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Expression and activity of lipid and oxidative metabolism enzymes following elevated temperature exposure and thyroid hormone manipulation in juvenile lake whitefish (*Coregonus clupeaformis*)

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ABSTRACT

Temperature has unequivocal effects on several aspects of fish physiology, but the full extent of its interaction with key endocrine signaling systems to influence metabolic function remains unknown. The aim of the current study was to assess the individual and combined effects of elevated temperature and hyperthyroidism on hepatic metabolism in juvenile lake whitefish by quantifying mRNA abundance and activity of key metabolic enzymes. Fish were exposed to 13 (control), 17 or 21 °C for 0, 4, 8 or 24 days in the presence or absence of low-T_4 (1 μg × g body weight^{-1}) or high-T_4 (10 μg × g body weight^{-1}) treatment. Our results demonstrate moderate sensitivity to elevated temperature in this species, characterized by short-term changes in mRNA abundance of several metabolic enzymes and long-term declines in citrate synthase (CS) and cytochrome *c* oxidase (COX) activities. T_4-induced hyperthyroidism also had several short-term effects on mRNA abundance of metabolic transcripts, including depressions in *acetyl-coA carboxylase β (accβ)* and *carnitine palmitoyltransferase 1β (cpt1β)*, and stabilization of *cs* mRNA levels; however, these effects were primarily limited to elevated temperature groups, indicating temperature-dependent effects of exogenous T_4 treatment in this species. In contrast, maximal CS and COX activities were not altered by hyperthyroidism at any temperature. Collectively, our data suggest that temperature has the potential to manipulate thyroid hormone physiology in juvenile lake whitefish and, under warm-conditions, hyperthyroidism may suppress certain elements of the β-oxidation pathway without substantial impacts on overall cellular oxidative capacity.

1. Introduction

Temperature directly impacts nearly all aspects of physiology and is, consequently, one of the most pervasive and influential stressors encountered in the environment. In recent decades, thermal shifts associated with climate change have 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). Climate change models predict an increase in global air temperatures between 1.4 and 3.1 °C without intervention to reverse the current rates of greenhouse gas emissions (IPCC, 2014). Furthermore, temperate regions in the northern hemisphere are anticipated to be heavily impacted by these climate shifts (IPCC, 2014). When combined with seasonal variability experienced in mid-range northern latitudes, thermally-sensitive, cool-water fish species such as the lake whitefish (*Coregonus clupeaformis*), may be placed at significant risk of physiological stress associated with exposure to chronic elevated temperatures or those approaching the extreme ends of their preferred temperature range.

Fish are ectothermic and do not actively regulate body temperature through endogenous mechanisms. Thus, metabolic enzymes are directly influenced by ambient thermal conditions, which can have a profound impact on metabolic pathways regulating energy supply and demand (Blier and Guderley, 1988; Hardewig et al., 1999; Schnurr et al., 2014). Metabolic remodeling following long-term exposure to high or low temperatures has been observed in several fish species (Johnston and Dunn, 1987), but is primarily observed in those that inhabit temperate regions (Battersby and Moyes, 1998; Blier and Guderley, 1988; Duggan et al., 2011; Little et al., 2013; Lucassen et al., 2006; Sidell, 1980). Effects of temperature on fish are complex and diverse ranging from those on whole-body parameters to individual tissues and cells (Johnston and Dunn, 1987). Some compensatory responses to cold

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acclimation include increases O_2 consumption rates (Guderley and Johnston, 1996; Little et al., 2013; Moon et al., 1985), metabolic scope (Little et al., 2013), mitochondrial density (Dhillon and Schulte, 2011; Guderley, 2004) and the activity of certain oxidative enzymes (Battersby and Moyes, 1998; McClelland et al., 2006). These increases have primarily been attributed to compensation for the loss of enzyme activity as temperatures decline, with the aim of maintaining cellular homeostasis (Johnston and Dunn, 1987). The majority of studies examining phenotypic plasticity under chronic thermal stress conditions have focused on oxidative metabolism associated with the carbohydrate metabolism by glycolysis, the tricarboxylic acid cycle (TCA) and electron transport chain (ETC) (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; McClelland et al., 2006; O'Brien, 2011; Windisch et al., 2011). However, secondary pathways of intermediary metabolism, such as lipid oxidation and alternate carbohydrate pathways, play a pivotal role in overall energy consumption by supplying substrates for subsequent oxidative processes. Studies in fish have demonstrated significant metabolic shifts with respect to the preference of lipid and carbohydrate pathways following temperature change (Way-Kleckner and Sidell, 1985; Sephton and Driedzic, 1991; Bailey et al., 1999; 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 full adaptive potential for metabolic remodelling in response to elevated temperatures in cool-water fish is unknown, as are the mechanisms responsible for initiating and mediating these responses. Although metabolic control occurs to some extent through allosteric regulation (Brownsey et al., 2006; Cohen, 1991) and sensing of energy stores (Mullur et al., 2014), there is also evidence that these pathways are under both thermal (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; Gupta and Thapliyal, 1991; Kao et al., 1999; Leary et al., 1996; Little et al., 2013; Peter and Oommen, 1993; Peter et al., 1996; Shivakumar and Jayaraman, 1984; Zak et al., 2017). 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; Zak et al., 2017). To date, documented effects of THs on metabolic processes in fish range from whole-body responses, such as O_2 consumption (Leary et al., 1996; Little et al., 2013; Peter and Oommen, 1993) and increases in blood triglyceride levels (Plisetskaya et al., 1983; Vargas-Chacoff et al., 2016), to cellular-level effects such as the activity of key enzymes (Chen et al., 2015; Kao et al., 1999; Peter and Oommen, 1993; Peter et al., 1996). For instance, exogenous THs transiently decrease the activity of the rate-limiting enzyme of fatty acid biosynthesis, acetyl-coA carboxylase (ACC), in sea lamprey (*Petromyzon marinus*) liver (Kao et al., 1999) and increase cytochrome *c* oxidase (COX) activity in both climbing perch (*Anabas testudineus* Bloch; Peter and Oommen, 1993; Peter et al., 1996) and tilapia (*Oreochromis mossambicus*; Shivakumar and Jayaraman, 1984). Hypothyroidism has also been linked to decreases 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), but an increase in the rate-limiting enzyme of mitochondrial β-oxidation, carnitine palmitoyltransferase 1 (CPT1) in yellow catfish (*Pelteobagrus fulvidraco*; Chen et al., 2015). The presence of inter- and intra-species variability in these TH responses (Etkin et al., 1940; Gupta and Thapliyal, 1991; Leary et al., 1996; Little et al. 2013; Peter and Oommen 1993; Umminger, 1978) suggests metabolic effects in fish could also be under the influence of additional environmental factors, such as temperature (Gupta and Thapliyal, 1991).

From an ecological perspective, studies on the effects of environmentally-relevant temperatures and their potential regulation via THs are best suited for temperate species that routinely experience large annual thermal fluctuations. The 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 the rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). However, compared to other cool-water species that inhabit a similar geographic range such as the northern pike (*Esox lucius*) and coho salmon (*Oncorhynchus kisutch*), lake whitefish prefer cooler temperatures and have reduced tolerance to elevated temperatures (Wismer and Christie, 1987). The preferred 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 between 24 and 27 °C (Edsall and Rottiers, 1976; Zak and Manzon, unpublished). However, lake whitefish is known to exhibit phenotypic plasticity following long-term exposure to temperatures outside the thermal preferendum, both with respect to cellular stress responses (Zak and Manzon, unpublished) and metabolism (Blier and Guderley, 1988; Zak et al., 2017). The aim of the current study was to characterize the extent of hepatic metabolic remodeling induced by long-term exposure to elevated temperatures in this species and assess the capability of TH, via exogenous TH treatment, to modulate these acclimation responses. To this end, we quantified the mRNA transcript abundance of key metabolic enzymes and CS and COX activities in juvenile lake whitefish exposed to elevated temperatures either in the presence or absence of exogenous T_4 treatment. Our results demonstrate effects on several metabolic enzymes following elevated temperature exposure which are further modified by exogenous T_4 treatment at elevated temperatures.

