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Research paper

Exogenous thyroid hormones regulate the activity of citrate synthase and cytochrome *c* oxidase in warm- but not cold-acclimated lake whitefish (*Coregonus clupeaformis*)

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ABSTRACT

Thermal acclimation is known to elicit metabolic adjustments in ectotherms, but the cellular mechanisms and endocrine control of these shifts have not been fully elucidated. Here we examined the relationship between thermal acclimation, thyroid hormones and oxidative metabolism in juvenile lake whitefish. Impacts of thermal acclimation above (19 °C) or below (8 °C) the thermal optimum (13 °C) and exposure to exogenous thyroid hormone (60 μg T_4/g body weight) were assessed by quantifying citrate synthase and cytochrome *c* oxidase activities in liver, red muscle, white muscle and heart. Warm acclimation decreased citrate synthase activity in liver and elevated both citrate synthase and cytochrome *c* oxidase activities in red muscle. In contrast, induction of hyperthyroidism in warm-acclimated fish stimulated a significant increase in liver citrate synthase and heart cytochrome *c* oxidase activities, and a decrease in the activity of both enzymes in red muscle. No change in citrate synthase or cytochrome *c* oxidase activities was observed following cold acclimation in either the presence or absence of exogenous thyroid hormones. Collectively, our results indicate that thyroid hormones influence the activity of oxidative enzymes more strongly in warm-acclimated than in cold-acclimated lake whitefish, and they may play a role in mediating metabolic adjustments observed during thermal acclimation.

1. Introduction

Temperature is an abiotic factor affecting all aquatic organisms. The ability to detect changes in ambient conditions and translate this information into appropriate biochemical and physiological responses is a requirement of all organisms that inhabit a dynamic thermal environment. In temperate regions, surface water temperatures can fluctuate more than 20 °C over an annual cycle (Bremer and Moyes, 2011; Dr. Rebecca North, 2017, personal communication). Thermal stratification in deep lakes can ease the level of thermal stress experienced by fish in temperate regions by providing thermal refuge in both summer and winter months. However, effective mechanisms to cope with long-term thermal stress are still required, particularly for fish inhabiting shallow lakes which do not stratify. Exposure to variable temperature conditions presents a unique challenge for ectothermic species which lack endogenous mechanisms to maintain a constant internal body temperature. Shifts in body temperature due to changes in ambient conditions may disrupt thermally-sensitive biochemical pathways that lose efficiency as temperature declines.

Physiological responses to thermal acclimation have been widely studied in teleosts, typically demonstrating compensatory increases in metabolic activity following cold acclimation at multiple levels of biological organization (Battersby and Moyes, 1998; Blier and Guderley, 1988; Duggan et al., 2011; Little et al., 2013; Lucassen et al., 2006; Sidell, 1980). Mitochondrial function, which can be partly assessed through the activity of oxidative enzymes such as citrate synthase (CS) and cytochrome *c* oxidase (COX), is of particular interest in metabolic studies due to its relationship to electron transport and ATP production. In rainbow trout (*Oncorhynchus mykiss*), cold acclimation to 4 °C has been shown to increase CS activity by approximately 40% in red muscle and 70% in white muscle (Battersby and Moyes, 1998). Likewise, increases in CS activity as great as 150% have been reported following similar long-term cold exposure in Atlantic cod (*Gadus morhua*; Lucassen et al., 2006). While CS activity tends to exhibit expected responses to temperature change, acclimation effects on COX activity are less

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consistent. For instance, Battersby and Moyes (1998) reported significant increases in red and white muscle COX activities in rainbow trout with cold acclimation, while Guderley and Gawlicka (1992) observed no change in COX enzyme capacity in either tissue following long-term cold exposure in the same species. Furthermore, a significant reduction in COX enzyme capacity was identified in lake whitefish (*Coregonus clupeaformis*) red and white muscle with cold acclimation (Blier and Guderley, 1988), rather than the increases anticipated by attempts to compensate for reduced enzyme efficiency at low temperatures. Variable responses to thermal acclimation observed among multiple fish species suggests that these metabolic adjustments may be influenced by additional internal and/or environmental cues (Bremer and Moyes, 2011).

Although numerous studies have examined the impact of thermal acclimation on aspects of physiology and metabolism in fish, little is known about the contributions endocrine signaling pathways make to these observed adjustments. However, studies in mammalian systems have identified thyroid hormones (THs) as key regulators of oxidative metabolism and mitochondrial biogenesis (Cioffi et al., 2013; Hulbert, 2000; Sheehan et al., 2004; Weitzel and Iwen, 2011). These effects are thought to be mediated primarily by 3′,5,3-triiodothyronine (T_3, Hulbert, 2000), which is metabolized from its precursor, thyroxine (T_4), via enzymatic removal of the 5′ iodide. As described in recent reviews (Cioffi et al., 2013; Weitzel and Iwen, 2011), T_3 most commonly interacts with DNA-bound retinoid-X-receptor/TH-receptor heterodimers to alter gene transcription. Activation of the receptor complex by T_3 leads to an increase in expression of the transcription factor peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α), which coordinates the expression of numerous additional genes involved in mitochondrial biogenesis (Cioffi et al., 2013; Weitzel and Iwen, 2011). While the effects of THs on mammalian metabolism are thought to occur through these mitochondrial biogenetic pathways (Cioffi et al., 2013; Weitzel and Iwen, 2011), further research is necessary to evaluate the effects of THs on mitochondrial function in teleosts. Given the strong association between THs and mammalian metabolism, we hypothesized that THs may also be involved in mediating temperature-induced metabolic adjustments in ectotherms, either by similar or alternative mechanisms. Furthermore, changes in CS and COX activities have previously been correlated with modifications in TH status and PGC1α expression in some fish species (LeMoine et al., 2008; Sheehan et al., 2004).

