Molecular drivers of mitochondrial membrane proliferation in response to cold acclimation in threespine stickleback

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1 **Abstract**

2 Little is known about how the synthesis of mitochondrial phospholipids is integrated into
3 mitochondrial biogenesis in fish or mammals. Glycerol-3-phosphate acyltransferase
4 (GPAT; EC 2.3.1.15) catalyzes the addition of fatty acyl CoA to the *sn*-1 position of
5 glycerol-3-phosphate, in what is considered the rate-limiting step in phospholipid
6 biosynthesis. Previous studies have shown that mitochondrial volume density increases
7 in oxidative skeletal muscle but not liver of *Gasterosteus aculeatus* (threespine
8 stickleback) in response to cold acclimation. We hypothesized that maximal activity of
9 GPAT would increase in oxidative skeletal muscle but not liver during cold acclimation,
10 coinciding with mitochondrial biogenesis. GPAT activity was measured in liver and
11 oxidative skeletal (pectoral adductor) muscle of threespine stickleback acclimated to 8 °C
12 or 20 °C. In addition, mRNA levels of enzymes involved in phospholipid synthesis,
13 including cytidine diphosphodiacylglycerol synthase-1 (CDS1), CDS2, GPAT1, GPAT2
14 and 1-acylglycerol 3-phosphate acyltransferase-2 (AGPAT2) were quantified in liver and
15 pectoral muscle of stickleback harvested during cold acclimation. GPAT activity and
16 transcript levels of AGPAT2 increased in response to cold acclimation in pectoral muscle
17 but not liver. Transcript levels of GPAT1 increased in liver but not pectoral muscle.
18 Overall our results suggest that the activity of GPAT, and possibly AGPAT as well,
19 increase during cold acclimation and may contribute to mitochondrial phospholipid
20 biosynthesis required for mitochondrial biogenesis.

21 Key Words: mitochondrial biogenesis, cold acclimation, threespine stickleback,
22 membrane biosynthesis

Abbreviations: AGPAT, 1-acylglycerol 3-phosphate acyltransferase; CDP-DAG, cytidine diphosphodiacylglycerol; CDS, cytidine diphosphodiacylglycerol synthase; CL, cardiolipin; DAG, diacylglycerol; EF-1 α , elongation factor-1 alpha; G3P, glycerol 3-phosphate; GPAT, glycerol 3-phosphate acyltransferase; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; mTOR, mechanistic target of rapamycin; mtDNA, mitochondrial DNA; NRF-1, nuclear respiratory factor 1; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TAG, triacylglycerol; TFAM, mitochondrial transcription factor A

1. Introduction

Mitochondrial density increases in response to cold acclimation in oxidative skeletal muscle of many cold-active teleosts, including goldfish, striped bass, zebrafish and stickleback (Egginton and Sidell, 1989; Johnston and Maitland, 1980; Orczewska et al., 2010; Tyler, 1984). Mitochondria are chimeric organelles, possessing proteins encoded in both the mitochondrial and nuclear genomes. Thus, mitochondrial biogenesis requires coordination of the two genomes to synthesize mitochondrial proteins and lipids, and to replicate the mitochondrial genome. In mammals, the co-transcriptional activator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) is considered the master regulator of mitochondrial biogenesis. In addition to regulating the transcription of nuclear-encoded genes destined for mitochondria, it also transactivates the expression of mitochondrial transcription factor A (TFAM), which is then translated on cytosolic ribosomes and imported into the mitochondrion where it induces mitochondrial DNA (mtDNA) replication and transcription, leading to an increase in mtDNA and proteins (Scarpulla, 2008). Less is known about mitochondrial biogenesis in fishes but several studies suggest that nuclear respiratory factor-1 (NRF-1) and PGC-1 β , rather than PGC-1 α , orchestrate the process (Bremer et al., 2015; Bremer et al., 2012; Orczewska et al., 2010). While mammalian studies have clearly delineated how mtDNA is replicated and mitochondrial proteins are synthesized during mitochondrial biogenesis, the molecular drivers mediating the synthesis of mitochondrial phospholipids remain largely unknown (O'Brien and Mueller, 2010).

Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the first, committed step in glycerolipid synthesis (Wendel et al., 2009). GPAT catalyzes the addition of fatty acyl coenzyme A (CoA) to the *sn*-1 position of glycerol-3-phosphate (G3P), producing lysophosphatidic acid (LPA). Downstream of GPAT, 1-acylglycerol-3-phosphate acyltransferase (AGPAT) [or lysophosphatidic acid acyltransferase (LPAAT)] catalyzes the addition of fatty acyl CoA to the *sn*-2 position of LPA, producing phosphatidic acid (PA). PA is then hydrolyzed to form diacylglycerol (DAG). PA is the precursor for synthesizing phosphatidylinositol (PI), phosphatidylglycerol (PG) and cardiolipin (CL); DAG is the precursor for synthesizing the most abundant membrane phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) (Takeuchi and Reue, 2009). The synthesis of CL, an essential phospholipid associated with the complexes of the mitochondrial respiratory chain, is

synthesized from PA by cytidine diphosphodiacylglycerol (CDP-DAG) synthase (CDS) (Horvath and Daum, 2013; Ren et al., 2014). There are two isoforms of CDS and both are localized to the endoplasmic reticulum (ER) (D'Souza et al., 2014). Recent studies suggest Tam41, also a CDS but localized to the mitochondrion, may play a more prominent role in the synthesis of CL than CDS1 or CDS2 (Tamura et al., 2013). There are four isoforms of GPAT, localized to the outer mitochondrial membrane (GPAT 1 and 2) and the ER membrane (GPAT 3 and 4) (Bell and Coleman, 1980). AGPAT1 and AGPAT2 are localized to the ER and have been well characterized, but as many as 11 isoforms have been reported in mammals (Takeuchi and Reue, 2009). Recent studies have revealed the first linkages between the synthesis of mitochondrial proteins and phospholipids. Knocking out PGC-1 α and PGC-1 β in mice results in altered mitochondrial morphology and a decrease in mRNA levels of CDS1 and levels of CL, PC and PE in hearts (Lai et al., 2014). Overexpression of PGC-1 α in extensor digitorum longus (EDL) muscle of mice alters phospholipid profiles, increasing content of 18:0/22:6 PC and 18:0/22:6 PE, similar to changes observed in response to exercise, and these changes are prevented by knocking out PGC-1 α (Senoo et al., 2015). Transcript levels of GPAT1, GPAT3, and LPAAT2 (AGPAT2) also increased in EDL of PGC-1 α null mice, suggesting one more of these enzymes may drive phospholipid remodeling, although principle component analysis did not support this (Senoo et al., 2015).

To shed light on how mitochondrial membrane synthesis is stimulated during cold-induced mitochondrial biogenesis in fish, we measured the maximal activity of GPAT in liver and oxidative pectoral adductor muscle of threespine stickleback held at 20 °C and cold-acclimated to 8 °C. Previous studies in our lab have shown that mitochondrial density increases in pectoral adductor muscle but not liver in response to cold acclimation (Orczewska et al., 2010). We anticipated that if GPAT drives increases in mitochondrial membrane biosynthesis, GPAT activity would increase in oxidative muscle but not liver. Additionally, we measured transcript abundance of key genes of the phospholipid biosynthetic pathway: CDS1, CDS2, GPAT1, GPAT2 and AGPAT2.

2. Materials and methods

2.1 Animal care

Threespine stickleback, *Gasterosteus aculeatus* were collected in 2013 from Kashwitna Lake, AK (61°50' N, 150°00' W; 13.1 °C) using minnow traps. Fish were transported to the University of Alaska Fairbanks, housed in environmental chambers, and maintained on a 10:14 hr light:dark cycle in aerated, filtered 114 L aquaria filled with 0.35% Instant Ocean in distilled water. Animals were held at 20 °C for 20 weeks then half of the animals (~60) were cold acclimated by decreasing the temperature in the environmental chamber to 15 °C on day 1, 10 °C on day 2, and 8 °C on day 3. Warm-acclimated fish were maintained at 20 °C for the duration of the experiment. Fish were held at 8 °C or 20 °C for an additional 11 weeks for measuring GPAT activity. For measuring transcript levels, fish from 8 °C and 20 °C were harvested 1, 4, and 9 weeks after the start of cold acclimation. Animals were harvested prior to feeding at the same time each morning. Fish were euthanized with an overdose of tricaine methanesulfonate (250 mg L⁻¹; pH 7) followed by cervical cord transection for assaying GPAT activity, and flash frozen in liquid nitrogen for measuring mRNA levels. All protocols were approved by the UAF Institutional Animal Care and Use Committee (135490-2 and 135490-7).