2. Materials and methods

2.1. Animal husbandry

Spawning adult male and female lake whitefish (*C. clupeaformis*) were collected from Lake Huron, ON 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 min 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 fibreglass tanks were performed three times per week using a flow through system and dechlorinated City of Regina water and light:dark photoperiod was 12:12. Fish were fed *ad libitum* with Aqueon mini cichlid sinking pellets (PetSmart) beginning 14 months post-hatch. 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. 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-T_4 and highT_4) receiving exogenous T_4 (Sigma Aldrich, Oakville, ON, Canada) to manipulate circulating TH levels. Final T_4 dosages were 0 μg T_4 × g^{-1} body weight (TH control), 1 μg T_4 × g^{-1} body weight (low-T_4) and 10 μg T_4 × g^{-1} body weight (high-T_4) 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 immediately before temperature change to provide baseline data on effects of hormone manipulation alone on metabolic activity (*day 0*). 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 × acclimation temperature) were sampled 4, 8 and 24 days following the initiation of temperature change (*days 4, 8* and *24*). Food was withheld for 12 h prior to sampling. Sample size for each treatment group ranged between 7 and 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 × g for 5 min and 3000 × g for 3 min) and stored at −80 °C for measurement of serum T_4 concentration. Whole livers were excised rapidly from animals following euthanasia via transection of the spinal cord and weighed for calculation of the hepatosomatic index (HSI) before being snap frozen on liquid N_2 and stored at −80 °C until mRNA and protein analyses.

2.3. Serum thyroxine (T_4) quantification

Total serum T_4 concentrations in juvenile lake whitefish were quantified in duplicate using the T_4 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-T_4 treated fish and 25 μl serum for high-T_4 treated fish. This kit uses a competitive binding assay to quantify unknown total T_4 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. 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}. ^{125}I 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 μg × dl^{-1}) counts were > 20,000.

To confirm kit suitability for 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 × g for 10 min), transferred to a fresh tube of water-equilibrated resin and shaken overnight at 4 °C. Stripped serum was collected following a final centrifugation step (21000 × g for 30 min) and stored at −86 °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 μg dl^{-1} and run simultaneously with comparison kit standards. 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 [Concentration (μg × dl^{-1})]. The results of these experiments confirmed linearity and parallelism between the two curves indicating lake whitefish serum does not interfere with the immunoassay.

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 18S and 28S rRNA bands and RNA was subsequently stored at −86 °C until required. mRNA transcripts were reverse-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 sequences *accα, accβ, cpt1α, cpt1β, acox1*, a*cox3, cs, cox4* and *ef1α* were amplified, sub-cloned and sequenced using PCR-based sequencing methods and submitted to GenBank (*accα*, accession no. MH744405; *accβ*, accession no. MH744406; *cpt1α*, accession no. MH744409; *cpt1β*, accession no. MH744410; *acox1*, accession no. MH744407; *acox3*, accession no. MH744408; *cs*, accession no. MH744411, *cox4*, accession no. MH744412; *ef1α*, accession no. MH744413). 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 (Supplemental Table S1). 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 *accα, accβ, cpt1α, cpt1β, acox1*, a*cox3, cs, cox4* and *ef1α* and partial lake whitefish cDNA sequence information available on the NCBI database for *cox1* (GenBank accession no. JX960883.1, Supplemental Table S2). Primers for lake whitefish *β-actin* (GenBank accession no. KP893542.1) were obtained from previous studies in our laboratory (Stefanovic et al., 2016). 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 (Supplemental Table S2). Standard curves were also used to ensure amplification efficiency of each primer pair ranged between 90 and 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 (Supplemental Table S2). 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.

Cross-amplification checks were performed between *accα* and *accβ*, and *cpt1α* and *cpt1β* 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 Biotek, 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. No cross-amplification was detected between primers and non-target isoforms.

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

The activity of liver CS and COX was determined spectrophotometrically using chemicals purchased from Sigma Aldrich, unless otherwise noted. Assays were performed as described by Zak et al. (2017) and read at 25 °C using a Synergy HTX multi-mode spectrophotometer (BioTek).

2.6. Data handling and statistical analysis

2.6.1. Hepatosomatic index

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

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

where liver weight and body weight were in common units. A two-way ANOVA (GraphPad Prism 6, La Jolla, CA, USA) was used to test for effects of temperature and time on HSI, and any interaction between these two factors. Pairwise differences in HSI among temperature groups were identified using the Bonferroni's multiple comparison test. Similarly, a two-way ANOVA was also used to test for effects of TH treatment and time on HSI as well as any interaction between these two factors with pairwise differences in HSI among TH treatment groups were identified using the Bonferroni's multiple comparison test. Statistical analyses were accepted as statistically significant when $P \leq 0.05$.

2.6.2. Serum T_4 concentrations

Interpolation and statistical analysis of serum T_4 concentrations was completed in GraphPad Prism 6 statistical software. Total T_4 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 T_4 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 T_4 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 T_4 treatment were analyzed separately for low- and high-T_4 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-T_4 treatment groups to detect significant differences in serum T_4 levels among temperature groups. All differences were considered significant when $P \leq 0.05$.

2.6.3. RT-qPCR mRNA transcript abundance

Statistical analysis of liver *acca, accβ, cpt1a, cpt1β, acox1*, a*cox3, 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 Core Team; R Studio version 1.0.136; R Development Core and Team (2013)). 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 45,000 iterations using a thinning value of 20 and a burn-in value of 5000. Impacts of temperature were modeled using temperature, time and temperature × time as fixed factors. Impacts of TH manipulation were modeled separately for each temperature using hormone treatment (sham, low-T_4 or high-T_4), time and treatment × time as fixed factors. Models were run without priors since use of the reference genes *ef1α* and *β-actin* did not alter model fit or data interpretation. Posterior mean estimates of target transcript levels are plotted as $\log_2$ 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.

2.6.4. CS and COX activities

Activity rates for CS and COX activity are presented as μmol/mg tissue/min. 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. 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 $OD \times mM^{-1} \times min^{-1}$, respectively.

A two-way ANOVA (GraphPad Prism 6) was used to test for effects of temperature and time on CS and COX activities, and any interaction between these two factors. Pairwise differences in enzyme activities among temperature groups were identified using the Bonferroni's multiple comparison test. Similarly, a two-way ANOVA was also used to test for effects of TH treatment and time on CS and COX activities as well as any interaction between these two factors. Pairwise differences in enzyme activities among TH treatment groups were identified using the Bonferroni's multiple comparison test. Statistical analyses were accepted as statistically significant when $P \leq 0.05$.

3. Results

3.1. Temperature effects

A time course of temperature-induced effects on hepatic metabolism was examined in juvenile lake whitefish at 0, 4, 8 and 24 days following exposure to 13 (control), 17 or 21 °C. The hepatosomatic index (HSI), which provides an indication of hepatic energy reserves, was used as an indicator of broad metabolic shifts in the liver. Overall, we observed mild temperature sensitivity in the HSI of juvenile lake whitefish. There were no significant differences among temperature groups within individual time points, but the HSI in 21 °C-acclimated fish increased significantly between *day 4* and *day 24* (Fig. 1; Supplemental Table S3). Neither temperature nor thyroid hormone treatment altered length, weight or condition factor (data not shown).

A more detailed analysis of potential metabolic remodelling following temperature change was performed via mRNA and activity quantification of enzymes associated with lipid and oxidative metabolic pathways. Changes in the balance between fatty acid biosynthesis and mitochondrial β-oxidation were estimated by mRNA abundance of the *acc* isoforms, *accα* and *accβ*, and *cpt1* isoforms, *cpt1α* and *cpt1β*, respectively. Both *accα* and *accβ* were sensitive to temperature but were

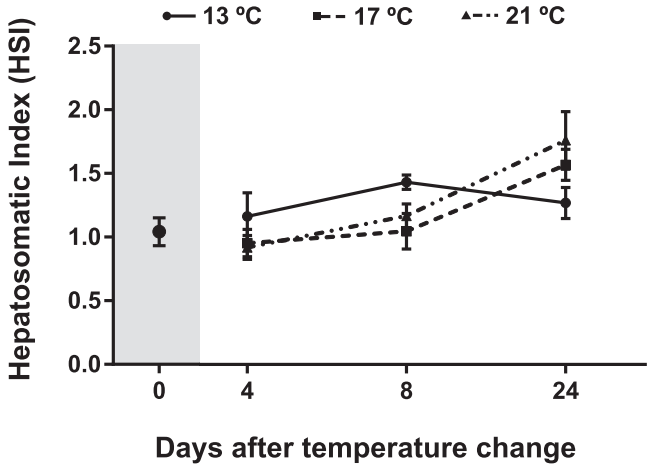

Fig. 1. Hepatosomatic index (HSI) of juvenile lake whitefish exposed to 13 (control), 17 or 21 °C for 0, 4, 8 or 24 days. Data points represent the mean HSI of 7–10 fish ± SEM, with day 0 representing the mean HSI from a single group of animals (n = 10) sampled immediately prior to the initiation of temperature change. Data for days 4, 8 and 24 days were analyzed using a two-way ANOVA to detect effects of temperature, time and their interaction on the HSI. No significant pair-wise differences ($P \leq 0.05$) were observed between treatments within each time point. Data for day 0 is for reference purposes and was not included in statistical analyses.

