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Original Communication

Tissue-specific acclimation of enzyme activity in the Eastern red spotted newt (*Notophthalmus viridescens viridescens*)

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ABSTRACT

The Eastern red spotted newt is an ectotherm that is active in winter and acclimation or acclimatization from summer to winter conditions results in behavioral, biochemical and metabolic modifications. Cytochrome c oxidase (CCO) is embedded in the inner mitochondrial membrane and in skeletal muscle, its activity increases in winter. The activity of citrate synthase (CS), a mitochondrial matrix enzyme, also increases in winter. The present study had two main purposes: 1) to include liver in our analysis; and 2) to determine the mechanisms by which the changes in enzyme activity are accomplished. To address goal 1 we measured the activity of CCO, CS and lactate dehydrogenase in liver tissue from summer- and winter-acclimatized newts. To address goal 2, in liver and skeletal muscle of summer- and winter-acclimatized newts we a) determined mitochondrial volume density and cristae surface area via TEM; b) measured the expression of genes encoding CCO and CS via rt-PCR; c) determined membrane fatty acid composition via gas chromatography of fatty acid methyl esters. We found: 1) CCO activity was higher in summer than winter in liver; 2) mitochondrial volume density decreased in muscle in winter while cristae surface area increased in winter in liver; 3) CCO activity was correlated with changes in membrane composition, not gross mitochondrial characteristics or gene expression; 4) CCO activity in liver did not correlate well with the parameters we measured; 5) direct quantification of mitochondrial properties by TEM is important.

KEYWORDS: acclimation, amphibian, transmission electron microscopy, enzyme activity, membrane fatty acids

ABBREVIATIONS

CCO - cytochrome c oxidase enzyme; COX1 - cytochrome c oxidase subunit 1 gene; COX5a - cytochrome c oxidase subunit 5a gene; CS - citrate synthase (enzyme and gene); FA - fatty acid; FAME - fatty acid methyl ester; LDH - lactate dehydrogenase; MUFA - monounsaturated fatty acid; PUFA - polyunsaturated fatty acid; rt-PCR - real-time polymerase chain reaction; SFA - saturated fatty acid; TEM - transmission electron microscopy

INTRODUCTION

The rates of biochemical reactions are affected by temperature, making environmental temperature an important variable in the lives of ectothermic vertebrates. Eurythermic ectotherms employ a number of mechanisms to contend with the challenges of a changing thermal environment. Behavioral thermoregulation is a widespread phenomenon designed to avoid thermal extremes [1]. When thermal avoidance is no longer an option, many ectotherms down-regulate metabolic enzyme activity to become inactive [2, 3] or become freeze tolerant [4]. Thermal acclimation

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[reversible phenotypic change also known as phenotypic plasticity or flexibility (not developmental plasticity)] allows some ectotherms to remain active even as seasonal environmental temperatures decrease, up-regulating processes to offset the dampening effect of lowered temperature [5, 6, 7].

A variety of mechanisms have been described for thermal acclimation. Increases in the activity of mitochondrial enzymes are ascribed to increases in mitochondrial volume density [8, 9, 10, 11, 12] or cristae surface area [13, 14] in some fishes. Increases in the expression of genes that code for key metabolic enzymes has been correlated with acclimation and increased enzyme activity in fishes and alligators [12, 15, 16]. Finally, remodeling of membrane phospholipid fatty acids toward the inclusion of more polyunsaturated fatty acids (PUFA) is a common phenomenon in cold acclimated ectotherms [16, 17, 18] and the activity of some membrane-bound enzymes is affected by membrane remodeling whereby higher activity is induced in more polyunsaturated membranes after cold acclimation [19, 20].

The Eastern red spotted newt (*Notophthalmus viridescens viridescens*) is an ectotherm that is active in winter. We have previously shown that acclimation (in the laboratory) and acclimatization (in the field) from summer to winter conditions results in behavioral, biochemical and metabolic modifications that overall could contribute to the ability of this species to remain active in the cold. The activities of enzymes involved in aerobic metabolism (cytochrome c oxidase, CCO, and citrate synthase, CS) are upregulated in skeletal muscle in winter, whereas lactate dehydrogenase (LDH), a key indicator of anaerobic metabolic capacity does not change [21]. The increases in aerobic enzyme activities are reflected at the whole animal level by higher metabolic rates in winter acclimated newts [22].