Lake whitefish is a cold-adapted species that inhabits freshwater systems across North America and is exposed to a wide range of temperatures across its natural geographical range. The preferred temperature of juvenile lake whitefish ranges from 12.7 to 16.8 °C (Holmes et al., 2002), but this species is known to inhabit shallow inland lakes with seasonal temperatures that fall outside this range. For instance, lake whitefish can be found in Blackstrap Lake in Dundurn, SK (51 °N, 106.4 °W), which is a man-made reservoir with an average depth of 5.15 m and maximum depth of 9.4 m (Hwang et al., 1975). Winter water temperatures of Blackstrap Lake can fall below 4 °C (Pernica et al., in press) and peak summer water temperatures exceeding 23 °C have been recorded for up to 7.7 m (Smith, 1978; Dr. Rebecca North, 2017, personal communication). Furthermore, the shallow depth profile of this lake precludes thermal stratification and the potential for thermal refuge, even at its deepest point. Due to temperature fluctuations faced in its native environment and previous reports on metabolic remodelling following thermal acclimation in this species (Blier and Guderley, 1988), we predicted lake whitefish would provide a suitable candidate to study the combined effects of thermal acclimation and TH manipulation. To date, relatively few studies have looked at the role of THs on the activity of CS and COX in cool-water fish. Little et al. (2013) examined the effects of hypothyroidism on zebrafish (*Danio rerio*), showing an increase in COX activity in the tail muscle of warm-acclimated animals. Likewise, T_4 treatment has been associated with increases in liver COX activity in climbing perch (*Anabas testudineus* Bloch; Peter and Oommen, 1989b; Peter et al., 1996) and gill COX activity in tilapia (*Oreochromis mossambicus*; Shivakumar and Jayaraman, 1984). While these studies indicate a relationship between TH and the regulation of oxidative metabolism in fish, no studies have yet been conducted on temperate species that experience large annual thermal fluctuations. The aim of the current study was to determine the extent to which THs mediate oxidative metabolism in cold-adapted, temperate fishes during long-term thermal acclimation events. To this end, we examined CS and COX enzyme capacity (activity) in liver, heart, red muscle and white muscle in lake whitefish following a multifactorial experimental protocol combining acclimation above or below thermal optimum with exogenous T_4 treatment. Our results highlight temperature- and tissue-specific changes in metabolic activity in response to both thermal acclimation and hormone manipulation, and could indicate potential shifts in metabolic processes due to hyperthyroidism in this species.

2. Materials and methods

2.1. Animal husbandry

Lake whitefish (*C. clupeaformis*) were collected from Lake Huron, ON, Canada in the winter of 2012 using 12–16 h gill net sets. Ova and milt were stripped from multiple spawning fish, pooled and ova were dry fertilized *in vitro* for 4 min. Fertilized ova were subsequently placed in 0.5% iodine in lake water for 30 min to disinfect, then thoroughly rinsed three times with additional fresh lake water. Embryos were placed in 1 L bottles (<10,000 embryos L^{-1}), packed on ice and shipped by same day air transport to the University of Regina Aquatics facility. Following fertilization, embryos were maintained at 3–4 °C in 2 L mini-hatching bell jars (Aquatic Ecosystems Inc. Apopka, Florida, USA) either until hatch, or until 60 d post fertilization (dpf) where embryos were transferred to petri dishes and raised at 3–4 °C until hatch. Synchronous hatching of all embryos was induced by increasing water temperature to 8 °C at a rate of approximately 0.5 °C h^{-1} at 145 dpf. Post-hatch, lake whitefish were maintained with a 12 h light:dark photoperiod in 60 L glass aquaria kept at 10 °C under continuous filtration and fed Otohime fish feed (Reed Mariculture, California, USA). Ninety-five days post-hatch, fish were transferred to 1700 L fiberglass holding tanks kept at 11–13 °C and fed *ad libitum* with a diet of EWOS Pacific Salmon fish feed (EWOS, BC, Canada). Water changes were completed three times per week using dechlorinated City of Regina water. Experiments were completed on fish 18 months post-hatch and all experimental procedures were carried out in accordance with the Canadian Council on Animal Care guidelines and approved by the University of Regina President's Committee on Animal Care.

2.2. Experimental design, hormone treatments and tissue collection

A two-factor experimental design involving TH status and temperature was used to determine if TH regulates mitochondrial (oxidative) metabolism and contributes to thermal acclimation. Juvenile lake whitefish were randomly divided into three temperature groups, 8 (cold), 13 (optimal) and 19 °C (warm). Fish in 8 and 19 °C temperature groups were further divided into two thyroid status experimental groups, control (euthyroid) and T_4 (hyperthyroid). Exogenous T_4 was administered to animals via coconut oil

implants. This approach allowed for long-term changes in TH status while avoiding daily water changes required for immersion methods and accompanying handling stress (Raine et al., 2010). The overall study used a total of 25 fish; each thyroid status group in both cold and warm acclimation groups consisted of a sample size of 5 (N = 20), with an additional 5 sham-injected control fish maintained at 13 °C. Control animals at all three temperatures received sham coconut oil implants containing vehicle (4.4% DMSO, BDH Chemicals, VWR International, Mississauga, ON, Canada) only. Thyroxine implants administered to cold- and warm-acclimated fish consisted of vehicle, plus 4 μg T_4 μl^{-1} coconut oil. Following anesthesia with 0.03% 2-phenoxyethanol (Sigma-Aldrich, Oakville, ON, Canada), implants were injected into the body cavity at a volume of 15 μl g^{-1} body weight (60 μg T_4 g^{-1} body weight). This dosage is in accordance with previous studies using implants to manipulate TH levels in juvenile rainbow trout (Raine et al., 2010). Following TH treatments, fish were maintained at 13 °C for two weeks, after which tank temperature was raised or lowered by 0.3 °C h^{-1} to reach the desired temperature. Fish were maintained at the final acclimation temperature for four weeks prior to sampling.

