating opposing effects of TH exposure on thermal acclimation responses in juvenile lake whitefish provides support for the role of THs in thermal regulatory responses and metabolic remodeling following long-term temperature change. However, it remains unclear whether THs simply mitigate the need for metabolic restructuring or actively suppress established metabolic responses to elevated temperatures. Furthermore, the compensatory responses of THs are not unanimously observed among all genes as *acox3* appears to act synergistically with temperature to be increased further when combined with exogenous T₄ treatment.

While a clear depression was observed in *cpt1β* mRNA abundance in response to exogenous T₄ exposure, *cpt1α* abundance remained unaffected except for a transient increase above control levels at *day 8* following exposure to 21 °C (Figure 15). These

results indicate distinct regulatory differences between *cpt1 α* and *cpt1 β* with respect to regulation via THs at temperatures within the optimal range of juvenile lake whitefish as well as with respect to elevated temperature-TH interactions. In most vertebrates, *cpt1 β* expression occurs almost exclusively in cardiac and skeletal muscle (Leaver et al., 2008; Boukouvka et al., 2010), but studies conducted in salmonids (Morash et al., 2010) as well as other more distantly-related species such as yellow catfish (*Pelteobagrus fulvidraco*; Zheng et al., 2013) have identified diversification of the *cpt1* gene family as well as extensive cross-expression of *cpt1 α* and *cpt1 β* isoforms between several different tissues. For salmonids, the unique tissue expression profiles of *cpt1 α* and *cpt1 β* isoforms relative to other vertebrate species has been attributed to recent genome duplication events in the salmonid lineage (Crête-Lafrenière et al., 2012; Morash et al., 2010). Such changes have the potential to greatly increase the complexity of *cpt1* function and regulation, via sub- and neo-functionalization of *cpt1* isoforms (Morash et al., 2010). Currently, little information on the functional differences between CPT1 α and CPT1 β activities exists in the literature, aside from notable differences in their sensitivity to allosteric inhibition by malonyl-coA and carnitine binding affinity (McGarry and Brown, 1997). In mammals, CPT1 β is approximately 80 times more sensitive to allosteric inhibition by malonyl co-A and is able to bind carnitine more effectively, resulting in a lower half maximal inhibitory concentration of malonyl-coA for muscle than liver (McGarry and Brown, 1997). In rainbow trout (*Oncorhynchus mykiss*), tissue-specific sensitivities to malonyl-coA are reversed and exhibit increased inhibition by malonyl-coA in liver than skeletal or cardiac muscles, likely due to differences in *cpt1 α* and *cpt1 β* expression profiles (Morash et al., 2008). TH-induced changes in the relative proportions of CPT1 α and CPT1 β in fish liver

have the potential to regulate overall β -oxidation rates. This could ultimately result in different functional associations between ACC and CPT1, particularly since malonyl-coA is considered one of the primary regulatory of mitochondrial β -oxidation (Boukouvala et al., 2010).