Table 1
Effects of elevated temperature exposure on mean mRNA abundance (log_2) of *acca*, *accβ*, *cpt1a*, *cpt1β*, *acox1*, *acox3* and *cox4* 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 (greyed row) within a time point.

Gene	Temp (°C)	Time		
		Day 4	Day 8	Day 24
acca	**13**	14.56	13.76	14.04
	17	13.55	12.83	14.67
	21	**12.37**^{a}	14.09^{ab}	14.70^{b}
accβ	**13**	8.77^{a}	7.29^{b}	7.62^{b}
	17	8.87^{a}	7.96^{ab}	7.75^{b}
	21	9.22	**9.15**	**8.65**
cpt1α	**13**	8.80^{a}	7.16^{b}	8.20^{ab}
	17	7.71	8.27	**6.80**
	21	8.06	7.37	7.44
cpt1β	**13**	7.32^{a}	5.78^{b}	5.71^{b}
	17	8.02^{a}	6.73^{ab}	6.19^{b}
	21	**8.85**^{a}	7.03^{b}	6.74^{b}
acox1	**13**	11.67	11.01	11.47
	17	11.22	11.41	11.22
	21	11.27	11.64	11.33
acox3	**13**	11.78^{a}	10.85^{b}	11.88^{a}
	17	11.54	11.66	11.49
	21	**12.83**^{a}	**12.02**^{ab}	11.62^{b}
cox4	**13**	14.58	14.62	14.86
	17	14.36	14.46	14.43
	21	13.93^{a}	14.79^{ab}	14.90^{b}

driven in opposite directions and on different time scales following the initiation of temperature change. On *day 4* following the onset of 21 °C, mean liver *acca* mRNA abundance was 4.6-fold lower than 13 °C controls, but recovered to control levels by *day 8* (Table 1 Supplemental Table S4). In contrast, mean *accβ* mRNA levels were elevated 3.6-fold in fish exposed to 21 °C, but not until *day 8* post-temperature-change (Table 1). *accβ* remained elevated on *day 24*, demonstrating long-term effects of elevated temperature on its abundance in liver. Exposure to the intermediate temperature of 17 °C had no significant effects on either *acca* or *accβ* at any time point. *cpt1α* mRNA abundance fluctuated in 13 °C control fish over the course the experimental period but was still depressed in 17 °C fish at *day 24* relative to controls (Table 1); a similar depression was not observed in fish exposed to 21 °C. In contrast, *cpt1β* was elevated over controls by exposure to 21 °C, but only at *day 4* post temperature change (Table 1). The remaining time points showed a decline in *cpt1β* abundance by either *day 8* or *24* in all three temperature groups.

We also examined the mRNA abundance of the peroxisomal β-oxidation enzymes, *acox1* and *acox3*, to investigate potential shifts in lipid pathways independent of mitochondria quantity. Overall, *acox1* was unaffected by exposure to either 17 or 21 °C (Table 1), while mean *acox3* abundance was elevated 2.1-fold following the onset of 21 °C on *day 4* and 2.3-fold on *day 8* before returning to control levels by *day 24* (Fig. 2F). No effect was observed on *acox3* following exposure to 17 °C.

Elevated temperature had mixed effects on mRNA abundance and activity of the oxidative enzymes citrate synthase (CS) and cytochrome *c* oxidase (COX) in juvenile lake whitefish. Relative to control fish maintained at 13 °C, mean *cs* mRNA abundance declined 2.3-fold on *day 4* following the onset of 21 °C (Fig. 2A; Supplemental Table S4). 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*. CS activity was also assessed to detect functional shifts in enzyme capacity associated with temperature change. Unlike *cs* mRNA levels, CS activity did not change in the short-term. 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*. (Fig. 2B, Supplemental Table S5). In addition to *cs*, we quantified mRNA abundance of two COX subunits. Abundance of the mitochondrial-encoded subunit, *cox1*, exhibited a 3.7-fold increase at *day 8* following the onset of 21 °C exposure (Fig. 2C; Supplemental Table S4). However, like *cs*, there were no long-term changes in steady state *cox1* mRNA abundance observed at *day 24*. Transcript abundance of the nuclear-encoded subunit, *cox4*, was not altered by temperature at any time point (Figure S1; Supplemental Table S4). Similar to CS, COX activity, which was used as a marker for overall functional capacity of the electron transport chain, sustained long-term changes in response to 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 (Fig. 2D; Supplemental Table S5). However, this effect was only significant for fish exposed to 21 °C.

3.2. Thyroid hormone effects

To examine the role of THs in mediating the temperature responses in juvenile lake whitefish liver, coconut oil implants containing either low-T_4 (1 μg T_4 × g body weight^{-1}) or high-T_4 (1 μg T_4 × g body weight^{-1}) were used to manipulate TH status in juvenile lake whitefish. T_4 implants were administered 7 days prior to the onset of temperature change and serum T_4 levels were quantified to confirm manipulation of TH status. While circulating serum T_4 levels were stable in sham-injected fish across all temperature groups, levels in low- and high-T_4 treated fish exhibited variability with respect to both temperature and time (Fig. 3, Supplemental Table S6). Serum T_4 levels were significantly elevated relative to sham-injected controls at all three temperatures in high-T_4 treated fish (Fig. 3B) and were also higher than sham levels in low-T_4 treated fish in some temperature groups (Fig. 3A). Although low-T_4 had no effect on serum T_4 levels in 13 °C groups at any of the 3 sampling time points or in 17 and 21 °C groups on *day 24*, elevations in T_4 levels among the low-T_4 fish were evident in 17 and 21 °C temperature groups on *days 4* and *8* post temperature change.

The mRNA levels of several lipid enzyme isoforms were altered by

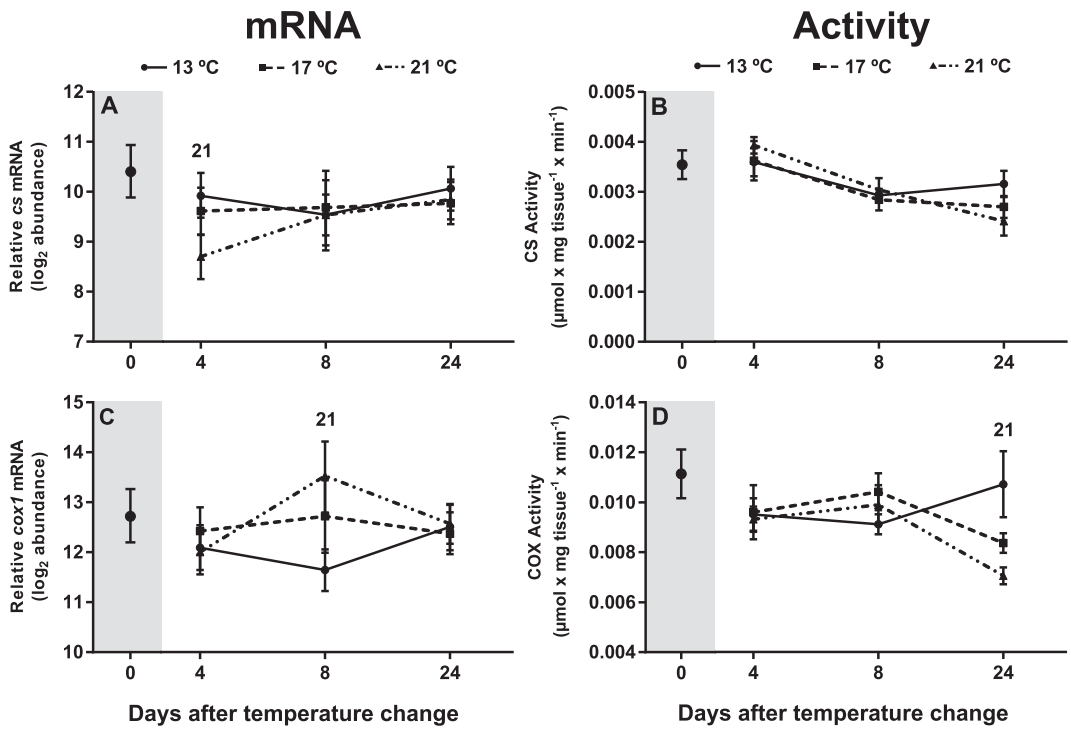

Fig. 2. Effects of elevated temperature exposure on *citrate synthase* (*cs*) mRNA (A), CS activity (B), *cytochrome c oxidase 1* (*cox1*) mRNA (C) and COX activity (D) in juvenile lake whitefish. mRNA and enzyme activities in in liver were quantified 0, 4, 8 or 24 days following exposure to 13, 17 or 21 °C. *cs* and *cox1* mRNA abundance are reported as $\log_2$ abundance and were modelled using a Bayesian MCMC sampling scheme (See methods). Plots represent posterior mean estimates ± 95% credible intervals. Mean (± SEM) CS and COX enzyme activities are reported as µmol × mg tissue^{-1} × min^{-1} and were analyzed using two-way ANOVA followed by Tukey post-hoc comparisons (see methods). Significant differences from fish maintained at 13 °C are indicated by the corresponding temperature being noted above the relevant data points. All data displayed for day 0 is for reference purposes and was not included in the displayed statistics.