We undertook the present study with two goals in mind. We wished to extend our study of acclimation in this model animal to include liver, as it is a large internal organ and metabolically active, and therefore contributes significantly to whole animal metabolic rate. Thus, we measured CCO, CS and LDH activities in liver tissue from summer- and winter-acclimatized newts. Second, we wished to begin to determine the mechanism(s) by which the changes in mitochondrial enzyme activity take place, and compare these mechanisms between tissue types. Because CCO activity is upregulated to a greater degree than CS activity in skeletal muscle [21], we suspected that different mechanisms may be responsible for regulating these enzymes. Specifically, a change in mitochondrial volume density should affect both enzymes equally, but an increase in cristae surface area would affect CCO specifically. Therefore we compared these mitochondrial properties in summer- and winter-acclimatized newts, in both skeletal muscle and liver using transmission electron microscopy (TEM). The increases in activity of CCO and CS could be due to increases in the expression of the genes encoding these proteins. We compared the expression of subunits of CCO encoded by mitochondrial (COX1) and nuclear (COX5a) genes, and the expression of CS in skeletal muscle and liver in winter- versus summer-acclimatized newts. Finally, because CCO is embedded in the inner mitochondrial membrane, whereas CS is found in the mitochondrial matrix, modification of the membrane milieu such as increasing the polyunsaturation of the membrane fatty acids would also preferentially affect CCO activity. For that reason we compared the membrane phospholipid fatty acid content of skeletal muscle and liver tissue in winter- versus summer-acclimatized newts.

MATERIALS AND METHODS

Animals

Adult Eastern red spotted newts (*Notophthalmus viridescens viridescens*) were collected locally in Sewanee, Franklin County, TN (88°55’0” x 35°12’15”) during January (winter) and June/July (summer), taken to the laboratory, sacrificed, and tissues were collected. Tissues for TEM were immediately fixed in 2.5% glutaraldehyde in 0.1 M cacodylic acid at room temperature, incubated overnight and then stored in 0.1 M cacodylic acid at 4°C for later embedding and analysis. For rt-PCR tissues were stored at -20°C in RNAlater Stabilization Reagent (Qiagen). Tissues to be used for enzyme assays and fatty acid analysis were immediately frozen in liquid nitrogen and stored at -80°C under N_2 gas. Use of newts was approved by the University of the South IACUC.

Enzyme assays

Assays for CCO, CS and LDH activity were performed on liver homogenates (n = 8 summer and winter newts) based on methods previously described [21, 22]. Briefly, a single liver was weighed and 9x volumes of homogenization buffer (50 mM imidazole, 2 mM $MgCl_2$, 5 mM EDTA, 1 mM glutathione (reduced), 0.1% Triton X-100, and 0.1 mM PMSF, pH 7.5) were added. Tissue was homogenized and centrifuged at 4°C, and the supernatant used for enzyme assays. Enzyme assays were performed on all samples in duplicate at 8, 20 and 26°C using a PerkinElmer Lambda 14 UV/Vis (Waltham, MA, USA) spectrophotometer with a 6 cell changer, and the temperature was controlled via a Fisher Scientific (Pittsburgh, PA, USA) Isotemp 1016S circulating refrigerated water bath. The calculation of enzyme activity was performed over the period (at least 2 minutes) of linear absorbance change. Reactions were followed for 5 minutes. One unit of activity is the amount of enzyme producing 1 µmol of product per minute. Enzyme activities were expressed in terms of units/g wet tissue.

Citrate synthase (EC 4.1.3.7)

This assay followed the transfer of sulphydryl groups to 5,5'dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm. Specific conditions were: 100 mM Tris-HCl, 0.1 mM DTNB, 0.15 mM acetyl-CoA, 0.15 mM oxaloacetate (omitted for the control), pH 8.0.

Cytochrome C oxidase (EC 1.9.3.1)

The oxidation of reduced cytochrome c was followed at 550 nm. The extinction coefficient for cytochrome c is 19.1 $ml \bullet cm^{-1} \bullet \mu Mol^{-1}$. Specific conditions were: 100 mM potassium phosphate, 0.075 mM reduced cytochrome c, pH 7.5. The reactions were run against a reference of 0.075 mM cytochrome c oxidized with 0.33% (w/v) potassium ferricyanide. Reduction of cytochrome c was accomplished by adding a small amount of sodium hydrosulfite, which was subsequently removed by bubbling with air for 20 - 30 min.

Lactate Dehydrogenase (EC 1.1.1.27)

This assay followed the disappearance of NADH at 340 nm. Specific conditions were: 100 mM potassium phosphate, 0.16 mM NADH, 0.4 mM pyruvate (omitted for control), pH 7.0.