2.2 Maximal activity of GPAT (EC 2.3.1.15)

Liver and oxidative pectoral adductor muscle were excised on an ice-cold stage. Tissues from two individuals were pooled and homogenized in 8 volumes of buffer (10 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, 1 mM DTT; pH 7.4 at 4 °C) using a Tenbroeck homogenizer. Protein concentrations were determined using a Bradford assay with bovine serum albumin (BSA) as a standard (Bradford, 1976). Initial assays were carried out in a 500 µl reaction mixture containing 40 mM Tris-HCl pH 7.5, 2 mM MgCl₂, 2 mg ml⁻¹ BSA (essentially fatty acid-free), 2 mM KCN, 100 µM palmitoyl-CoA or palmitoleoyl-CoA, 0.1-10 mM G3P and 0.1 µCi [¹⁴C] glycerol-3-phosphate (Perkin Elmer, Waltham MA, USA) (Vancura and Haldar, 1994). Reactions were initiated by the addition of 150-400 µg of protein. Maximal GPAT activity was linear for 20 minutes and proportional to the amount of protein added. These assays indicated that 5 mM G3P was sufficient to saturate the enzyme and activities were equivalent using either palmitoyl-CoA or palmitoleoyl-CoA as a substrate (data not shown). GPAT activity in warm- and cold-acclimated stickleback was assayed at 14 °C in a 500 µl reaction mixture containing 40 mM Tris-HCl pH 7.5, 2 mM MgCl₂, 2 mg ml⁻¹ BSA (essentially fatty acid-free), 2 mM KCN, 100 µM palmitoleoyl-CoA, 5 mM G3P, 0.1 µCi [¹⁴C] glycerol-3-phosphate, and 400 µg protein.

Reactions were stopped after 20 minutes by the addition of 0.5 ml H₂O-saturated 1-butanol. Samples were centrifuged at 12,100 g for 5 minutes at room temperature. A 200 µL aliquot of the butanol layer was added to 10 mL liquid scintillation fluid (Ecolite MP Biomedicals, LLC, Solon, OH, USA). ¹⁴C was counted using an LS 6500 multi-purpose scintillation counter (Beckman Coulter, Fullerton CA, USA). Maximal GPAT activity was measured in triplicate in warm- and cold-acclimated animals (*N* = 6). Background activity was measured in reaction mixtures lacking palmitoleoyl-CoA and subtracted from activity in the presence of substrate. Specific activity of GPAT was calculated as nmol G3P acylated per min per mg protein (nmol min⁻¹ mg⁻¹).

2.3 Gene expression

RNA was isolated from 10-30 mg of pectoral adductor muscle and liver tissue from 6-8 individuals harvested at each time point during cold and warm acclimation (described above) using the RNeasy Fibrous Tissue Mini-kit (Qiagen, Valencia, CA, USA) as described previously (Orczewska et al., 2010). RNA levels were quantified by measuring the absorbance at 260 nm with a NanoDrop® (ND-1000) spectrophotometer. RNA integrity was verified by mixing 2 µl RNA with loading buffer (5% glycerol, 0.04% bromophenol blue, 0.1 mM EDTA, pH 8.0) and separating it on a 2% agarose gel. Total RNA was reverse transcribed to complementary DNA (cDNA) using TaqMan® reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) as described previously (Orczewska et al., 2010). Briefly, cDNA was synthesized in a 10 µl reaction containing 5.5 mM MgCl₂, 2.5 µM random hexamers, 2 mM deoxynucleotides (dNTPs), 4 U of RNase inhibitor, 37.5 U reverse transcriptase and 200 ng RNA. Reactions were performed using an iCycler (Bio-Rad Laboratories) programmed at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min.