4. LITERATURE CITED

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5. APPENDIX

5.1. Appendix Figures

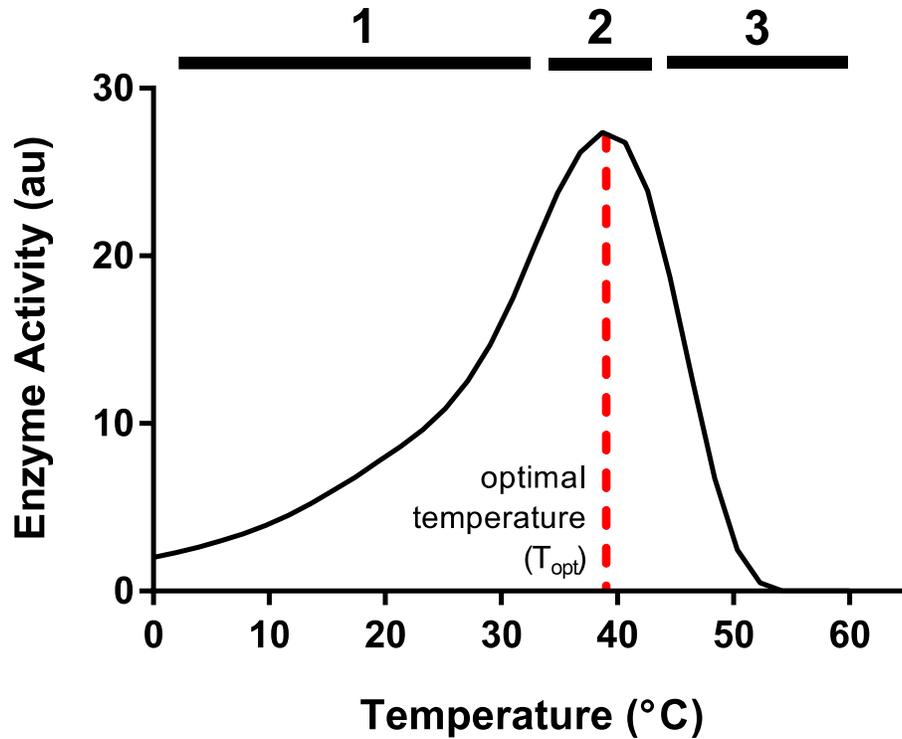


Figure A1. Schematic diagram depicting temperature versus enzyme activity. Typical enzymes respond to temperature in three phases, whereby, activity rate increases with temperature when below optimal (Phase 1), briefly plateaus at the optimal temperature (T_{opt} ; Phase 2) and declines rapidly as temperatures exceed optimal (Phase 3). Each phase is indicated by numbers above the figure and the schematic models the activity of an enzyme that has a Q_{10} value of ~ 2 at temperatures below optimal. The dotted, vertical line represents the optimal temperature of this theoretical temperature-activity curve. Figure adapted from Campbell and Reece (2005) and Elliot and Elliot (2005).

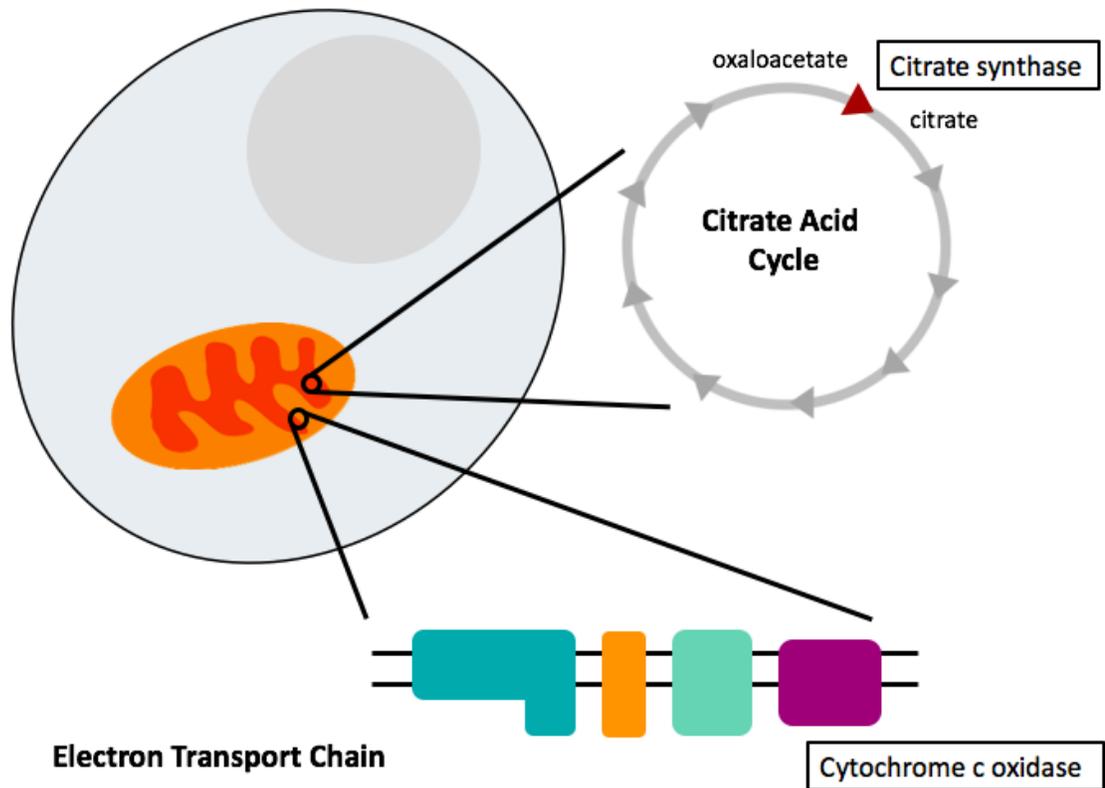


Figure A2. Schematic diagram depicting the function and location of the oxidative enzymes citrate synthase and cytochrome c oxidase. Citrate synthase is located in the mitochondrial matrix and catalyzes the formation of citrate from oxaloacetate and acetyl-coA. Cytochrome c oxidase is the terminal enzyme of the electron transport chain and catalyzes the transfer of four electrons to oxygen (O_2) to form two molecules of water (H_2O). Four protons are also consumed in this reaction. Cytochrome c oxidase is located on the inner mitochondrial membrane.