Transmission electron microscopy

After the fixation described above, newt skeletal muscle and liver tissue was embedded in epoxy resin and thin sectioned. The percentage of the total cell volume occupied by mitochondria (mitochondrial volume density) was computed from 10 arbitrarily selected fields of view (at 11,500X) chosen from at least 3 separate thin sections for each animal. The mitochondrial volume density (V_v) was determined using point count stereology methods [23]. The mitochondrial volume density (V_v) was computed for each separate animal (n = 5 for each season) and the mean and standard deviation of the V_v for all 5 animals was then determined. In all, greater than 250 square micrometers of cell area were quantified for each tissue from each animal. In a similar manner, the surface area of mitochondrial cristae per volume of mitochondria (surface area density) was determined by point count stereology methods [23]. Greater than 75 square micrometers of mitochondrial area was examined for the cristae surface area density (S_v) analysis. This represented 10 fields of view (at 21,000X) from at least 3 separate sections from each tissue for each animal.

Gene expression

RNA was extracted from tissue samples stored in RNAlater using PerfectPure RNA Tissue kit (5 Prime). Skeletal muscle and liver were ground in liquid N_2 with a mortar and pestle, and the lysate was further homogenized using an 18.5 gauge needle and syringe. Isolated RNA was stored at -80°C. After a supplemental incubation with DNase I Amplification grade (Sigma-Aldrich), purified RNA was converted into cDNA using iScript (BioRad) reverse transcriptase reaction kit. Using this cDNA the target gene was amplified using specific oligonucleotide (25 - 30 bases) primers (synthesized by Integrated DNA Technologies) designed to specific sequences within the target transcript. Sybr-green (BioRad) reactions were run on a MyiQ™ Real-time PCR thermal-cycler (BioRad, Hercules, CA, USA). PCR cycles were 95°C for 3 minutes, 40 cycles at 95°C for 30 sec, annealing temperature (55 or 60°C) for 30 sec and 72°C for 30 sec. Assays were performed in triplicate (n = 4 or 5 for muscle and n = 5 for liver for each season). The results were analyzed by

comparing the cycle threshold values of the genes of interest against the housekeeping gene (28s rRNA), and calculating a fold change in winter tissues using summer as the control [16, 24]. To ensure that each RNA sample was not contaminated with DNA, control rt-PCR was performed using the isolated RNA with no reverse transcription.

rt-PCR primers (Table 1) were designed from mitochondrial sequences obtained on GenBank for *N. viridescens* for 16s rRNA and COX1. Nuclear gene sequences are not available for *N. viridescens*. In these cases (28s rRNA, COX5a and CS) we aligned sequences for other vertebrates (available on GenBank) and used conserved regions within the gene to design sequencing primers. The sequencing primers were used to amplify the gene of interest in *N. viridescens* by traditional PCR using GoTaq Flexi PCR Kit (Promega; Madison, WI) or Amplitaq Gold PCR Master Mix (Life Technologies; Carlsbad, CA). PCR products were electrophoresed to ensure a single PCR product of the expected size, were purified using QIAquick PCR Purification Kit (Qiagen; Valencia, CA) and sequenced at the DNA Analysis Facility at Yale University. This final *N. viridescens* sequence was aligned via BLAST to validate the sequence as the gene of interest and was used to design primers for rt-PCR. To further validate the rt-PCR product, traditional PCR products produced using the rt-PCR primers were also purified, sequenced, and the sequences aligned via BLAST. Melt curves ensured a single rt-PCR product.

Membrane phospholipid fatty acid analysis

Lipid extractions were performed as described previously [25]. All solvents contained 0.01% w/v butylated hydroxytoluene (BHT) as an antioxidant. Lipids were extracted from up to 0.2 g of tissue (n = 10 or 13 for skeletal muscle and n = 10 for liver) using chloroform:methanol (2:1 v/v; minimal solvent:sample ratio 20:1) and phases were separated using 1M H_2SO_4. The phospholipids were separated from neutral lipids by solid phase extraction on silicic acid columns from Phenominex, Inc. These phospholipids were transmethylated with acetyl chloride and the FA methyl esters (FAME) separated on a Varian 3900 (Palo Alto, CA, USA) gas chromatograph with an autoinjector, WCOT fused silica column (100 m x 0.25 mm) with select FAME coating tailor made, and a flame ionization detector. Individual FAs were identified by comparing their peak's retention time with external standards, and FA composition was calculated as mol% of total FA. Only those comprising at least 0.5 mol% of the total were used in calculations.

Statistics

Statistical analyses were performed using SPSS 19.0 from IBM®. Enzyme activities were compared by univariate ANOVAs with season and assay temperature as factors. Individual means were compared by LSD (least significant difference) *post-hoc* tests only after the overall ANOVAs indicated that differences existed. TEM and fatty acid comparisons were performed by Student's t-tests. Differences were considered significant at $p \leq 0.05$.