Transcript levels of CDS1, CDS2, GPAT1, GPAT2, and AGPAT2 were measured in pectoral adductor muscle and liver tissue using quantitative real-time PCR (qRT-PCR) as described previously (Orczewska et al., 2010). Briefly, gene-specific primers were designed using sequence information obtained from Ensembl (www.ensembl.org) and the software Primer Express (Applied Biosystems) with at least one primer from each pair spanning a splice site to ensure that genomic DNA was not amplified (Table 1). Primers were synthesized commercially

(Invitrogen, Carlsbad, CA, USA). Transcript levels were measured using an ABI 7900HT sequence detection system (Applied Biosystems) in triplicate. 20 μ L reaction mixtures contained 5 ng cDNA, 10 μ L Power SYBR® Green Master Mix (Applied Biosystems) and 0.3 μ M of each forward and reverse primer, with the exception that only 1 ng of cDNA was used to quantify transcript levels of 18S. Optimal primer concentration was determined by measuring transcript levels using 0.2 μ M, 0.3 μ M and 0.4 μ M of each forward and reverse primer. A dissociation curve was analyzed for each reaction to ensure specificity of each primer set. Two controls were used to identify and omit samples with contaminating genomic DNA. One control replaced the cDNA template with an equal volume of Milli-Q H₂O. The second control was prepared for each sample during cDNA synthesis by omitting reverse transcriptase. Reaction efficiency and relative quantity of input RNA were determined using a standard curve for each gene prepared by pooling samples from warm- and cold-acclimated animals. The expression of each target gene was normalized to 18S rRNA in liver tissue and elongation factor-1 alpha (EF-1 α) in oxidative muscle; the expression of these genes was previously determined to remain constant in threespine stickleback during warm and cold acclimation (Orczewska et al., 2010).

2.4 Statistical analyses

Significant differences in mRNA levels, and mass and length of individuals used for measuring mRNA levels, were determined using a one-way ANOVA. Differences in GPAT activity among cold- and warm-acclimated animals and between tissue types were determined using a two-way ANOVA. ANOVAs were followed by a *post-hoc* Tukey's honestly significant difference test. Data were normally distributed as determined by a Shapiro-Wilk test. Equal variance was confirmed with Bartlett's test. A Student's t-test was used to determine significant differences in mass and length between cold- and warm-acclimated animals used for measuring GPAT activity. Data are presented as means $\pm$ s.e.m. Level of significance was set at $P < 0.05$. Statistical tests were performed using Sigma Plot V 11.2 (Systat Software, San Jose, CA, USA) and JMP7 (SAS, Cary, NC, USA).

3. Results

3.1 Physical characteristics

There was no significant difference in body length or mass between warm- and cold-acclimated stickleback used for measuring maximal GPAT activity (Table 2) or mRNA levels. (Table 3).

3.2 GPAT activity in warm- and cold-acclimated stickleback

There was a significant difference in GPAT activity between tissues ($F = 148.03$, $P < 0.001$) and between warm- and cold-acclimated stickleback ($F = 17.997$, $P < 0.001$). Maximal activity of GPAT was 2.9-fold higher in pectoral muscle ($0.29 \pm 0.01 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$) of cold-acclimated animals compared to those held at 20°C ($0.10 \pm 0.02 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$; $P < 0.001$) (Fig. 1). There was no difference in GPAT activity in liver tissue between warm- and cold-acclimated individuals ($P = 0.236$). Maximal GPAT activity in liver was $0.50 \pm 0.03 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ in warm-acclimated fishes and $0.55 \pm 0.04 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ in cold-acclimated fishes ($P > 0.05$). However, GPAT activity was higher in liver compared to pectoral muscle in both cold- and warm-acclimated animals ($P < 0.001$).

3.3 Expression of genes involved in lipid biosynthesis

Transcript levels of CDS1 and CDS2 did not change in response to acclimation in liver (Fig. 2A) and were undetectable in pectoral adductor muscle. GPAT1 transcripts increased in liver in animals held at 8°C for 1 week and then declined by week 4, and were equivalent to animals held at 20°C (Fig. 2B). AGPAT2 transcript levels did not change in liver during cold acclimation (Fig. 2B). In contrast, AGPAT2 mRNA levels increased in pectoral adductor in animals held at 8°C for 1 week and then declined and were equivalent to animals at 20°C by week 4 (Fig. 3). GPAT1 mRNA levels did not change in pectoral adductor muscle in response to cold acclimation (Fig. 3) and GPAT2 mRNA was undetectable in liver and oxidative muscle.