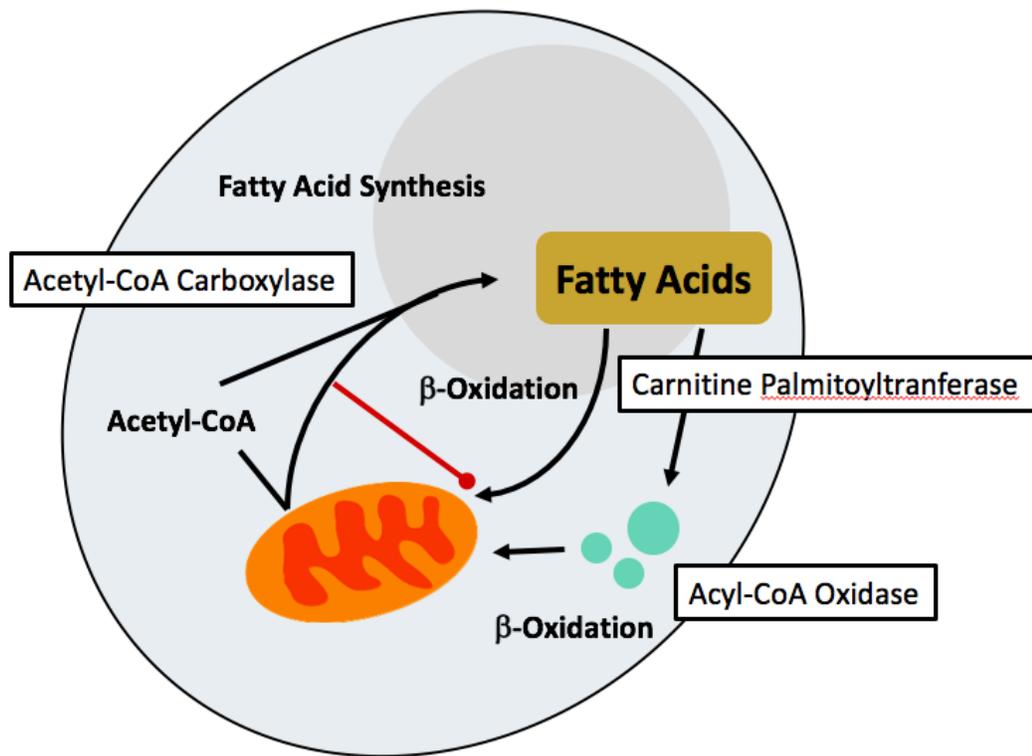


Figure A3. Schematic diagram depicting the function and location of the lipid enzymes acetyl-coA carboxylase, carnitine palmitoyltransferase and acyl-coA oxidase. Acetyl-coA carboxylase catalyzes the formation of malonyl-coA from acetyl-coA, which is the first committed step of fatty acid synthesis. Two isoforms of acetyl-coA carboxylase exist in the cell; the α isoform is present in the cytosol while the β isoform is situated on the outer mitochondrial membrane. Carnitine palmitoyltransferase is considered the rate-limiting enzyme of the mitochondrial β -oxidation pathway and shuttles fatty acids into the mitochondrial intermembrane space. Similarly, acyl-coA oxidase is considered the rate limiting enzyme of the peroxisomal β -oxidation pathway and catalyzes the first step of fatty acid breakdown in this organelle. The inhibitory action of malonyl-coA on carnitine palmitoyltransferase activity is depicted by the red line.