Tissue collection was performed following anesthesia with 0.03% 2-phenoxyethanol. Blood was collected from the caudal vein and permitted to clot overnight at 4 °C. Serum was collected following two centrifugation steps (4000 *g* for 5 min and 3000 *g* for 3 min) and stored at −80 °C. Remaining tissues (liver, heart, red muscle and white muscle) were excised rapidly following euthanasia via transection of the spinal cord, snap frozen on liquid N_2 and stored at −80 °C until use.

2.3. Serum thyroid hormone quantification

Serum T_4 and T_3 concentrations were quantified by direct radioimmunoassay as described by Dickhoff et al. (1978) and modified by McCormick et al. (1995). Samples were assayed in duplicate with statistics performed on the mean of technical replicates. Thyroid hormone concentrations exceeding 800 ng ml^{-1} were beyond the reliable detection limit of the radioimmunoassay standard curve and were, therefore, reported as 800 ng ml^{-1}. In total, this censoring was applied to four T_4 measurements from one cold-acclimated, T_4-treated fish and three warm-acclimated, T_4-treated fish.

2.4. Homogenization and enzyme assays

All chemicals used to complete enzymatic assays were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise specified. Tissues were suspended in 10 volumes of chilled homogenization buffer (50 mM Hepes, 1 mM EDTA, 0.1% Triton X-100, pH 7.4) and homogenized for 2 min in chilled 5 ml Wheaton glass/Teflon tissue homogenizers (Wheaton, Millville, NJ. USA). Liver, heart, and red muscle tissue were homogenized without prior processing; white muscle was pulverized using a stainless steel mortar and pestle submerged in liquid N_2 immediately before homogenization. Assays on each tissue type were optimized to 0.06–0.12 change in optical density per minute (ΔOD min^{-1}) for CS and 0.03–0.06 ΔOD min^{-1} for COX using serial dilution series to provide traces that were linear for the entire assay period and were completed in triplicate. Reactions were prepared on ice in 96-well plates and transferred to an EPOCH spectrophotometer (BioTek) housed at 21 °C for quantification. Assay methods were modified from published protocols (Moyes et al., 1997).

Cytochrome *c* oxidase activity was determined within 30 min of homogenization to avoid potential loss of activity over time. Homogenates were incubated in COX incubation solution (50 mM Tris, pH 8.0, 0.5% Tween 20) for 5 min to aid in membrane solubilization and the reaction was initialized by the addition of reduced cytochrome *c* to a final concentration of 0.048 mM and a final assay volume of 210 μl. Absorbance was read immediately at 550 nm to detect enzymatic oxidation of cytochrome *c*. Reduced cytochrome *c* (2 mM, Calzyme Laboratories, San Luis Obispo, CA, USA) was prepared in 50 mM Tris, pH 8.0 by addition of excess ascorbate followed by exhaustive dialysis in 50 mM Tris, pH 8.0 at 4 °C using 32 mm standard regenerated cellulose dialysis tubing (6–8 kDa MWCO, Spectrum Laboratories, CA, USA). Dialysate was replaced every 8–16 h. Optical density ratios for 280:550 nm and 550:565 nm between 1.1–1.3 and 10–20, respectively, were used to ensure full reduction of cytochrome *c* prior to storage at −80 °C.

Citrate synthase activity was determined from homogenates that had undergone one freeze–thaw cycle following confirmation that it led to no appreciable change in enzyme activity. 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 using a no-OAA control well and was subtracted from experimental wells.

Enzyme activities are expressed as a function of total protein concentration using the equation,

$$Activity = \left(\frac{\Delta OD * min^{-1}}{(L * \epsilon * \rho)}\right)\left(\frac{V_a}{V_s}\right)$$

where L is optical path length (cm), ε is molar absorptivity (OD mM^{-1} cm^{-1}), ρ is protein concentration (mg ml^{-1}), V_a is total assay volume (ml) and V_s is homogenate volume (ml). Change in optical density per minute was determined using the final 240 s of CS assay traces and the first 150 s of COX assay traces. Molar absorptivity values used for CS and COX enzyme activity calculations were 13.6 and 28.5 OD mM^{-1} min^{-1}, respectively. Protein concentration of homogenates was determined using the Pierce bicinchononic acid protein assay kit (ThermoScientific, USA) according to manufacturer's directions using 5 μl homogenate and an A:B reagent ratio of 50:1. Enzyme activity from technical triplicates was averaged and replicate values falling outside one standard deviation from the triplicate mean were eliminated from the data set.