RESULTS

Liver enzyme acclimation

CCO activity was higher in summer than winter newts at all temperatures ($p = 0.016$ at 8°C,

Table 1. Primers used for real-time PCR.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Base pairs
16s rRNA	CTTGTACCTTTTGCATAATGGTCTA	GGCTCGTTAGGCTTATCACCTCTA	119
COX1	CTTTGGRGCCTGAGCTGGCATATT	CCGAATCCACCAATTATCACTGTA	126
CS	GTCACATTGGGCCAGGGTTCTTGG	TCAACTCTGGCAGGGTGGTTCCTG	198
COX5a	CCAGAACCAAAAATCATTGATGCAG	GGTCCAGCTTTATCCTTTACAGCTT	54
28s rRNA	GGCATCGTGAAGAGACATGAGCGGT	CGGCCTCGCCGGGTCAGTGAAAA	104

COX, cytochrome c oxidase; CS, citrate synthase; Base pairs is the amplicon size.

p = 0.006 at 20°C and p = 0.008 at 26°C; Figure 1A). CS activity was not different between summer and winter newts except at 8°C where activity was higher in winter newts (p = 0.050; Figure 1B). There were no significant differences in LDH activity between seasons at any assay temperature (Figure 1C).

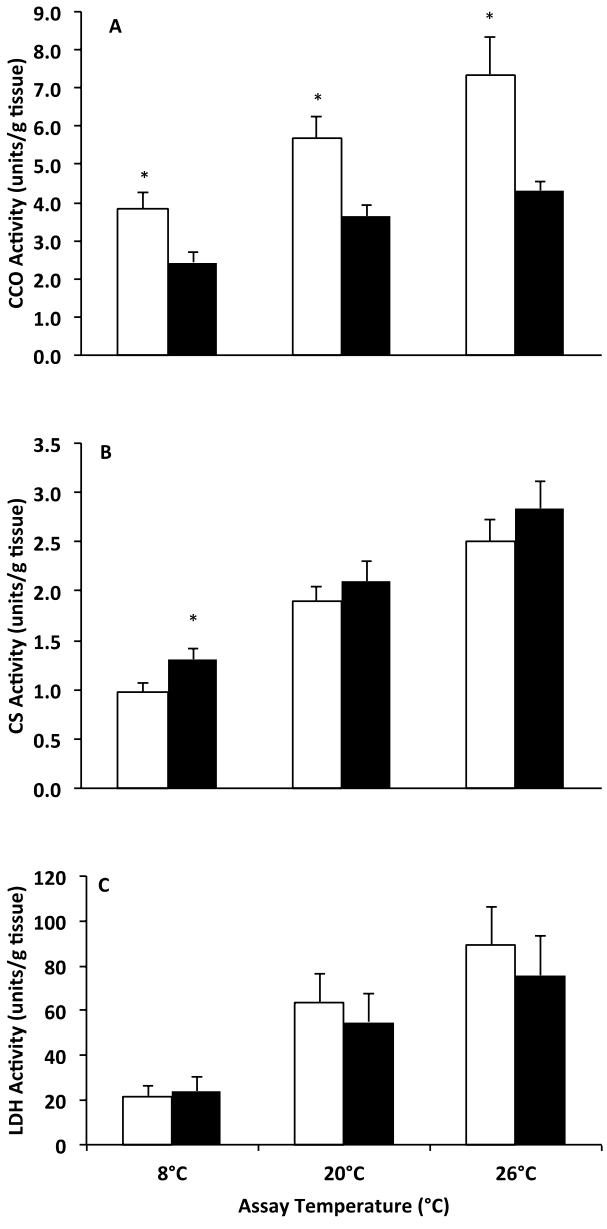

Figure 1. Mean ± S.E. enzyme activity (A = CCO; B = CS; C = LDH) in liver from summer (open bars) and winter (black bars) newts. *indicates significantly different between seasons at the same temperature.

TEM

Mitochondrial volume density was significantly lower in winter than in summer newts in skeletal muscle (Figure 2A, B) but liver tissue did not exhibit this seasonal adjustment (Table 2). On the other hand, cristae surface density of mitochondria in skeletal muscle was not seasonally different, but in liver cristae surface area was higher in winter than in summer newts (Figure 3A, B).

Gene expression

Skeletal muscle exhibited an increase in the expression of only 16s rRNA in winter versus summer (1.8 times; Figure 4), whereas in liver the expression of the 16s rRNA, COX1 and CS genes were elevated during winter 5.2, 6.0 and 2.5 times, respectively (Figure 4).

Lipid analysis

Skeletal muscle membranes exhibited significant differences in fatty acid (FA) makeup between summer- and winter-acclimatized newts. Muscle membranes from winter-acclimatized newts had lower saturated fatty acid (SFA) content and higher PUFA content than those from summer-acclimatized newts (Table 3). In addition, higher n-6 but lower n-3 PUFA content led to a lower ω-3 balance in muscle membranes of winter newts. Major fatty acids that were lower in muscle membranes of winter newts included 18:0 and 22:6n-3. Meanwhile, these winter membranes were higher in 20:4n-6 and had almost double the mol% content of 18:2n-6 compared to muscle membranes of summer newts.