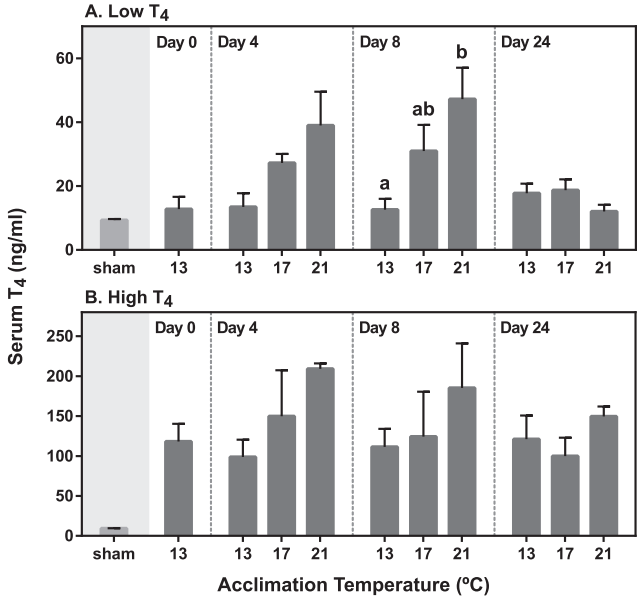

Fig. 3. Serum thyroxine (T_4) levels (ng × ml^{-1}) in juvenile lake whitefish treated with low-T_4 (A; 1 µg T_4 × g body weight^{-1}) or high-T_4 (B; 10 µg T_4 × g body weight^{-1}) coconut oil implants at 4, 8 and 24 d following exposure to 13, 17 or 21 °C. T_4 implants were administered 7 days prior to the onset of temperature change. Significant differences (Kruskal-Wallis and Dunn's multiple comparison tests) between temperatures within a time point are indicated by different lower-case letters above treatment groups. Plots represent mean of 3–8 serum samples ± SEM. Serum T_4 levels in sham-injected fish were not significantly different between temperature or time points (Supplemental Table S6) so were pooled and included in each plot for reference. Sham serum T_4 levels are not included in the statistics indicated on each plot.

TH, both in the presence and absence of elevated temperature stress. In fish acclimated to 13 °C, an increase in *accα* mRNA was observed on *day 0* in response to high-T_4 treatment only (Fig. 4A). Both low- and high-T_4 treatment increased *accα* mRNA abundance at this time point in fish at 17 and 21 °C (Fig. 4B and C; Supplemental Table S7). Additional short-term effects of exogenous T_4 treatment were observed for elevated temperature groups as both low- and high-T_4 treatment increased *accα* mRNA abundance on *day 8* in fish exposed to 17 °C (Fig. 4B) and low-T_4 treatment increased levels at *day 4* post temperature change at 21 °C (Fig. 4C). Likewise, *accβ* abundance was elevated by high-T_4 treatment at *day 0* and displayed temperature-dependent effects at subsequent time points. For instance, while neither low-T_4 nor high-T_4 treatment altered *accβ* transcript abundance at either of the two lower temperatures (Fig. 4D and E), low-T_4 treatment reduced *accβ* at both *day 4* and *day 8* in fish acclimated to 21 °C (Fig. 4F; Supplemental Table S7).

Of the two *cpt1* isoforms, *cpt1β* was more strongly affected by T_4 treatment than *cpt1α*. *cpt1α* mRNA abundance was stable in response to T_4 treatment at all temperatures except for low- and high-T_4 treated fish exposed to 21 °C on *day 8* where *cpt1α* mRNA abundance was elevated 3.0-fold and 7.4-fold above shams, respectively (Fig. 5C; Supplemental Table S7). In contrast, *cpt1β* exhibited short-term effects of TH treatment which were amplified at elevated temperatures. At 13 °C, mean *cpt1β* mRNA levels were 6.4-fold lower with high-T_4 treatment on *day 4* post-temperature change (Fig. 5D), but these declines expanded to 18.7-fold at 17 °C and 19.2-fold at 21 °C (Fig. 5E and F). Similarly, mean *cpt1β* mRNA levels in low-T_4 treated fish on *day 4* were also reduced relative to sham-injected fish by 7.0-fold and 16.9-fold at 17 and 21 °C, respectively. While the effects of TH on *cpt1β* in 13 °C fish were limited to *day 4*, effects of high-T_4 treatment could be observed at subsequent time points in elevated temperature groups. At 17 °C, mean *cpt1β* mRNA levels remained depressed on *day 8* post-temperature change, but recovered to sham levels by *day 24* (Fig. 5E). In contrast, mean *cpt1β* mRNA abundance in high-T_4 treated fish exposed to 21 °C was not

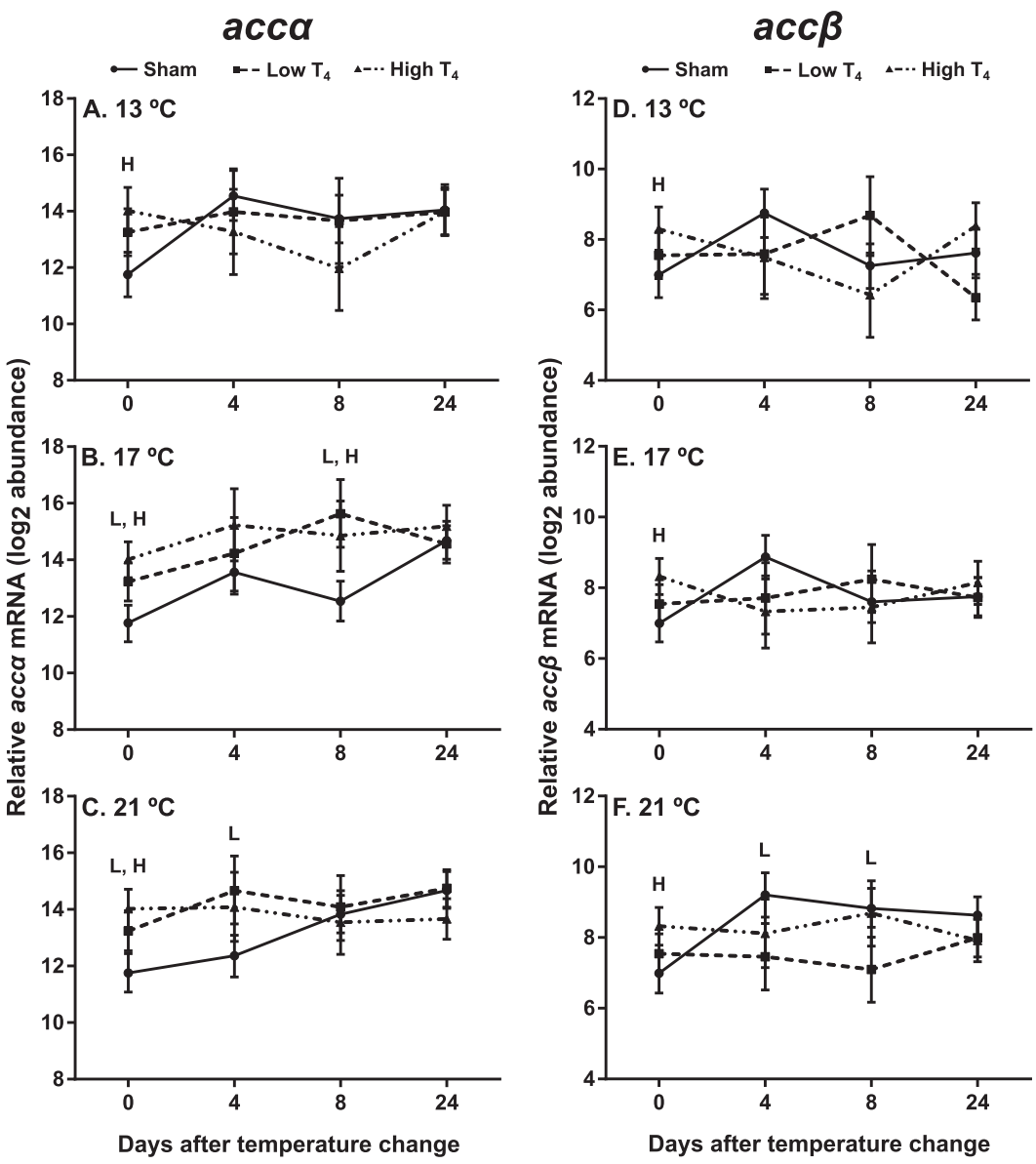

Fig. 4. Abundance of *acetyl-coA carboxylase* α (*accα*; A–C) and *acetyl-coA carboxylase β* (*accβ*; D–F) mRNA transcripts (log_2) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A, D), 17 (B, E) or 21 °C (C, F). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low-T_4 (1 μg T_4 × g body weight^{-1}) or high-T_4 (10 μg T_4 × g body weight^{-1}) 7 days prior to the onset of temperature change. Data was modelled within each temperature group using a Bayesian MCMC sampling scheme (see Methods). Data points represent posterior mean estimates from 7 to 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-T_4, H = high-T_4).