2.5. Data and statistical analysis

Normality and homoscedasticity of CS and COX activity data was confirmed in the R environment (R core team, R Foundation for Statistical Computing, Vienna, Austria) via Shapiro-Wilk and Bartlett's tests, respectively ("stats" package). Parametric analyses on CS and COX activity were conducted using GraphPad Prism 6 statistical software (La Jolla, CA, USA). One-way analysis of variance (ANOVA) was performed on data from sham-injected control fish from each acclimation temperature to determine effects of temperature alone on enzyme activity and serum T_3 and T_4 concentrations, followed by Tukey's post hoc comparisons. Two-way ANOVA was used to determine effects of temperature, hormone, and temperature × hormone interaction on enzyme activity in 8 and 19 °C acclimated fish. Two-way ANOVA was followed by Bonferroni's multiple comparison tests to detect pair-wise differences in enzyme activity of each tissue type following T_4 treatment. Since no interaction effects were observed using two-way ANOVA, multiple comparison tests were completed within a single acclimation temperature. Serum T_4 and T_3 data within each cold and warm acclimation temperature were analyzed with Mann Whitney (non-parametric) tests to determine the effect of thyroid hormone

treatment on serum T_3 and T_4 concentrations within a temperature group. All results were considered significant at $P < 0.05$. Data in figures are presented as box-plots depicting the minimum value, first quartile, median, third quartile and maximum value for each treatment group.

3. Results and discussion

3.1. Warm acclimation induces tissue-specific changes in CS and COX activities

Effects of thermal acclimation on oxidative metabolism in lake whitefish was assessed by acclimating fish to 8, 13 or 19 °C for four weeks and quantifying activity of CS and COX in liver, heart, red muscle and white muscle. We observed a 25% decrease in mean CS activity in liver following warm acclimation, relative to 13 °C control animals ($P < 0.01$, Fig. 1A, Table 1). This was in contrast to red muscle where mean CS activity increased by approximately 36% ($P < 0.01$) and mean COX activity increased by 23% ($P = 0.04$) under the same temperature conditions (Fig. 1, Table 1). According to the general understanding of enzyme kinetics, activity increases with temperature, thereby reducing the amount of enzyme required to generate a given end product. As a result, the warm-induced increase in CS and COX activities observed in red muscle is opposite to expected effects in response to a prolonged increase in ambient temperature. Nevertheless, similar results have been observed in previous studies conducted on lake whitefish white muscle (Blier and Guderley, 1988), goldfish liver (*Carassius auratus* L.; LeMoine et al., 2008), and the muscle of two stenothermal Antarctic fish, Antarctic eelpout (*Pachycara brachycephalum*; Windisch et al., 2011) and marbled rockcod (*Notothenia rossii*; Strobel et al., 2013), during long-term thermal acclimation experiments. These results suggest a substantial effect of high temperature on lake whitefish red muscle with a considerable increase in the capacity for oxidative metabolism and ATP production relative to fish maintained at thermal optimum. The primary function of red muscle is to power sustained swimming activity (Rome et al., 1988). Therefore, it is possible that the observed differences in this tissue following warm acclimation may be more related to increased energy demand resulting from elevated basal metabolic rate or locomotory and behavioural changes than to basic biochemical changes on the cellular level. Decreases in water temperature have been shown to reduce tail beat frequency and critical sustained swimming speed in fish (Bartolini et al., 2015; Little et al., 2013; Little and Seerbacher, 2013), so it is reasonable to predict the opposite trend as water temperature rises. Furthermore, anecdotal observations in the lab also strongly support increased swimming activity in warm-acclimated lake whitefish relative to fish maintained at optimal or cold temperatures (Zak and Manzon, personal observation). An increase in swimming activity could increase the demand for readily available energy, contribute to higher ATP requirements, and account for the increase in CS and COX activities observed in red muscle. It is interesting, however, that COX activity was not significantly altered following thermal acclimation in all other tissue types (Fig. 1B). This may reflect the relative insensitivity of COX to changes in ambient temperature compared to other metabolic enzymes (Blier and Guderley, 1988).

Numerous studies examining the effects of thermal acclimation on mitochondrial enzymes in teleosts have shown CS and COX exhibit a compensatory increase in activity following cold acclimation to accommodate a drop in enzyme efficiency at lower temperatures (Battersby and Moyes, 1998; Blier and Guderley, 1988; Bremer and Moyes, 2011; Duggan et al., 2011; Guderley and Gawlicka, 1992; Little et al., 2013; Lucassen et al., 2006). However, the results of the current study showed no effect of cold acclimation on either CS or COX activities in lake whitefish in any tissue type (Fig. 1A and B). Previous work to quantify Q_{10} values of metabolic enzymes in lake whitefish have indicated CS and COX activity rates show less dependence on temperature, even following thermal acclimation events, than other metabolic enzymes in the same species (Blier and Guderley, 1988). Thus, it is possible that lake whitefish are either well adapted to colder temperatures, or that the cold acclimation temperature chosen in the current study ($\Delta T = 5$ °C below optimal) was not sufficient to induce metabolic remodelling with respect to these two enzymes. Metabolic reorganization in fish requires a substantial energy investment (Blier and Guderley, 1988). Therefore, an increase in mitochondrial density may demand the presence of a threshold temperature that was not reached in this study. With respect to organismal physiology, these data suggest cellular level compensations in energy expenditure associated with long-term exposure to temperatures as low as