4. Discussion

Little is known about the molecular mechanisms governing phospholipid biosynthesis during mitochondrial biogenesis but our results indicate that GPAT activity increases in oxidative muscle of stickleback during cold acclimation, coinciding with mitochondrial biogenesis, and may contribute to the synthesis of mitochondrial phospholipids.

The maximal activity of GPAT significantly increased 2.9-fold in response to cold acclimation in oxidative muscle of stickleback (Fig.1) and was correlated with a 1.9-fold increase in mitochondrial volume density (Orczewska et al., 2010). In contrast, there was no change in GPAT activity in liver (Fig.1) where mitochondrial biogenesis does not occur (Orczewska et al., 2010). These results suggest that GPAT may contribute to mitochondrial membrane biogenesis in oxidative muscle during cold acclimation in stickleback.

GPAT activity was measured at a common temperature of 14 °C. The 2.9-fold increase in GPAT activity in pectoral adductor muscle in stickleback at 8 °C compared to those at 20 °C corresponds with a Q_{10} of 2.4. The average Q_{10} for many physiological processes is ~ 2 , suggesting stickleback have compensated or overcompensated for the effect of temperature on GPAT activity (Hochachka and Somero, 2002). The increase in GPAT activity is on par with the 3.3-fold increase in the activity of superoxide dismutase (SOD), measured in pectoral adductor of stickleback in response to cold acclimation (Kammer et al., 2011). For this study, SOD activity was also measured at the acclimation temperature of the animals (8 °C and 20 °C) and activity was indeed higher in cold-acclimated animals, indicating overcompensation for thermal effects (Kammer et al., 2011).

The higher maximal activity of GPAT in liver tissue compared to oxidative skeletal muscle is correlated with the higher TAG content of this tissue and likely reflects the dual role of GPAT in synthesizing both phospholipids and TAG (ie; Gimeno and Cao, 2008; Hammond et al., 2002; Igal et al., 2001). TAGs occupy 57 - 59% of hepatocyte volume compared to only 0.4 - 0.5% in oxidative pectoral adductor muscle of stickleback (Orczewska et al., 2010). The density of lipid droplets does not change in either pectoral adductor muscle or liver in response to cold acclimation (Orczewska et al., 2010), suggesting that the increase in GPAT activity in pectoral adductor likely contributes to an increase in mitochondrial density in oxidative skeletal muscle rather than increased synthesis of TAGs.

Although GPAT activity increased in response in pectoral muscle after 11 weeks of cold acclimation, GPAT1 mRNA levels did not change and GPAT2 mRNA was undetectable for up to weeks 9 of cold acclimation. These data suggest several possibilities. First, because we did not measure mRNA levels at week 11, we may have missed an increase in transcript levels at this time, although typically increases in mRNA levels precede increases in protein. Alternatively,

the microsomal isoforms of GPAT (GPAT3 and GPAT4) may drive the increase in GPAT activity. In most mammalian tissues, mitochondrial GPATs account for only 10% of total GPAT activity; the exception is in liver where it accounts for 30-50% (Coleman and Lee, 2004). Although GPAT3 and GPAT4 are expressed in skeletal muscle (Cao et al., 2008; Chen et al., 2008; Shan et al., 2010), we measured mRNA levels of the mitochondrial isoforms because we thought they might be more important in synthesizing phospholipids during mitochondrial biogenesis. In support of this, the mitochondrial isoforms have been shown to alter membrane composition more so than the microsomal isoforms. GPAT1 null mice have altered phospholipid composition in hearts with lower amounts of palmitate in PE, PC and a mixture of PS and PI and higher levels of arachidonate in PC and PE (Lewin et al., 2008). Livers of GPAT1 null mice contain PE and PC with 21% less palmitate (the preferred fatty acid substrate of GPAT1) at the *sn*-1 position and 36% (in PE) or 40% (in PC) more arachidonate at the *sn*-2 position (Hammond et al., 2002). Alterations in diet that stimulate TAG synthesis and increase levels of GPAT1 and GPAT3 do not alter mRNA levels of GPAT2, consistent with our original hypothesis that GPAT2 may be more important for the synthesis of phospholipids than TAGs (Wang et al., 2007). Other studies however, suggest that microsomal GPATs may also contribute to membrane synthesis. Overexpressing PGC-1 α , which stimulates mitochondrial biogenesis, results in remodeling of phospholipids in skeletal muscle and is correlated with increases in both GPAT1 and GPAT3 mRNAs (Senoo et al., 2015). Similar to our results, GPAT2 mRNA was not detected in skeletal muscle, nor was GPAT4 in this study (Senoo et al., 2015). Additionally, overexpression of GPAT4 increases levels of PI and alters the abundance of some PC species but does not affect TAG level (Chen et al., 2008; Nagle et al., 2008).