lower on *day 8*, but once again fell below sham levels at *day* 24 (Fig. 5F).

Similar to effects of temperature alone, *acox1* mRNA abundance was not altered by either low- or high-T_4 treatment at any temperature, aside from a mild increase at *day 8* in low-T_4 fish exposed to 17 °C (Fig. 6A–C). For *acox3*, we observed no effects of exogenous T_4 treatment in fish maintained at 13 °C (Fig. 6D), but *acox3* mRNA abundance was elevated by low- and high-T_4 treatment on *day 8* post temperature change at 17 and 21 °C, respectively (Fig. 6E and F).

Changes in TH status had temperature-dependent effects on *cs* mRNA abundance in juvenile lake whitefish liver. While neither low-T_4 nor high-T_4 treatment altered *cs* mRNA abundance of fish maintained at 13 °C (Fig. 7A; Supplemental Table S7), transient effects of TH manipulation were observed in response to temperatures in both 17 and 21 °C fish. At 17 °C, low-T_4 increased *cs* mRNA levels 2.5-fold over sham-injected controls, 8 days following the onset of temperature change (Fig. 7B), but 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 3.0-fold on *day 4* in sham-injected fish in response to the increase in temperature to 21 °C, but remained elevated, and significantly higher than sham-injected, in fish when treated with high-T_4 (Fig. 7C). Moderate recovery of mRNA levels was also observed on *day 4* in fish treated with low-T_4, but 95% credible intervals overlapped with sham-injected fish and high-T_4 groups. Changes in *cs* mRNA abundance with TH treatment did not translate into effects on CS activity levels. Rather, CS activity in fish treated with either low- or high-T_4 closely reflected responses observed in sham-injected fish at all three temperatures (Fig. 7D–F; Supplemental Table S8). In contrast to the temperature-dependent effects of T_4 manipulation on *cs* mRNA, *cox1* and *cox4* transcripts in low- and high-T_4 treated fish did not differ from sham-injected controls at any temperature (Fig. 8A–C and S2; Supplemental Table S7). Like CS, TH

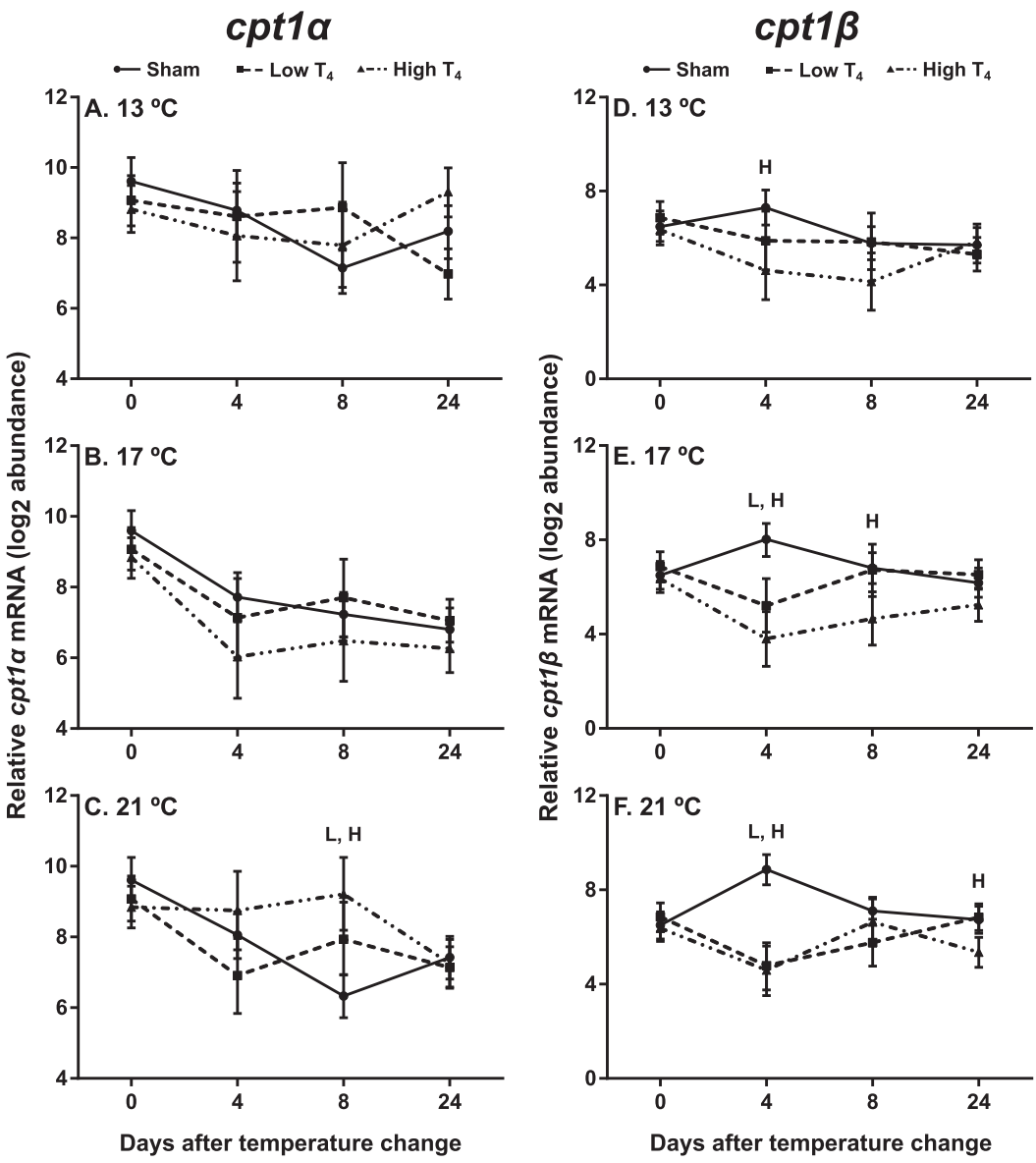

Fig. 5. Abundance of *carnitine palmityoltransferase 1α* (*cpt1α*; A–C) and *carnitine palmitoyltransferase 1β* (*cpt1β*; D–F) mRNA transcripts (log_2) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A, D), 17 (B, E) or 21 °C (C, F). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low-T_4 (1 μg T_4 × g body weight^{-1}) or high-T_4 (10 μg T_4 × g body weight^{-1}) 7 days prior to the onset of temperature change. Data was modelled within each temperature group using a Bayesian MCMC sampling scheme (see Methods). Data points represent posterior mean estimates from 7 to 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-T_4, H = high-T_4).

manipulation had limited effects on COX activity (Fig. 8D–F; Supplemental Table S8). While activity was mildly depressed by 1.39-fold and 1.27-fold on *day 0* in low- and high-T_4 treatment groups, respectively, no difference from shams due to low- or high-T_4 treatment were observed throughout the period following temperature change.

4. Discussion

4.1. Thermal impact on metabolic pathways

Metabolic remodeling in response to temperature 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 (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, elevated temperatures are an important physiological stressor, particularly for cool-water fishes that are well-adapted to colder temperatures. Furthermore, increases in mean global water temperatures, such as those predicted by certain climate change models (IPCC, 2014), make the understanding of elevated temperature responses in fish a priority. Collectively, our data suggest that juvenile lake whitefish are capable of metabolic remodeling following exposure to elevated temperature and that these effects can be triggered in response to modest, environmentally relevant temperature shifts.