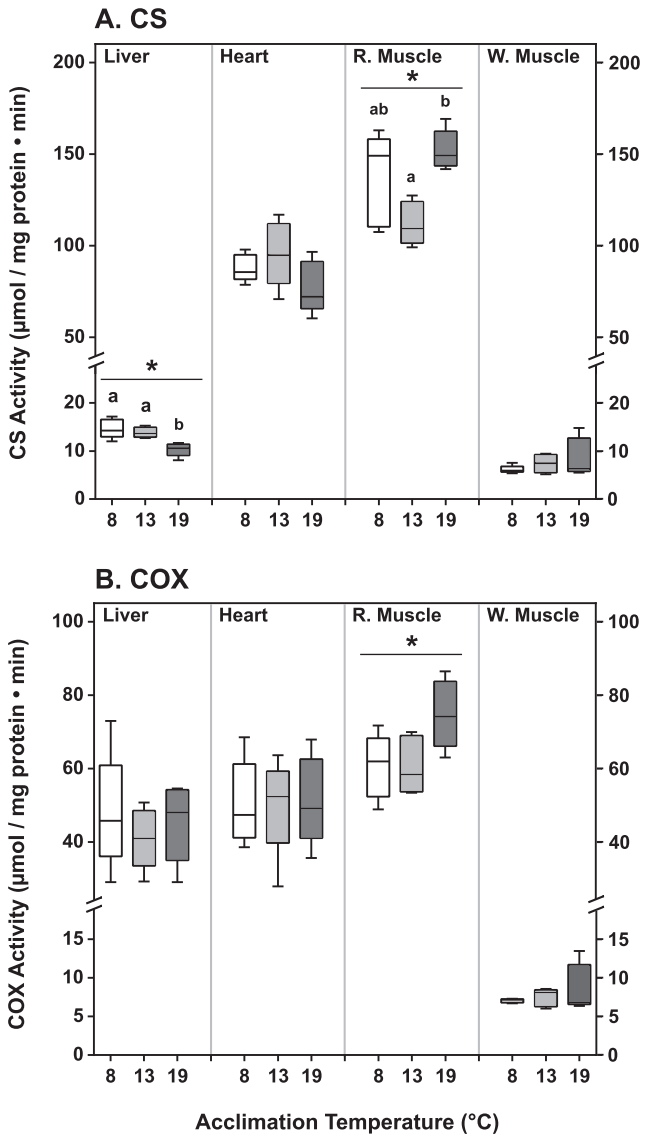

Fig. 1. Activity of CS (A) and COX (B) in lake whitefish liver, heart, red muscle and white muscle following thermal acclimation to 8, 13 or 19 °C. Enzyme activities were assayed at 21 °C from crude tissue homogenates and are expressed as µmol·mg protein^{-1} min^{-1}. Sample size for each treatment group is five with the exception of CS liver 13 °C where n = 4. Data were analyzed using one-way ANOVA followed by Tukey's post hoc analysis. Asterisks indicate significant differences in enzyme activity between temperature groups and significant pair-wise differences within each tissue are indicated by lowercase letters ($P < 0.05$). Boxes represent first and third quartiles and horizontal lines represent the median for each treatment group; whiskers indicate maximum and minimum values.

Table 1
One-way ANOVA statistics on CS and COX activity among fish acclimated to 8, 13 or 19 °C for four weeks.

	F	df_n, df_d	P
CS			
Liver	10.78	2, 11	<0.01**
Heart	2.26	2, 12	0.15
Red Muscle	7.00	2, 12	<0.01**
White Muscle	1.17	2, 12	0.34
COX			
Liver	0.42	2, 12	0.66
Heart	0.01	2, 12	0.99
Red Muscle	4.42	2, 12	0.04*
White Muscle	0.83	2, 11	0.46

Abbreviations: COX, cytochrome *c* oxidase; CS, citrate synthase, df_n, degrees of freedom (numerator); df_d, degrees of freedom (denominator); $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

8 °C are not required for lake whitefish. However, the lack of quantifiable thermal compensation in CS and COX activities between 8 and 13 °C acclimated fish in the current study does not eliminate the possibility that lake whitefish employ alternate mechanisms to compensate for a decrease in enzyme efficiency at colder temperatures. Increases in the hepatosomatic index have been reported following cold acclimation between 6 and 12 °C below thermal optimum in several fish species (Arjona et al., 2010; Foster et al., 1993; Larsen et al., 2001; Lucassen et al., 2006; Orczewska et al., 2010), including lake whitefish (Blier and Guderley, 1988). Changes in organ:body mass ratios in fish undergoing long-term acclimation events suggests thermal compensation in the liver may be controlled, in part, by an increase in tissue volume rather than changes in enzyme activities on the cellular level (Hardewig et al., 1999). From an ecological perspective, many of the physiological challenges imposed by temperature change may be at least partially offset though behavioural thermoregulation. Thermal stratification of deep lakes during winter months provides the opportunity for fish movement within the water column to escape extreme temperatures ordinarily requiring significant metabolic restructuring. Seasonal movements related to temperature have previously been described for lake whitefish living in the North American Great Lakes (Holmes et al., 2002). While these efforts have focused primarily on fish movements during spring and summer and shown migrations towards deeper water as temperatures increase, the presence of winter movements to accommodate thermal and/or spawning preferences has also been reported (Holmes et al., 2002). More severe metabolic challenges may be imposed on lake whitefish inhabiting shallow lakes or lakes that do not stratify due to temperature stress in both the winter and summer months, combined with limited thermal refuge.