Liver membranes also exhibited significant differences in FA makeup between summer- and winter-acclimatized newts. While SFA content did not differ, liver membranes from winter newts were lower in MUFA and higher in PUFA content than liver membranes from summer-acclimatized newts (Table 3). Liver membrane from winter newts also had higher n-6 PUFA content, but as there was no difference in n-3 PUFAs, the ω-3 balance was not significantly different. Liver membranes from winter newts were lower in 18:0, 18:1n-9, 18:1n-7, and 20:4n-6, but higher in 16:0, 22:6n-3, and again almost double the mol% content of 18:2n-6 compared to liver membranes from summer newts.

Table 2. TEM analysis of skeletal muscle and liver mitochondrial parameters in winter- and summer-acclimatized newts.

Mitochondrial variable	Winter (n = 5)	Summer (n = 5)	P-value
Muscle			
Vv (mt,f)	1.44 ± 0.30	3.18 ± 0.28	0.0033
Sv (cr,mi)	0.01566 ± 0.0008	0.01876 ± 0.0015	0.1075
Liver			
Vv (mt,f)	5.66 ± 0.41	5.14 ± 0.51	0.4646
Sv (cr,mi)	0.004225 ± 0.000138	0.003342 ± 0.00018	0.0028

Mean ± S.E.M.; mt,f = volume density of total mitochondria (%); cr,mi = surface density of mitochondrial cristae (um^2/um^3).

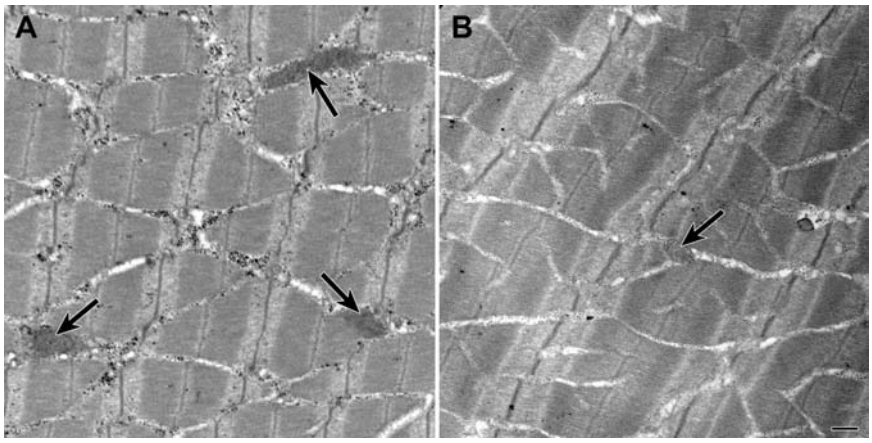

Figure 2. TEM of *N. viridescens* skeletal muscle from summer (A) and winter (B) newts. Arrows indicate mitochondria. Magnification = 11,350x; Bar = 500 nm.

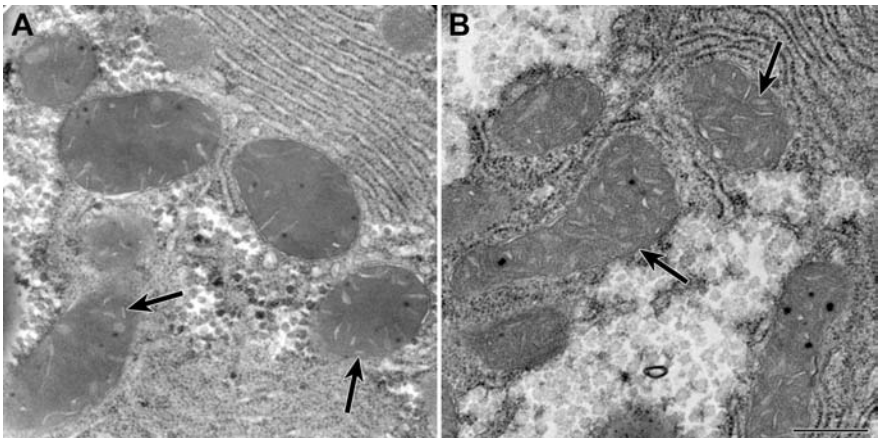

Figure 3. TEM of *N. viridescens* liver from summer (A) and winter (B) newts. Arrows indicate cristae inside mitochondria. Magnification = 30,400x; Bar = 500 nm.