Thermal preferences of lake whitefish decline towards adulthood (Holmes et al., 2002), but they are typically between 12.7 and 16.8 °C

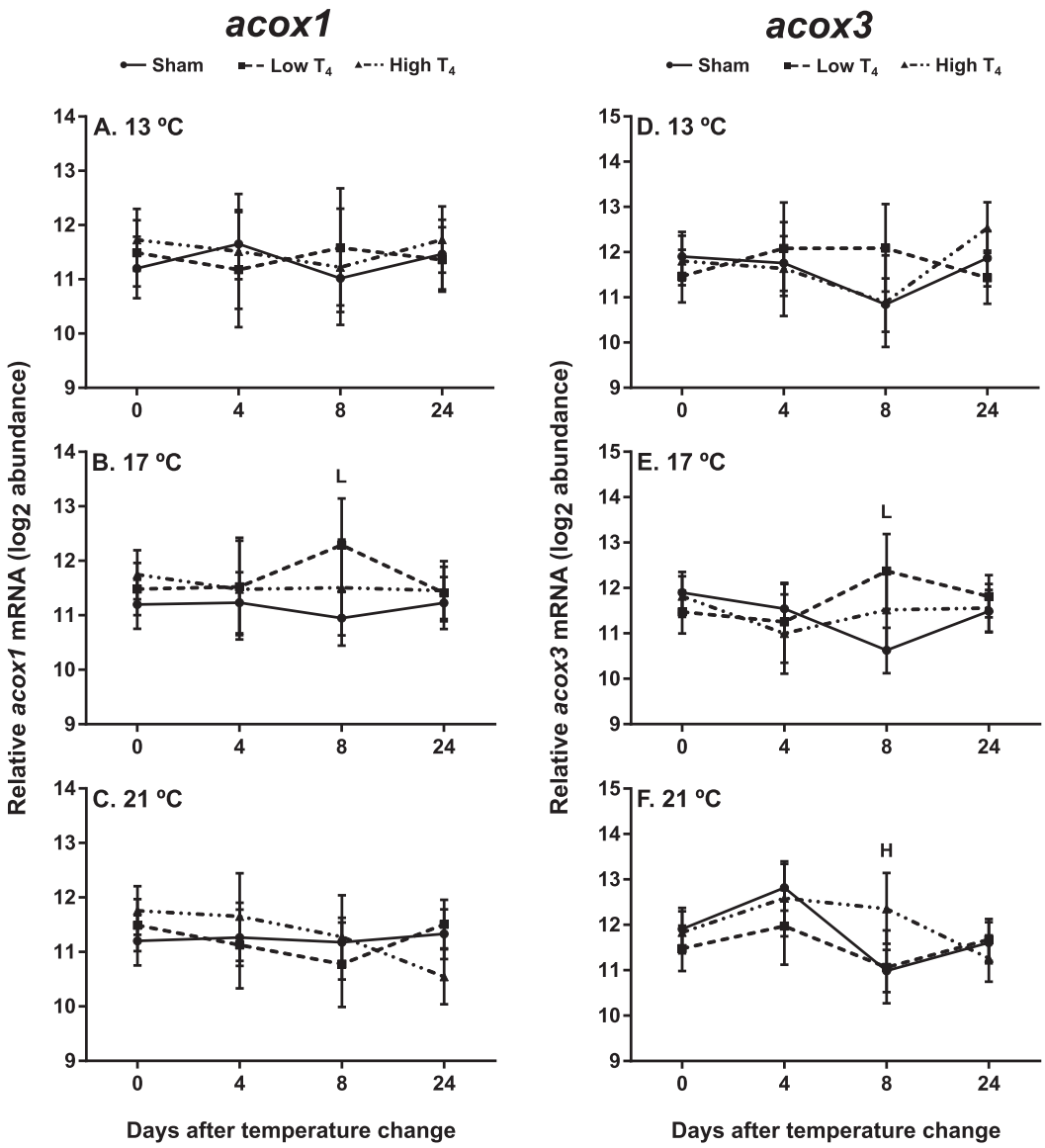

Fig. 6. Abundance of *acyl-coA oxidase 1* (*acox1*; A–C) and *acyl-coA oxidase 3* (*acox3*; D-F) mRNA transcripts (log_2) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A, D), 17 (B, E) or 21 °C (C, F). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low-T_4 (1 μg T_4 × g body weight^{-1}) or high-T_4 (10 μg T_4 × g body weight^{-1}) 7 days prior to the onset of temperature change. Data was modelled within each temperature group using a Bayesian MCMC sampling scheme (see Methods). Data points represent posterior mean estimates from 7 to 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-T_4, H = high-T_4).

for juveniles (Holmes et al., 2002, Wismer and Christie, 1987). As such, the temperatures chosen for this study represent the lower (13 °C), upper (17 °C) and extension (21 °C) of the preferred physiological range for juveniles. However, they are still below the lethal limits of this species, which vary between 24 and 26 °C in laboratory-acclimated juveniles (Zak and Manzon, unpublished) and 26.7 °C for certain wild lake whitefish populations (Edsall and Rottiers, 1976). With the exception of a mild depression in *cpt1α* mRNA at *day 24*, exposure to 17 °C had little effect on the transcription and/or stability of the mRNA transcripts we examined. In contrast, 21 °C had an effect on nearly all other transcripts, suggesting the need for metabolic compensation as temperatures extend beyond the preferred range of this species. The full extent of downstream metabolic remodeling associated with these types of transient fluctuations in mRNA abundance is unknown and, as such, the possibility of long-term impacts on enzyme synthesis and biological function remains to be determined. Unlike *accα*, *cpt1β*, *acox3*, *cs* and *cox1*, exposure to 21 °C resulted in sustained effects on *accβ* mRNA abundance as well as CS and COX activities. The observed depression in CS and COX activities by *day 24* is consistent with metabolic responses predicted by thermal enzymatic reaction rates whereby, as catalytic rate increases with temperature, less enzyme is required to achieve an equivalent physiological rate of activity. Interpreted alone, the observed changes in CS and COX activities suggest that exposure to 21 °C either does not alter overall metabolic demands in liver or a shift in metabolism to alternate fuel sources occurs which in part compensates for the decrease CS and COX activity.

The presence of warm-induced metabolic shifts in lake whitefish is supported by the mild increase in HSI between *day 4* and *day 24* in fish exposed to 21 °C. These results suggest an increase in hepatic glycogen (carbohydrate) and/or triglyceride (lipid) reserves (Johnston and Dunn, 1987) following exposure to elevated temperatures. However, increases in HSI following warm acclimation are unusual among fish species as most studies describe a higher HSI in cold- versus warm-acclimated fish (Arjona et al., 2010, 2011; Foster et al., 1993; Kullgren et al., 2013;