Another possibility for the discordance between mRNA levels of GPAT1 and GPAT2 and GPAT activity may be due to phosphorylation of GPAT, which regulates its activity. In rats, a decrease in GPAT activity is correlated with an increase in cAMP kinase activity in liver in response to exercise, although phosphorylation of GPAT was not confirmed in this study (Park et al., 2002). It is thought that under these conditions, a decrease in GPAT activity might divert fatty acyl CoAs to carnitine palmitoyl transferase-1, also localized the outer mitochondrial membrane, for fatty acid oxidation during exercise (Park et al., 2002). Stronger evidence for phosphorylation of GPAT comes from a study in adipocytes, where phosphorylation of GPAT3

and GPAT4 is required for an insulin-stimulated increase in GPAT activity and is prevented when PI-3 kinase is inhibited (Shan et al., 2010).

While neither GPAT1 nor GPAT2 increased in response to cold acclimation in oxidative muscle, AGPAT2 mRNA levels did. Similarly, AGPAT2 mRNA increased in response to exercise in skeletal muscle of mice, which stimulates mitochondrial biogenesis (Senoo et al., 2015). AGPAT2 is highly expressed in adipose tissue and mutations in AGPAT2 cause congenital generalized lipidodystrophy 1, characterized by a near complete absence of body fat and impaired insulin signaling (Agarwal et al., 2002). High activity of AGPAT2 is also linked to cancer (Coon et al., 2003). Recently, it has been shown that AGPAT2 regulates mTOR signaling and knocking out AGPAT2 reduces mTOR signaling (Blaskovich et al., 2013). This is interesting because mTOR positively regulates mitochondrial activity (Albert and Hall, 2015), and inhibiting mTOR activity decreases levels of PGC-1 α and NRF-1 and cellular respiration rates in muscle (Cunningham et al., 2007). It is conceivable that the upregulation of AGPAT1 in oxidative muscle of stickleback stimulates mitochondrial biogenesis through an mTOR signaling pathway.

It is noteworthy that mRNA levels of GPAT1 in liver and AGPAT1 in pectoral adductor muscle both increased after one week of cold acclimation. The two enzymes may work in concert, with G3P being converted to LPA by GPAT1 in liver, which is then exported and taken up by muscle and converted to PA by AGPAT1. Prior studies of cold acclimation in stickleback also found increases in gene expression within the first week of cold acclimation, including citrate synthase in liver and pectoral adductor muscle, and PGC-1 β in liver, suggesting metabolic reorganization begins early in the cold acclimation process (Orczewska et al., 2010).

The lack of CDS1 and CDS2 mRNA in pectoral adductor muscle suggest that Tam41, localized to the mitochondrial inner membrane, likely synthesizes CL, as has been shown in yeast (Tamura et al., 2013). Originally, Tam41 was identified as a protein localized to the inner membrane essential for assembling and maintaining the TIM23 complex involved in importing proteins into mitochondria in yeast (Tamura et al., 2006). Later studies showed that knocking out Tam41 virtually eliminated CL and significantly reduced levels of PG, consistent with its function as a CDS (Kutik et al., 2008).