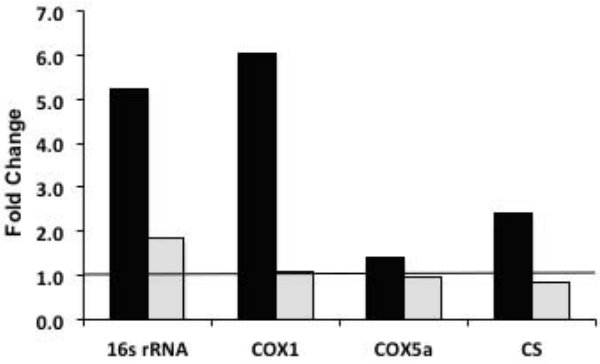

Figure 4. Fold change in gene expression in winter compared to summer in liver (black bars) and skeletal muscle (grey bars). Expression at or around the line at 1.0 indicates no significant difference in seasonal expression. All n = 5 for liver; in muscle, COX1 summer n = 5, all other summer n = 4, all winter n = 5.

DISCUSSION

These data indicate that the Eastern red spotted newt exhibits tissue specific seasonal biochemical acclimation. Previous studies have shown that CCO and CS activities are higher in skeletal muscle of winter than summer newts [21, 22]. In the present study, CCO activity in liver was higher in summer than in winter newts and CS activity generally did not differ with season, although it was higher from winter animals at 8°C. The higher CCO activity in summer was unexpected from an acclimation standpoint, which normally results in higher activity in winter. However, while surprising this is not unheard of in the literature and is likely due to the complex functions performed by the liver [26].

The changes in CCO activity in skeletal muscle were more easily explained by the changes in membrane composition than by mitochondrial characteristics or gene expression. Muscle had higher membrane PUFA and lower SFA contents in winter, which are common responses to cold acclimation [16, 17, 18]. More specifically, muscle had a lower ω-3 balance, which affects membrane function by increasing membrane Ca^{2+}-Mg^{2+} pump activity in the sarcoplasmic reticulum (SR), preserving muscle function at low temperature [27]. In addition, CCO associates with, and its activity is highly influenced by, cardiolipin [28, 29], a double phospholipid found in the inner mitochondrial membrane whose characteristic structure is high in 18:2n-6 [30]. The high 18:2n-6 content of the newt muscle membranes in winter could reflect the impact of this fatty acid on the function of both membrane systems, as the source for n-6 FAs in SR, and its incorporation into cardiolipin to control CCO activity in the inner mitochondrial membrane. One limitation of our study is that we used whole tissue membranes, not specifically mitochondrial membranes (due to the small size of this species), so that changes taking place at the mitochondrial level could be masked by phospholipid content in other membrane systems, or changes that we have suggested as significant may not be taking place at the mitochondrial level. Experiments that modify membrane structure through diet could test whether or not changes in membrane structure impact CCO activity.

Acclimation of enzyme activity in skeletal muscle did not appear to be driven either by gross mitochondrial characteristics, or changes in gene expression. During the winter when the activities of the CCO and CS increase, the mitochondrial volume density decreased and there was no change in cristae surface area. This result was unexpected not only due to the measured increases in enzyme activity, but because CS activity is often used as an indicator of mitochondrial density in tissues [31]. These data suggest that this may not be a good measure for all species under all conditions. In addition, there were no changes in the expression of genes encoding CCO (COX1 and COX5a) or CS. Thus, in muscle, the source for the increase in CS does not appear to be any of the parameters we measured. For CCO, it is possible that we simply did not measure expression of enzyme subunits that did vary, as we studied expression of only one of three mitochondrially encoded subunits and one of 10 nuclearly encoded subunits. In three spine sticklebacks in winter when there is an increase in CCO activity, expression of COXIII increased but COXIV did not [12]. Other studies have also shown variability in the correlation of COX subunit gene expression with activity [15, 16, 32, 33].

None of the parameters we measured directly reflect the winter decrease in CCO activity in the liver.

Table 3. Tissue membrane phospholipid fatty acid composition of newts collected during summer and winter.