Warm acclimation-induced impacts on CS and COX activities in liver and red muscle are not unexpected since both tissues are often associated with large-scale physiological changes following thermal acclimation in fish (Arjona et al., 2010; Foster et al., 1993; Larsen et al., 2001; Lucassen et al., 2006; Orczewska et al., 2010; Sidell, 1980). Analogous to effects on the hepatosomatic index, red muscle also experiences structural modifications with increasing water temperature, such as a reduction in mitochondrial volume and surface density (Orczewska et al., 2010), as well as declines in the proportion and diameter of red muscle fibres (Johnston and Lucking, 1978; Sidell, 1980). While no change in the proportion of red muscle fibres was noted in previous acclimation experiments using lake whitefish (Blier and Guderley, 1988), our study confirms that oxidative metabolism in red muscle is significantly affected at the enzyme level. The observed changes in liver and red muscle following long-term temperature shifts, in this and other studies (Battersby and Moyes, 1998; Foster et al., 1993; Guderley and Gawlicka, 1992; Hardewig et al., 1999; Orczewska et al., 2010; Strobel et al., 2013), suggest that these two tissues are largely responsible for mediating the physiological changes needed to maintain functional integrity of metabolic pathways under the studied thermal conditions (Hardewig et al., 1999).

3.2. Thyroxine exposure influences oxidative metabolism in warm-acclimated lake whitefish

An extensive body of research has been dedicated to understanding the effects of thermal acclimation on ectothermic metabolism, but there is little information the role of THs in these processes. Serum TH concentrations of lake whitefish were manipulated using coconut oil implants to assess the effects of hyperthyroidism on oxidative enzyme activities following thermal acclimation. Effectiveness of exogenous T_4 treatments were confirmed by quantifying serum T_3 and T_4 levels. Relative to control fish, treatment with exogenous T_4 implants resulted in a significant increase in mean serum T_4 concentrations of both cold- and warm-acclimated fish (Table 2). Serum T_3 concentrations were also significantly elevated following exogenous T_4 treatments in both temperature groups, but to a lesser extent than T_4 with mean serum T_3 concentrations in cold- and warm-acclimated fish increasing by 8-fold and 17-fold, respectively. Although T_4 treatments administered to lake whitefish resulted in supraphysiological serum T_4 levels, the levels of the more biologically-active T_3 hormone largely fall within the high physiological range reported for teleost fish (Dickhoff et al., 1978; Eales and Shostak, 1987). Even relatively high levels of T_3 and T_4 are relevant to the understanding of metabolic regulation by THs since administration of high TH concentrations are representative of effects elicited at lower concentrations in fish (García-G et al., 2004, 2007; Schmid et al., 2003). For instance, previous work in tilapia (*O. mossambicus*) showed a plateau in IGF-1 mRNA expression changes spanning a thousand-fold increase in administered T_3 (Schmid et al., 2003). Similarly, García-G et al. (2007) demonstrated that physiologically relevant concentrations of T_2 and T_3 induced similar effects on mRNA abundance of deiodinase enzymes in hypothyroid killifish (*Fundulus heteroclitus*) previously described for supraphysiological TH levels in the same species (García-G et al., 2004). The consistent effects of a wide range of TH concentrations suggested by these studies indicate that physiological effects induced by high TH concentrations are qualitatively similar to those induced at lower concentrations.

As previously described, warm acclimation alone decreased CS activity in liver and increased both CS and COX activities in red muscle (Fig. 1). However, in the presence of exogenous T_4 implants, warm acclimation resulted in a mean 43% increase in liver CS activity ($P < 0.01$) compared to sham-injected controls (Fig. 2A). Mean CS and COX activities in red muscle of T_4-treated, warm-acclimated fish decreased by 29% ($P = 0.01$) and 28% ($P = 0.02$), respectively (Fig. 2A and B). Thus, the same tissues and enzymes affected by warm acclimation are also those that are impacted, in the opposite direction, when combined with elevated THs. Impacts of THs on oxidative processes during thermal acclimation have previously been identified in other fish species (Little et al., 2013). Little et al. (2013) demonstrated that, relative to euthyroid fish, COX activity in zebrafish tail muscle was significantly elevated under hypothyroid conditions in warm-acclimated individuals. This was despite no initial effects of acclimation alone on COX activity in the euthyroid state. While the absence of THs appears to trigger more extensive remodelling of COX activity in response to warm acclimation, elevated TH levels, such as those achieved in the current study, seem to coincide with reduced metabolic adjustments following thermal acclimation in lake whitefish. This suggests a scenario in which oxidative enzymes are less sensitive to thermal acclimation in the presence of elevated THs and

Table 2
Mean ± SEM serum T_4 and T_3 concentrations (ng/ml) and Mann-Whitney statistics (U) in control and T_4-treated fish acclimated to 8 or 19 °C.

	Control	n	T_4-treated	n	U	P
T_4						
8 °C	2.79 ± 0.94	5	489.56 ± 124.42	5	0	0.01**
19 °C	4.62 ± 0.81	5	774.00 ± 26.00	4	0	0.02*
T_3						
8 °C	9.44 ± 1.37	5	77.05 ± 41.09	5	1	0.02*
19 °C	8.42 ± 1.10	5	143.48 ± 37.01	4	0	0.02*

Abbreviations: T_4, thyroxine; T_3, triiodothyronine; SEM, standard error of the mean; U, Mann-Whitney U statistic; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