In mice, knocking out PGC-1 α and PGC-1 β led to downregulation of CDS1, and a decrease in CL in cardiac myocytes, suggesting contrary to studies in yeast, that CDS1 may be involved in synthesizing CL, although levels of Tam41 were not measured (Lai et al., 2014). The lack of change in CDS mRNA levels in stickleback, particularly in muscle where mitochondrial biogenesis occurs, may be due to differences in the mitochondrial biogenic pathway between fish and mammals. In contrast to mammals, PGC-1 α is not the master regulator of mitochondrial biogenesis in fish. PGC-1 α levels do not change in response to cold acclimation in muscle of goldfish (Bremer et al., 2012; LeMoine et al., 2008), zebrafish (McClelland et al., 2006) or stickleback (Orczewska et al., 2010), nor in response to exercise in zebrafish (McClelland et al., 2006). Comparison of PGC-1 α sequences among vertebrates revealed a polyserine insertion within the NRF-1 binding domain of PGC-1 α in teleosts, which may eliminate its capacity to mediate mitochondrial biogenesis (LeMoine et al., 2008). Indeed, NRF-1 and PGC-1 β appear to play a more prominent role in inducing mitochondrial biogenesis in fish (Bremer et al., 2012; LeMoine et al., 2008; McClelland et al., 2006). Future studies might investigate the role of NRF-1 and PGC-1 β in regulating the transcription of GPAT and AGPAT isoforms to further elucidate how mitochondrial phospholipid biosynthesis is integrated into mitochondrial biogenesis in fish.

Acknowledgements

Funding for this research was provided by a grant from the *National Science Foundation* to K.M.O. (IOS-0643857).

FIGURE LEGENDS

Figure 1. GPAT activity in liver and oxidative muscle of stickleback held at 8 °C and 20 °C. Significant differences are indicated with asterisks ($P < 0.05$). Values represent the means $\pm$ s.e.m. $N = 6$.

Figure 2. Relative transcript levels of genes involved in membrane biosynthesis in liver. Relative transcript levels of (A) CDS1 and CDS2 and (B) GPAT1 and AGAT2 measured in stickleback liver during cold and warm acclimation. Transcript levels were normalized to 18S mRNA. Different letters indicate significant differences among fish harvested at different times and temperatures during acclimation ($P < 0.05$). $N = 6-8$.

Figure 3. Relative transcript levels of genes involved in membrane biosynthesis in oxidative skeletal muscle. Relative transcript levels of GPAT1 and AGPAT2 were measured in pectoral adductor muscle of stickleback during cold and warm acclimation. Transcript levels were normalized to EF-1 α . Different letters indicate significant differences among fish harvested at different times and temperatures during acclimation ($P < 0.05$). $N = 6-8$.

Table 1.

Primers used for qRT-PCR

Gene	Sequence (5' to 3')	Primer Efficiency	Amplicon Size (bp)
AGPAT2 ENSGACG00000015898	F ATCATCTCCAACCACCAGAGCT	0.93 ± 0.05	51
	R GATCTCCATCAGGCCCAACA		
CDS1 ENSGACG00000014333	F CAGTTCCTGGCGCGCTAT	0.95 ± 0.04	51
	R TGCGAGGTACAAGGCGAAG		
CDS2 ENSGACG00000013234	F CTCTTCGCATCCTCAGCAAATAC	0.89 ± 0.03	51
	R GGTAGAGGGCAAAGGAGATGAAT		
GPAT1 ENSGACG00000006114	F ACAGCAATGGCCTTTTCCAC	0.86 ± 0.02	51
	R TGCAAGCAATGATTGCATCAG		
GPAT2 ENSGACG00000005887	F CCCGGATAGAAGCGTGAGG	0.75 ± 0.11	51
	R TCCTTGAGAGAAAAGGGCTGAG		

Ensembl accession numbers are shown below each gene; F = forward primer, R = reverse primer

Table 2. Effects of cold temperature on physical characteristics of *G. aculeatus* used for measuring GPAT activity.

Temperature	N	Length (cm)	Body Mass (g)
8 °C	12	5.59 ± 0.14	1.53 ± 0.08
20 °C	12	5.77 ± 0.12	1.65 ± 0.08

Values are means ± s.e.m. *N* = number of individuals

Table 3. Effects of cold temperature on physical characteristics of *G. aculeatus* used for measuring mRNA levels of genes involved in phospholipid biosynthesis.

Temperature and Time Point of Harvest	<i>N</i>	Length (cm)	Body Mass (g)
8 °C week 1	16	4.94 ± 0.06	1.24 ± 0.06
8 °C week 4	11	5.14 ± 0.13	1.44 ± 0.11
8 °C week 9	9	5.41 ± 0.07	1.60 ± 0.08
20 °C week 1	10	5.18 ± 0.13	1.41 ± 0.10
20 °C week 9	10	5.45 ± 0.10	1.59 ± 0.10

Values are means ± s.e.m. *N* = number of individuals

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Figure 1

Figure 2

Figure 3