	Skeletal muscle		Liver	
Fatty acid	**Summer (n = 13)**	**Winter (n = 10)**	**Summer (n = 10)**	**Winter (n = 10)**
Saturates				
C16:00	19.65 ± 0.44	18.63 ± 0.73	10.10 ± 0.50	15.11 ± 0.54*
C17:00	0.88 ± 0.16*	ND	0.91 ± 0.05	0.87 ± 0.04
C18:00	14.72 ± 0.24*	11.08 ± 0.23	19.00 ± 0.27*	12.35 ± 0.42
∑ Saturates	35.86 ± 0.70*	30.55 ± 0.81	30.81 ± 0.80	29.09 ± 0.47
Monosaturates				
C16:1 n7	ND	ND	1.11 ± 0.06	1.17 ± 0.16
C18:1 n-9	8.43 ± 0.40	8.90 ± 0.34	11.77 ± 0.60*	9.20 ± 0.71
C18:1 n-7	3.93 ± 0.13*	3.26 ± 0.20	3.80 ± 0.14*	2.95 ± 0.16
C22:1 n-9	0.80 ± 0.13*	ND	ND	ND
∑ Monounsaturates	13.68 ± 1.10	12.76 ± 0.49	17.61 ± 0.54*	14.14 ± 0.82
n-3 Polyunsaturates				
C18:3 n-3	0.58 ± 0.13	0.96 ± 0.17	1.65 ± 0.12	2.46 ± 0.40
C20:5 n-3	5.53 ± 0.44*	4.40 ± 0.34	6.80 ± 0.43*	4.75 ± 0.51
C22:5 n-3	3.46 ± 0.16*	2.91 ± 0.12	2.56 ± 0.15	2.47 ± 0.17
C22:6 n-3	6.38 ± 0.25*	4.58 ± 0.44	2.94 ± 0.20	3.81 ± 0.22*
n-6 Polyunsaturates				
C18:2 n-6	7.61 ± 0.34	14.05 ± 0.68*	8.68 ± 0.37	15.66 ± 0.48*
C20:2 n-6	ND	0.84 ± 0.13*	ND	1.08 ± 0.03*
C20:3 n-6	2.38 ± 0.14	2.09 ± 0.11	1.63 ± 0.09	1.45 ± 0.09
C20:4 n-6	22.13 ± 0.46	25.03 ± 0.77*	25.58 ± 0.47*	23.84 ± 0.57
C22:4 n-6	1.22 ± 0.11	1.31 ± 0.11	1.01 ± 0.06	0.81 ± 0.05
C22:5 n-6	0.92 ± 0.12*	ND	ND	ND
∑ Polyunsaturates	50.45 ± 1.10	56.69 ± 1.04*	51.58 ± 0.89	56.77 ± 0.78*
Total Unsaturates	64.14 ± 0.70	69.45 ± 0.81*	69.19 ± 0.80	70.91 ± 0.47
Indexes				
∑n-9	9.24 ± 0.41	9.14 ± 0.37	12.65 ± 0.60*	10.01 ± 0.71
∑n-7	4.46 ± 0.26*	3.62 ± 0.26	4.96 ± 0.16*	4.13 ± 0.28
∑n-6	34.43 ± 0.52	43.85 ± 1.26*	37.65 ± 0.49	43.31 ± 0.95*
∑n-3	16.02 ± 0.78*	12.84 ± 0.92	13.95 ± 0.72	13.49 ± 0.77
ω-3 balance	31.61 ± 0.97*	22.67 ± 1.62	26.93 ± 1.03	24.56 ± 1.30
Unsaturation index	219.59 ± 4.5	223.7 ± 4.9	218.1 ± 3.8	219.1 ± 3.2

*indicates significant difference between seasons in the same tissue $p \leq 0.05$. ND = not detectible (lower than 0.5 mol%). ω-3 balance is the ratio of n-6/n-3 fatty acids. Unsaturation index is defined as the number of double bonds per 100 fatty acid molecules. Fatty acids are indicated by standard nomenclature: example 18:2n-6 :: 18 = number of carbons, 2 = number of double bonds, n-6 = the first double bond from the omega (free) end of the fatty acid is at the sixth carbon.

The liver mitochondrial cristae surface area increased in winter by 27%, as did COXI expression (6 times), both of which would be expected to correlate with an increase in CCO activity. Gross membrane characteristics were also in favor of higher CCO activity in winter rather than summer with the higher winter PUFA content. The one fatty acid that differed significantly in both tissues and correlated positively with CCO activity was 20:4n-6, arachadonic acid, which was higher in winter muscle and summer liver samples. However, previous studies on the effects of FAs on mitochondrial properties in fish have not identified arachadonic acid as highly influential [34, 35] where levels of 20:5n-3 seemed especially significant. The contrary nature of all the mechanistic data versus enzyme activity suggests there may be some parameter in the enzyme assay, such as pH, that needs adjustment. It is also important to remember that enzyme activities indicate the maximum activity possible, not *in vivo* activity.

Finally, our data underline the importance of TEM in directly quantifying mitochondrial volume density. While it is common for CS activity to be use as an indirect indicator of mitochondrial volume density [31], in our study, CS activity changes were not reflected in the direct TEM measurements. In addition, 16s rRNA expression (versus a nuclear housekeeping gene) has also been used as a marker for mitochondrial volume density [16] but in our study 16s rRNA expression increased in both liver (5x) and muscle tissue (2x) in winter compared to summer, but TEM of the tissues indicated either no change (liver) or a decrease (muscle) in mitochondrial volume density.