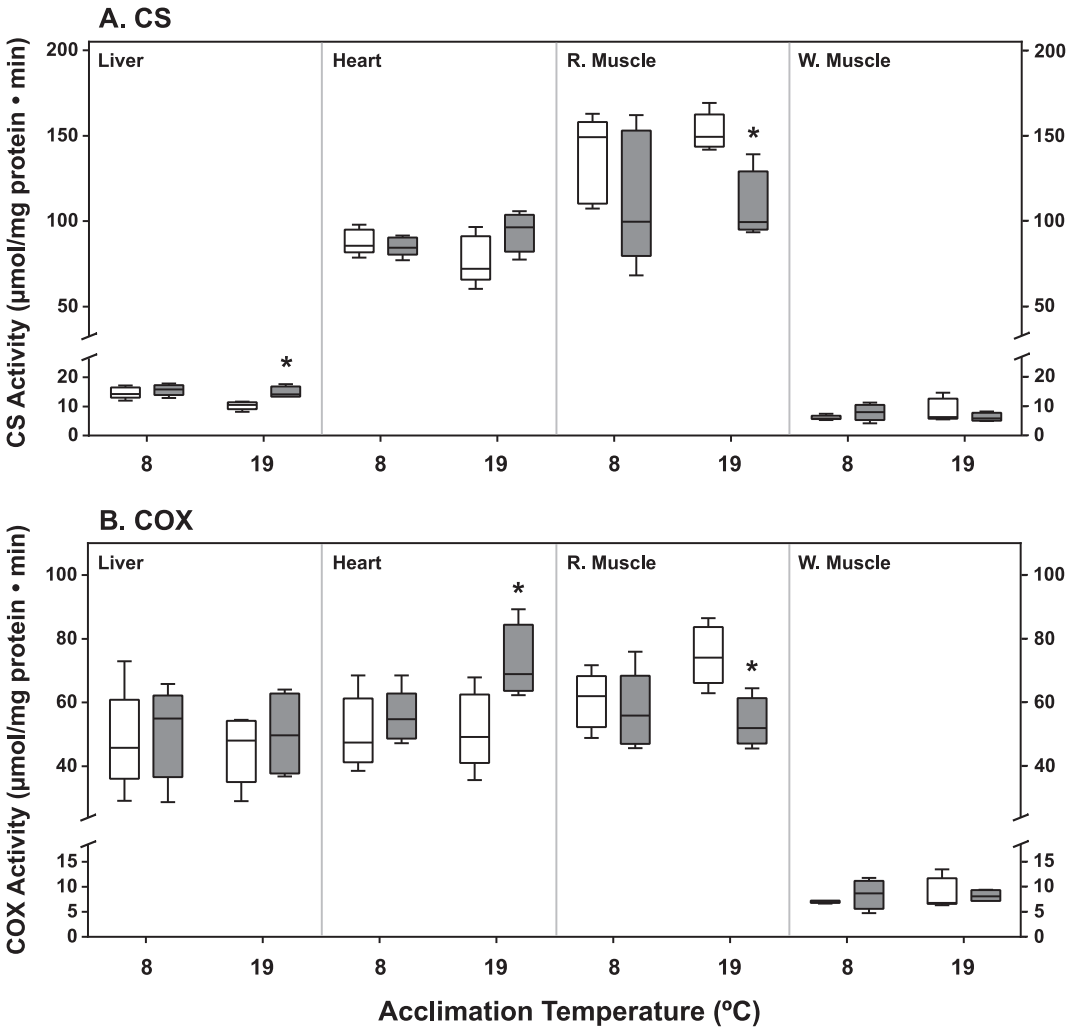

Fig. 2. Activity of CS (A) and COX (B) in lake whitefish liver, heart, red muscle and white muscle following hormone manipulation via coconut oil implants containing vehicle (control) or 60 µg T_4 g^{-1} of fish and thermal acclimation to 8 or 19 °C. Enzyme activities were assayed at 21 °C from crude tissue homogenates and are expressed as µmol·mg $protein^{-1}$ min^{-1}. Sample size for each treatment group is five with the exceptions of 8 °C white muscle COX control and all 19 °C T_4 fish, where n = 4. Data were analyzed using two-way ANOVA followed by Bonferroni multiple comparison tests. Significant pair-wise differences ($P < 0.05$) are indicated by asterisks above treatment groups and indicate differences between control and T_4-treated fish within a single acclimation temperature and tissue type. Boxes represent first and third quartiles and horizontal lines represent the median for each treatment group; whiskers indicate maximum and minimum values.

indicates that THs may play a role in maintaining metabolic homeostasis in fish subjected to prolonged temperature elevation. However, the biological mechanisms underlying this relationship are unclear. It is possible that THs participate in the regulation of oxidative enzymes either by eliminating the need to undergo metabolic restructuring triggered by long-term temperature shifts above thermal optimum, or by actively suppressing typical metabolic responses. In either case, elevated THs may confer a net energetic benefit in cool-water fish subjected to warmer temperatures by limiting energetic costs associated with extensive metabolic remodelling and mitochondrial biogenesis.

In addition to the observed effects of exogenous T_4 on CS and COX activities during warm acclimation in liver and red muscle, heart COX activity was also significantly increased by combined T_4 treatment and warm acclimation ($P = 0.02$, Fig. 2). In contrast, both CS and COX activities of white muscle were unchanged in response to both warm acclimation and hormone manipulation. These results highlight variable responses among tissue types

Table 3
Two-way ANOVA statistics on CS and COX activity in control and T_4-treated fish acclimated to 8 or 19 °C

	Acclimation Temperature			Hormone Treatment			Temperature x Hormone		
	F	df_n, df_d	P	F	df_n, df_d	P	F	df_n, df_d	P
CS									
Liver	9.90	1, 15	<0.01**	10.95	1, 15	<0.01**	4.32	1, 15	0.06
Heart	0.04	1, 15	0.85	2.30	1, 15	0.15	4.26	1, 15	0.06
Red Muscle	0.17	1, 15	0.69	7.99	1, 15	0.01**	0.69	1, 15	0.42
White Muscle	0.11	1, 15	0.74	0.05	1, 15	0.82	3.00	1, 15	0.10
COX									
Liver	0.06	1, 15	0.82	0.34	1, 15	0.57	0.03	1, 15	0.87
Heart	3.04	1, 15	0.10	6.72	1, 15	0.02*	2.50	1, 15	0.13
Red Muscle	1.30	1, 15	0.27	7.49	1, 15	0.02*	4.03	1, 15	0.06
White Muscle	0.40	1, 14	0.54	0.18	1, 14	0.68	0.70	1, 14	0.42