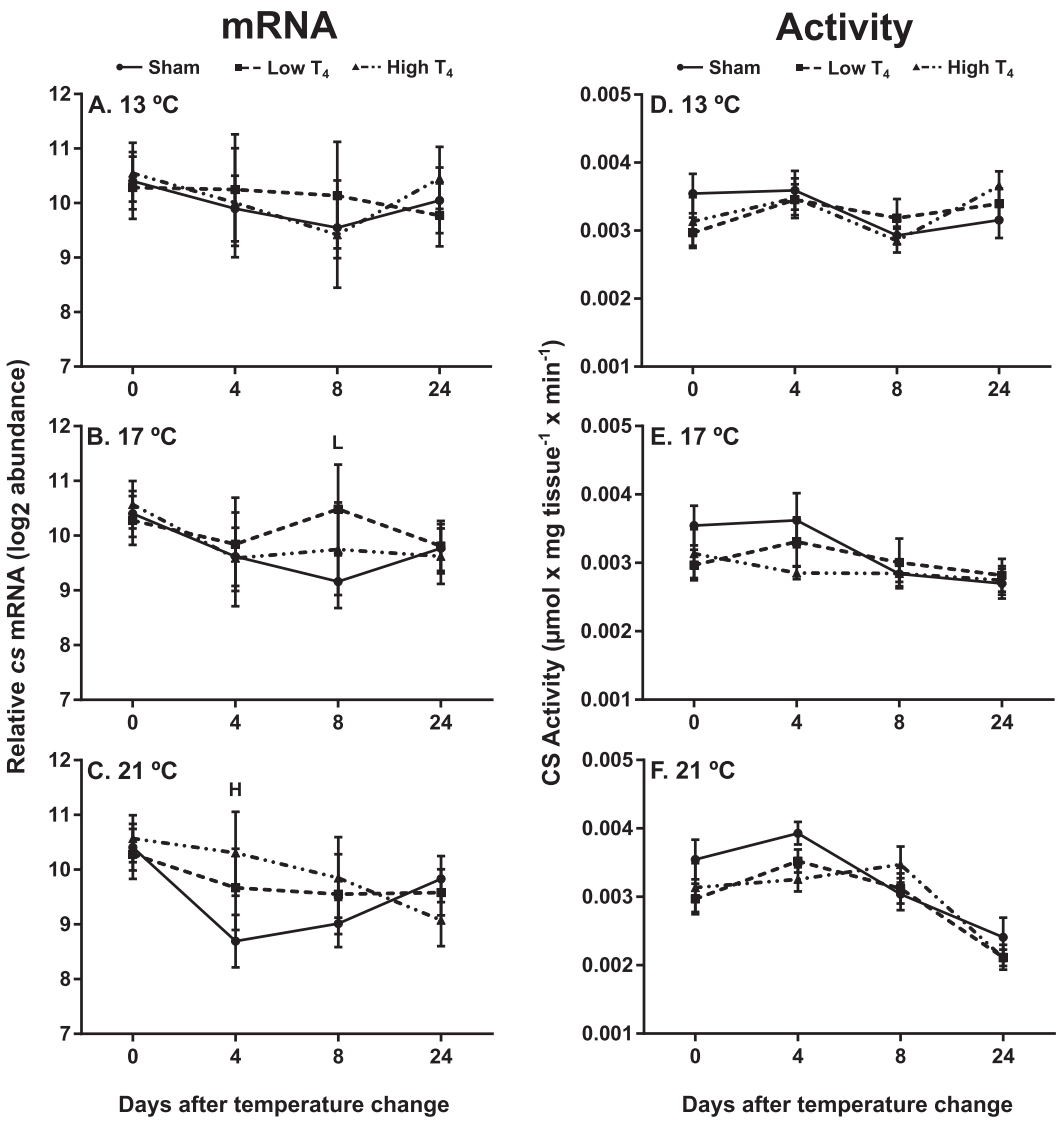

Fig. 7. Abundance of *citrate synthase* (*cs*) mRNA (A–C) and CS activity (D–F) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A, D), 17 (B, E) or 21 °C (C, F). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low-T_4 (1 µg T_4 × g body weight^{-1}) or high-T_4 (10 µg T_4 × g body weight^{-1}) 7 days prior to the onset of temperature change. mRNA abundance is reported as $\log_2$ abundance and were modelled using a Bayesian MCMC sampling scheme and the MCMC.qPCR package in R (Matz et al. 2013) with hormone treatment, time and treatment × time as fixed factors. Data points represent posterior mean estimates from 7 to 10 fish ± 95% credible intervals. Activity is reported as µmol × mg tissue^{-1} × min^{-1} and was analyzed using two-way ANOVA to examine effects of hormone treatment, time and treatment × time on enzyme activity followed by Tukey post-hoc comparisons. Data points represent mean activities of 7–10 fish ± SEM. Letters above treatment groups indicate the hormone treatment at which 95% credible intervals differed from sham-injected fish within each time point for *cs* mRNA and significant pair-wise comparisons ($P \leq 0.05$) between 13 °C and elevated temperature groups within a time point for CS activity (L = low-T_4, H = high-T_4).

Larsen et al., 2001; Lucassen et al., 2006; Orczewska et al., 2010), including previous studies in lake whitefish (Blier and Guderley, 1988). It is conceivable that in some species, acclimation both below and above typical preferred temperatures may increase HSI. For instance, increases in HSI following warm-acclimation have been observed in other laboratory-based experiments on certain salmonids, such as coho salmon (*Oncorhynchus kisutch*; Larsen et al., 2001). Variable responses, both between and within fish species, also suggests possible effects related to the duration of temperature exposure and/or nutritional status on regulation of the HSI following temperature change, or the presence of additional internal and/or environmental cues (Larsen et al., 2001). Increases in HSI and decreases in CS and COX activity with increased temperature could also reflect a preferential shift away from lipid β-oxidation toward carbohydrate metabolism. Northern temperate fish have been shown to enhance aerobic lipid β-oxidation when acclimated to cold winter temperatures relative to warm summer temperatures (Way-Kleckner and Sidell, 1985; Sephton and Driedzic, 1991; Bailey et al., 1999). Consistent with this were observations that warm water Amazonian teleosts preferential use anaerobic carbohydrate metabolism over aerobic lipid oxidation relative to cool water temperature species (Driedzik and Fonseca de Almeida-Val, 1996).

4.2. Thyroid hormone impact on metabolic pathways

Changes in metabolic function and performance elicited by THs in fish have not been unequivocally accepted. However, THs have been associated with numerous aspects of metabolic remodeling in mammalian species (Cioffi et al., 2013; Hulbert, 2000; Weitzel and Iwen, 2011) and remain promising candidates in developing a mechanistic understanding of metabolic changes induced by thermal acclimation. Many of the effects elicited by THs in fish and other ectotherms are temperature-dependent, whereby effects are observed at warm