CONCLUSION

While mitochondrial enzymes in muscle increase activity during winter, in liver the activity of CCO decreased in winter. The liver enzyme activity did not correlate well with any of the mechanistic parameters measured, all of which exhibited changes that would be expected to increase enzyme activity in winter. In skeletal muscle the modification of CCO activity was more easily explained by changes in membrane composition, and did not correlate well with gross mitochondrial characteristics or changes in gene expression. We were not able to determine the source for the increase in CS activity in newt skeletal muscle in winter. Our data underline this importance of using direct measures of mitochondrial properties by TEM.

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REFERENCES

1. Bicego, K. C., Barros, R. C. H. and Branco, L. G. S. 2007, Comp. Biochem. Physiol. A, 147, 616.
2. Pinder, A. W., Storey, K. B. and Ultsch, G. R. 1992, Estivation and hibernation in Environmental Physiology of the Amphibians, Feder, M. E. and Burggren, W. W. (Eds.), University of Chicago Press, Chicago, 250.
3. St. Pierre, J. and Boutilier R. G. 2001, Physiol. Biochem. Zool., 74, 390.
4. Storey, K. B. 1987, Am. J. Physiol., 253, R292.
5. Seebacher, F., Guderley, H., Elsey, R. M. and Trosclair III, P. L. 2003, J. Exp. Biol., 206, 1193.
6. Wilson, R. S., James, R. S. and Johnston, I. A. 2000, J. Comp. Physiol. B, 170, 124.
7. Wilson, R. S. and Johnston, I. A. 2004, International Congress Series, 1275, 201.
8. Johnston, I. A. and Maitland, B. 1980, J. Fish Biol., 17, 113.
9. Egginton, S. and Johnston, I. A. 1984, J. Therm. Biol., 9, 165.
10. Tyler, D. D. and Sidell, B. D. 1984, J. Exp. Zool., 232, 1.

11. Egginton, S. and Sidell, B. D. 1989, Am. J. Physiol., 256, R1.
12. Orczewska, J. I., Hartleben, G. and O'Brien, K. M. 2010, Am. J. Physiol., 299, R352.
13. Kilarski, W. M., Romek, M., Kozlowska, M. and Gorlich, A. 1996, J. Fish Biol., 49, 1280.
14. St. Pierre, J., Charest, P. and Guderley, H. 1998, J. Exp. Biol., 201, 2961.
15. Lucassen, M., Koschnick, N., Eckerle, L. G. and Portner, H. O. 2006, J. Exp. Biol., 209, 2462.
16. Seebacher, F., Murray, S. A. and Else, P. L. 2009, Physiol. Biochem. Zool., 82, 766.
17. Wodtke, E. 1978, Biochim. Biophys. Acta, 529, 280.
18. Grimm, J. M., Miles, D. R. B. and Crockett, E. L. 2010, J. Exp. Biol., 213, 445.
19. Hazel, J. R. 1972, Comp. Biochem. Physiol. B, 43, 863.
20. Hazel, J. R. 1995, Ann. Rev. Physiol., 57, 19.
21. Berner, N. J. and Bessay, E. P. 2006, Comp. Biochem. Physiol. A, 144, 429.
22. Berner, N. J. and Puckett, R. E. 2010, J. Exp. Zool., 313A, 231.
23. Weibel, E. R., Kistler, G. S. and Scherle, W. F. 1966, J. Cell Biol., 30, 23.
24. Pfaffl, M. W. 2001, Nucl. Acids Res., 29, 2002.
25. Berner, N. J., Else, P. L., Hulbert, A. J., Mantle, B., Cramp, R. L. and Franklin, C. E. 2009, J. Comp. Physiol. B., 179, 857.
26. Bremer, K. and Moyes, C. D. 2011, J. Exp. Biol., 214, 1888.
27. Ruf, T. and Arnold, W. 2008, Am. J. Physiol., 294, R1044.
28. Robinson, N. C. 1993, J. Bioenerg. Biomembr., 25, 153.
29. Schlame, M., Rua, D. and Greenberg M. L. 2000, Progress in Lipid Research, 39, 257.
30. Hoch, F. L. 1992, Biochim. Biophys. Acta, 1113, 71.
31. Dalziel, A. C., Moore, S. E. and Moyes, C. D. 2005, Am. J. Physiol., 288, R163.
32. Frick, N. T., Bystriansky, J. S., Ip, Y. K., Chew, S. F. and Ballantyne, J. S. 2010, Am. J. Physiol., 298, R608.
33. Duggan, A. T., Kocha, K. M., Monk, C. T., Bremer, K. and Moyes, C. D. 2011, J. Exp. Biol., 214, 1880.
34. Guderley, H., Kraffe, E., Bureau, W. and Bureau, D. P. 2008, J. Comp. Physiol., 178, 385.
35. Kraffe, E., Marty, Y. and Guderley, H. 2007, J. Exp. Biol., 210, 149.