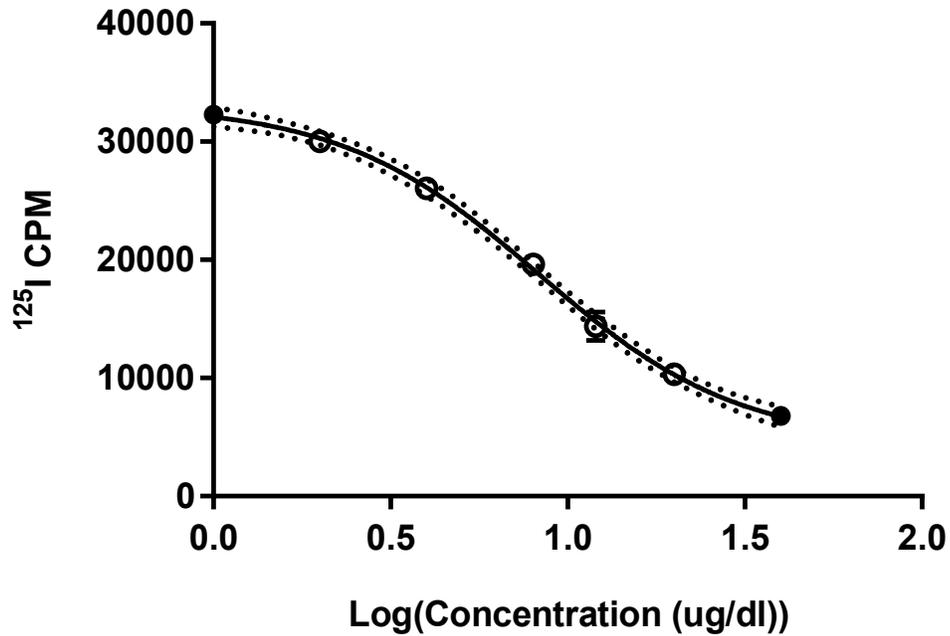


Figure A4. Raw ¹²⁵I counts per minute (CPM) of base (2, 4, 8, 12 and 20 μg/dl; open circles) and supplemental (1 and 40 μg/g; closed circles) standards used to construct radioimmunoassay standard curves for serum T₄ quantification. Good conformity of supplemental standards were observed with respect to the expected sigmoid-curve, validating addition of supplemental standards into the standard curve for calculation of T₄ levels in juvenile lake whitefish serum samples.

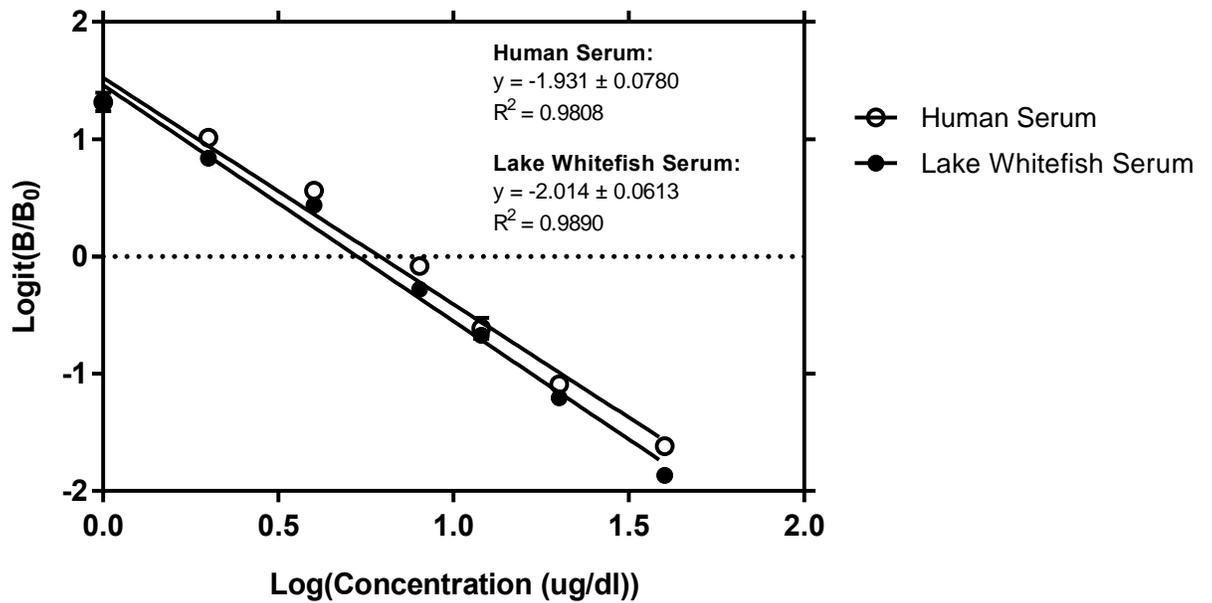


Figure A5. Lake whitefish serum reactivity check with MP BioMedicals T₄ Monoclonal Solid Phase Radioimmunoassay kit (Catalog No. 06B-254030). To confirm antibody reactivity with juvenile lake whitefish serum, kit standards (open circles) were run alongside stripped lake whitefish serum spiked with known concentrations of T₄ (closed circles). Linear regression analysis shows no difference in slopes ($P = 0.41$), confirming no difference in kit reactivity between human and lake whitefish serum samples.