Abbreviations: COX, cytochrome *c* oxidase; CS, citrate synthase; df_n, degrees of freedom (numerator); df_d, degrees of freedom (denominator); $^*P \leq 0.05$; $^{**}P \leq 0.01$

following elevated serum T_4 and T_3 levels in lake whitefish. Much of this tissue-specificity may be attributed to upstream regulatory processes controlling TH availability and bioactivity such as hormone uptake or deiodination (Eales, 1984). For fish in particular, deiodination plays a large role in controlling local T_4 and T_3 concentrations within tissues and represents a strategic control point for physiological effects on target tissues (Eales, 1984; Peter, 2011). Despite the ability to transport both TH types from circulation into target tissues, approximately 50% of hepatic T_3 in fish is predicted to be derived from intracellular T_4 to T_3 conversion, while this value is greater than 75% in gill (Eales and Brown, 1993). Peripheral control of deiodination facilitates the formation of distinct T_3 and T_4 profiles among tissue types (Eales, 1984; Eales and Brown, 1993; Hulbert, 2000; Little et al., 2013) and could account for many of the tissue-specific effects of exogenous T_4 treatments observed in our study.

The molecular mechanism relating THs and mitochondrial oxidative enzymes in lake whitefish and other ectotherms remains elusive. This is, in part, because THs have been shown to target multiple cellular compartments (Hulbert, 2000). The best-studied mechanism of TH action is the regulation of gene expression through binding and activation of DNA-bound transcription factors (i.e. RXR-TR heterodimer). However, additional studies have suggested THs can exert effects via mitochondrial-based binding proteins and assimilate into lipid membranes (Hulbert, 2000), among other signal transduction pathways (Corderio et al., 2013). Peter and Oommen (1989b) provided evidence for transcriptional regulation of oxidative enzymes via THs in fish by demonstrating that exposure to protein synthesis inhibitors actinomycin D and chloramphenicol prevented T_4-induced stimulation of COX activity in climbing perch. Similar responses to T_4, both alone and in combination with protein synthesis inhibitors, has been reported for CS activity in catfish (*Clarias batrachus*; Tripathi and Verma, 2003). Furthermore, Little et al. (2013) observed an increase in transcript levels for the central metabolic coordinator, PGC1α, following T_2 and T_3 exposure in zebrafish. These studies indicate that the changes in metabolic enzyme activity following exogenous TH exposure in lake whitefish observed in the present study are most likely driven by changes at the transcriptional level rather than via protein modification and/or activation and are consistent with effects on oxidative enzyme activities following exposure to other nuclear receptor-mediated hormones such as testosterone and estradiol-17β in fish (Peter et al., 1996; Peter and Oommen, 1989a). However, information on how THs act to mediate fish metabolism in the context of thermal acclimation is limited and, currently, it is unclear if TH-induced changes in metabolic pathways might confer beneficial or detrimental effects. Additional characterization of TH-induced effects on respiration and oxidative enzymes is necessary to understand the full implications of exogenous T_4 exposure on oxidative metabolism in cool-water adapted fish species.

Consistent with numerous studies examining the effects of THs on cellular respiration (O_2 consumption) in various ectothermic species (Gupta and Thapliyal, 1991), metabolic adjustments induced by THs in the current study were restricted to warm-acclimated animals. Increased sensitivity to THs during warm acclimation was an organism-wide response since combined cold acclimation to 8 °C with exogenous T_4 exposure had no significant effect on the activity of either CS or COX in any tissue type (Fig. 2, Table 3). The absence of T_4-induced changes on the activity of aerobic enzymes in cold-acclimated lake whitefish suggests reduced sensitivity to hyperthyroidism during long-term exposure to low temperatures, possibly due to the initiation of a cold-induced static state that limits responses to endocrine signalling events (Gupta and Thapliyal, 1991). A reduction in thyroidal activity during cold acclimation has previously been observed in multiple fish species, including killifish and brook trout (*Salvelinus fontinalis*; Umminger, 1978). Similarly, circulating levels of free THs in fish tend to decrease with temperature (Eales, 1984), suggesting large-scale effects on hormone bioavailability. However, generalization of this response should be cautioned since other studies have shown certain fish species are sensitive to TH levels in cold. For instance, Little et al. (2013) observed increased sensitivity to hyperthyroidism in cold-acclimated versus warm-acclimated zebrafish. Regardless, the unique physiological responses to exogenous T_4 treatment we observed in warm-acclimated lake whitefish suggest a relationship between the thermal environment and TH action in lake whitefish and are consistent with earlier reports suggesting temperature and thermal acclimation play a critical role in the TH physiology of ectothermic species, particularly with respect to oxidative metabolism (Gupta and Thapliyal, 1991). The findings of our study may have consequences for lake whitefish and other cool-water fish species that are forced to venture outside their thermal preference in response to more extreme temperatures associated with climate change (Meisner et al., 1987). Further research should be conducted to determine the nature of the relationship between thermal acclimation and THs in ectotherms and to identify whether similar regulatory effects are observed in other aspects of metabolic function. Such information will be valuable in assessing physiological responses associated with long-term temperature shifts, such as those predicted by a changing global climate, as well as evaluating the role of THs in cell and organism homeostasis.

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