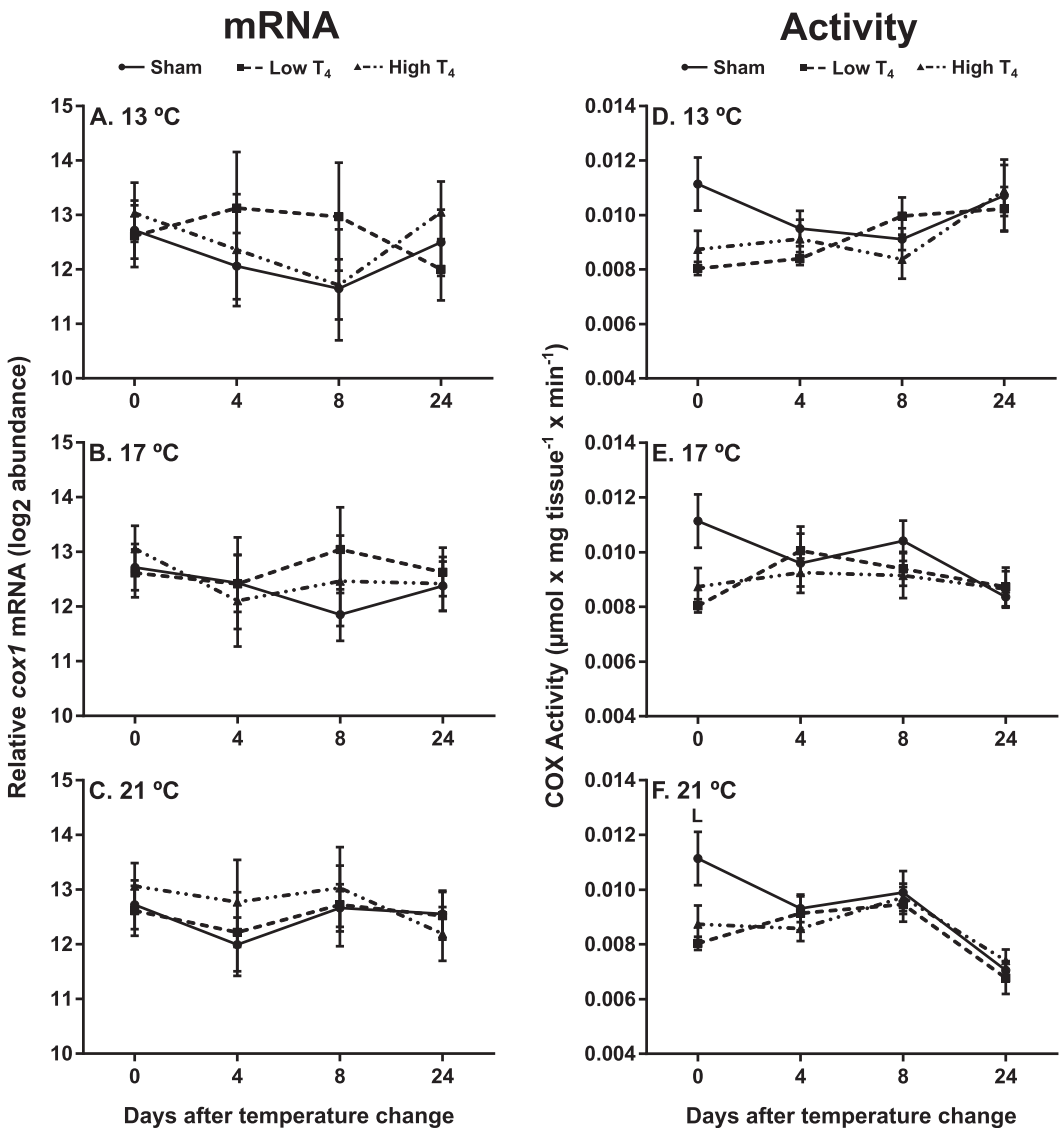

Fig. 8. Abundance of *cytochrome c oxidase 1* (*cox1*) mRNA (A–C) and COX activity (D–F) in juvenile lake whitefish following thyroid hormone manipulation and exposure to 13 (A, D), 17 (B, E) or 21 °C (C, F). Hormone status was manipulated via coconut oil implants containing vehicle (sham), low-T_4 (1 µg T_4 × g body weight^{-1}) or high-T_4 (10 µg T_4 × g body weight^{-1}) 7 days prior to the onset of temperature change. mRNA abundance is reported as $\log_2$ abundance and were modelled using a Bayesian MCMC sampling scheme and the MCMC.qPCR package in R (Matz et al. 2013) with hormone treatment, time and treatment × time as fixed factors. Data points represent posterior mean estimates from 7 to 10 fish ± 95% credible intervals. Activity is reported as µmol × mg tissue^{-1} × min^{-1} and was analyzed using two-way ANOVA to examine effects of hormone treatment, time and treatment × time on enzyme activity followed by Tukey post-hoc comparisons. Data points represent mean activities of 7–10 fish ± SEM. Letters above treatment groups indicate the hormone treatment at which 95% credible intervals differed from sham-injected fish within each time point for *cox1* mRNA and significant pair-wise comparisons ($P \leq 0.05$) between 13 °C and elevated temperature groups within a time point for COX activity (L = low-T_4, H = high-T_4).

temperatures, but not at cold (Gupta and Thapliyal, 1991). Our results largely support these observations and demonstrate a range of temperature-dependent effects of TH on mRNA abundance of metabolic enzymes in juvenile lake whitefish. Many studies examining the effects of THs on metabolic function in fish have focused on oxidative enzymes tightly associated with the TCA cycle and ETC (e.g., CS and COX). However, temperature-dependent effects of TH on metabolism were observed in transcript abundance of several key enzymes involved in lipid oxidative metabolism, suggesting these effects extend beyond those of the TCA and ETC to influence several aspects of metabolic function in juvenile lake whitefish.

Of the nine transcripts we examined, *acca*, *accβ*, *cpt1α*, *cpt1β*, *acox3* and *cs* all exhibited temperature-dependent expression in response to exogenous T_4 treatment. In all cases, effects of T_4 were largely absent at lower temperatures but became evident as temperature increased. The most consistent 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, *cpt1β*. High-T_4 treatment depressed *cpt1β* transcript abundance at *day 4* in fish maintained at 13 °C while effects of high-T_4 treatment at this time point were exaggerated at elevated temperatures and accompanied by effects of low-T_4 treatment (Fig. 5). These results depict strong short-term down-regulation of *cpt1β* by elevated THs in juvenile lake whitefish, which becomes more pronounced at higher temperatures. Since CPT1 activity is thought to be largely regulated at the transcriptional level in fish (Leaver et al., 2008), these results could be interpreted as a potential, biologically-relevant reduction in lipid oxidative capacity and preferential reliance on carbohydrate fuel sources which is further enhanced at elevated temperatures. Under most circumstances, mammalian studies suggest a pronounced catabolic effect of THs on both lipid

(Mullur et al., 2014; Pucci et al., 2000) and oxidative carbohydrate (Barker and Klitgaard, 1952; Cioffi et al., 2013; Etkin et al., 1940; Hulbert, 2000; Pucci et al. 2000) pathways. Furthermore, the majority of reports on TH and cellular metabolism in fish and other ectotherms also suggest the effects of TH are calorgenic in nature (Chen et al., 2015; Gupta and Thapliyal, 1991; Little et al., 2013; Peter and Oommen, 1993; Peter et al., 1996). Although the decrease in *cpt1β* may seem inconsistent with expected effects of TH on metabolic processes relative to mammalian models it might simply represent a shift in fuel substrate associated with the temperature increase that is enhanced by exogenous TH exposure. Fish have been shown to preferentially shift towards aerobic lipid oxidation with cold acclimation and warm water species tend to preferentially rely on anaerobic carbohydrate pathways relative to cool water species (Way-Kleckner and Sidell, 1985; Sephton and Driedzic, 1991; Bailey et al., 1999; Driedzik and Fonseca de Almeida-Val, 1996). Given the complexity and interconnected nature of these processes future work should take a systems based approach and make use of a transcriptomic, proteomic or metabolomic technologies to better decipher the key metabolic pathways regulated by TH and temperature.

While a clear depression was observed in *cpt1β* mRNA abundance in response to exogenous T_4 treatment, *cpt1α* abundance remained unaffected except for a transient increase above control levels at *day 8* following exposure to 21 °C (Fig. 5A). These results indicate distinct directional and temporal regulatory differences between *cpt1α* and *cpt1β* via THs. In most vertebrates, *cpt1β* expression occurs almost exclusively in cardiac and skeletal muscle (Leaver et al., 2008; Boukouvala et al., 2010), so the presence of detectable changes in hepatic *cpt1β* mRNA abundance in lake whitefish is particularly noteworthy. Studies conducted in other 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 *cpt1α* and *cpt1β* isoforms between several different tissues. For salmonids, the unique tissue expression profiles of *cpt1α* and *cpt1β* isoforms have 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 CPT1α and CPT1β 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, CPT1β 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 to exhibit increased inhibition by malonyl-coA in liver than skeletal or cardiac muscles. These observations are likely attributed to differences is *cpt1α* and *cpt1β* expression profiles (Morash et al., 2008). TH-induced changes in the relative proportions of hepatic CPT1α and CPT1β in lake whitefish are plausible given the results of the present study and could have the potential to regulate overall β-oxidation rates. This could ultimately result in altered functional associations between ACC and CPT1, particularly since malonyl-coA is considered one of the primary regulators of mitochondrial β-oxidation (Boukouvala et al., 2010).

Previous studies in our laboratory have demonstrated modest increases in liver CS activity with exogenous T_4 treatment in fish exposed to elevated temperatures (Zak et al., 2017) suggesting TH could have a biological effect on overall oxidative capacity by the TCA cycle and ETC in juvenile lake whitefish. However, neither low- nor high-T_4 treatment altered CS or COX enzyme activity in the present study, despite coinciding with exposure to a higher acclimation temperature (Fig. 6D–F and 7D-F). The most likely explanation for this disparity is due to differences in T_4 dose administration and the resulting serum T_4 concentration. Serum T_4 levels in our previous study were approximately 165- to 175-fold higher than sham injected controls (Zak et al., 2017) while those in the present study were elevated by both low- and high-T_4 treatment, but no more than 2.3-fold higher than the observed physiological range for salmonids (Dickhoff et al., 1978). As such, the results of the present study are likely reflective of physiological, rather than supraphysiological, responses. Despite the absence of distinct changes in CS and COX activities associated with physiological TH treatment, the results in our previous study do suggest regulatory potential via THs. It is possible that TH-induced effects on transcription and activity of these enzymes could become more pronounced as temperatures continue to increase, but this remains to be explicitly tested. 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, 2011) and TH action (Gupta and Thapliyal, 1991) are becoming increasingly relevant due to rising global temperatures associated with climate change.

Several studies have linked THs to a number of metabolic effects at elevated temperatures that are consistent with increased energy production in fish (Gupta and Thapliyal, 1991), including changes in O_2 consumption (Little et al. 2013; Peter and Oommen, 1993). While the measurement of maximal CS and COX activities provides an estimate of cell mitochondrial content (Battersby and Moyes 1998; Dalziel et al., 2005; O'Brien, 2011) and in some cases overall ETC capacity (Dalziel et al., 2005; O'Brien, 2011), it may not provide an accurate assessment of substrate turnover, also known as metabolic flux, or *in vivo* O_2 consumption rates (Schulte, 2015; Suarez et al., 1997). As such, the absence of direct changes in CS and COX activities does not rule out the possibility of changes in metabolic pathways upstream of the citric acid cycle and ETC, such as those involved in lipid catabolism. Furthermore, since certain rate-limiting enzymes we examined, such as *cpt1*, are thought to be regulated at the transcriptional level (Leaver et al., 2008), there is reason to predict that changes in mRNA abundance could have biologically-relevant effects on metabolic flux that are independent of overall ETC capacity. The full extent which these processes are also controlled by internal endocrine factors is unknown. However, the results of our study indicate some level of regulatory potential via THs on the abundance of rate-limiting enzymes of lipid pathways, particularly at elevated temperatures, and warrant further research directed towards understanding changes in both *in vivo* metabolic processing and the potential for TH influence in mediating these processes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.02.001.

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