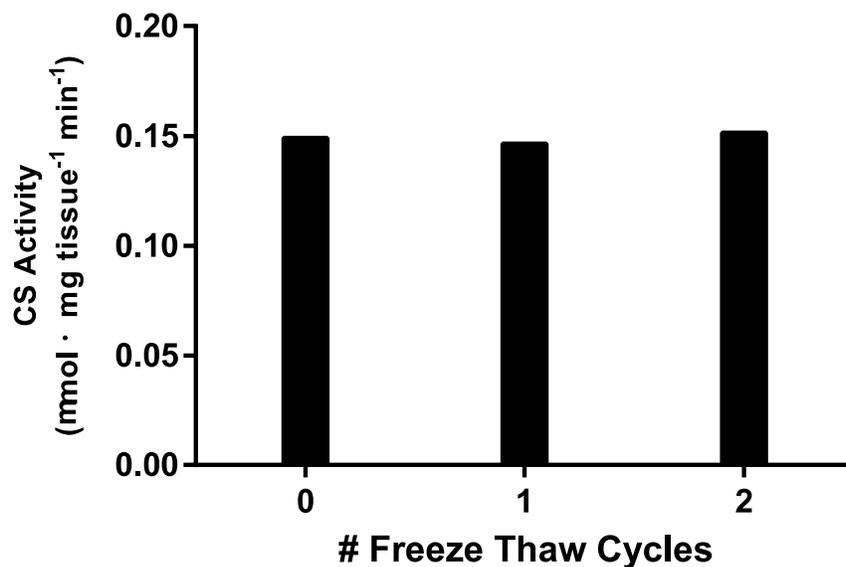


Figure A6. Effects of freeze-thaw on CS activity in juvenile lake whitefish liver homogenates. CS assays were performed using standard assays on a single homogenate immediately following homogenization (0 freeze thaw cycles), or following 1 or 2 freeze thaw cycles at -86 °C to assess the effect of repeated freeze thaw on CS stability. No loss of activity was detected due to freeze-thaw.

5.2. Appendix Tables

Table A1. Critical thermal maximum (CTMax) of thermally-acclimated juvenile lake whitefish. Fish were acclimated to their respective acclimation temperature for several months. The CTMax was determined separately for each acclimation temperature by gradually increase water temperature at a rate of 0.2 °C min⁻¹ and averaging the temperature at which the first five fish lost the ability to maintain dorsoventral orientation. Data is obtained from Zak et al., unpublished.

Acclimation Temperature (°C)	CTMax (°C)
6	24
12	25
18	26

Table A2. Summary of cross-amplification checks between target and non-target gene isoforms for each primer pair.

Primer Pair	Template	Mean Cq at specified plasmid dilution		
		10⁻⁶	10⁻⁷	10⁻⁸
<i>accα</i>	<i>accα</i>	22.73	26.09	29.17
	<i>accβ</i>	nd	nd	nd
<i>accβ</i>	<i>accα</i>	nd	nd	nd
	<i>accβ</i>	23.82	27.04	30.67
<i>cpt1α</i>	<i>cpt1α</i>	22.40	DNQ	28.79
	<i>cpt1β</i>	nd	DNQ	nd
<i>cpt1β</i>	<i>cpt1α</i>	nd	nd	38.73*
	<i>cpt1β</i>	24.34	28.04	31.74
<i>acox1</i>	<i>acox1</i>	23.09	26.38	29.77
	<i>acox3</i>	nd	39.07*	nd
<i>acox3</i>	<i>acox1</i>	nd	nd	nd
	<i>acox3</i>	22.88	26.06	29.49

nd = no amplification detected; DNQ = did not quantify; * Detection of amplified product believed to be the source of primer or template contamination since no amplification was observed at the highest plasmid concentration