



# Identification and characterization of two isoforms of acyl-coenzyme A oxidase 1 gene and their expression in fasting-induced grass carp *Ctenopharyngodon idella* adipocyte lipolysis

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**Abstract** Acyl-coenzyme A oxidases 1 (ACOX1) is the first rate-limiting enzyme responsible for peroxisomal  $\beta$ -oxidation. In the present study, two mRNA variants, ACOX1a and ACOX1b, transcribed from a single gene, were for the first time isolated and characterized from grass carp *Ctenopharyngodon idella*, both encoding putative peptides of 660 amino acids. Analysis of the exon-intron structures clarified that grass carp ACOX1a and ACOX1b comprise 14 coding exons and correspond to 3a and 3b isoforms of exon 3 splicing variants. Both ACOX1a and ACOX1b mRNAs were expressed in a wide range of tissues, but the abundance of each ACOX1 mRNA showed the tissue-dependent expression patterns. Time-course analysis of ACOX1 expressions indicated that the level of ACOX1a mRNA reached an almost maximal level at day 2, while that of ACOX1b mRNA reached an almost maximal level at day 8 during grass carp primary preadipocyte differentiation. In fasting-induced adipocyte lipolysis, only ACOX1a showed a significant increase in adipocyte, indicating that two ACOX1 isoforms may serve somewhat different roles in the peroxisomal  $\beta$ -oxidation. These results suggested that grass carp ACOX1a and

ACOX1b were differently modulated by fasting in adipocyte. In addition, we found that mitochondrial  $\beta$ -oxidation might dominate at the early stage of fasting in adipocytes, indicating that mitochondria and peroxisomes might possess different capacities in fasting-induced adipocytes fatty acid oxidation.

**Keywords** *Ctenopharyngodon idella* · ACOX1 · Adipocyte · Differentiation · Fasting

## Introduction

In aquaculture, many fish species suffer from metabolic disorders resulting from excessive fat accumulation (Tacon 1996); however, the mechanisms involved in fat metabolism in fish have not been revealed clearly. Adipose tissue functions as an endocrine organ at the center of energy homeostasis, thereby playing an important role in nutrient homeostasis (Rosen and Spiegelman 2014). Because research shows that metabolic disease is attributed to the excessive fat deposited in the adipose tissue (Eckel et al. 2005), adipocyte lipid metabolism in

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fish has been given more and more research concerns in recent years.

Evidence from many studies suggests that modulation of fatty acid oxidation is a new strategy to treat obesity (Herrero et al. 2013). Peroxisomes are involved in the  $\beta$ -oxidation chain shortening of long-chain and very-long-chain fatty acyl-coenzymes (CoAs) (Tugwood et al. 1992). The first step of peroxisomal  $\beta$ -oxidation is catalyzed by the enzyme acyl-CoA oxidase (ACOX) (Inestrosa et al. 1979; Tugwood et al. 1992). Acyl-coenzyme A oxidase 1 (ACOX1) is a homodimer catalyzing the first, rate-limiting step of  $\beta$ -oxidation in the peroxisomes of all eukaryotes. Dysfunction of ACOX1 gene causes hepatic steatosis (Fan et al. 1996). In humans, two isoforms resulted from alternative splicing with different nucleotide sequences for exon 3 (3a or 3I and 3b or 3II) have been described (Oaxaca-Castillo et al. 2007). The alternative transcripts of ACOX1 have also been observed in some fish, e.g., zebrafish (Morais et al. 2007), Nile tilapia (He et al. 2014), and brown trout (Madureira et al. 2016). Although these studies found that ACOX1 was modulated by nutritional stage or hormone, the role of ACOX1 remains poorly characterized in fish adipocytes.

Besides the peroxisomes, mitochondria are also one of organelles to oxidize fatty acids. The CPT1 is a rate-limiting step in mitochondrial  $\beta$ -oxidation and has a pivotal effect on catalyzing the synthesis of long-chain fatty acylcarnitine (McGarry and Brown 1997). The peroxisomal and mitochondrial  $\beta$ -oxidation capacities are different. Peroxisomal  $\beta$ -oxidation is the predominant fatty acid catabolic pathway in plants and several fungi (Hooks et al. 1999; Kunau et al. 1995; Pedersen and Henriksen 2005). In vertebrates, the domination role of mitochondrial and peroxisomal  $\beta$ -oxidation systems depends on tissues and physiological state. For example, mitochondrial  $\beta$ -oxidation showed a response to fasting in rat liver and white adipose tissue, while peroxisomal  $\beta$ -oxidation only showed a response to fasting in rat liver (Palou et al. 2008). Mitochondrion is the principal target for nutritional and pharmacological control of triglyceride metabolism (Frøyland et al. 1997). A study in Atlantic salmon found that peroxisomal  $\beta$ -oxidation dominates fatty acid oxidation in the liver (Frøyland et al. 2000). However, little is known about the difference of mitochondrial and peroxisomal  $\beta$ -oxidation in fish adipocytes.

Grass carp (*Ctenopharyngodon idella*) is an important farmed fish in China for its delicious meat and high

market value (Wang et al. 2015). It is considered a good model for the study of lipid metabolism because grass carp store excess fat in liver and visceral adipose tissue. Additionally, the draft genome of grass carp (Wang et al. 2015) released recently is a useful tool for identifying genomic structure of genes involved in lipid metabolism. In this study, two different ACOX1 genes were cloned and their tissue-specific expressions and mRNA levels in preadipocytes during differentiation were determined in grass carp. Furthermore, to determine the role of mitochondria and peroxisomes in fatty acid catabolism of adipocytes, the CPT1 and ACOX1 mRNA expression were evaluated and compared in adipocytes during fasting. The present study will extend our understanding on the physiological function of ACOX1 gene in the adipocyte of fish.

## Materials and methods

### Fish culture and sampling

Experimental grass carp were obtained from the local fish farm. Animals were acclimated to laboratory conditions for at least 4 weeks prior to experiments. Fish were assigned randomly to one of six treatment groups in 150-L circular tanks (approximately 16–20 fish per tank) with a flow-through water supply at 28 °C under a 12L:12D photoperiod. They were fed a commercial pellet diet (crude protein, 35%; crude lipid, 7%) three times a day and provided with continuous aeration to maintain the dissolved oxygen level near saturation. For cDNA cloning and analysis of tissue distribution, grass carp were euthanized by immersion in MS-222 (50 mg/L) after 24 h post feeding (Sigma, St. Louis, MO, USA). Tissues including the spleen, brain, kidney, abdominal fat, heart, white muscle, red muscle, and liver were rapidly dissected, frozen in liquid nitrogen, and stored at – 80 °C until used. All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals and approved by the Northwest A&F University Institutional Animal Care and Use Committee.

### Identification and cloning of ACOX1

The complete CDS of tilapia ACOX1 were used as queries for a BLASTN search to acquire sequences of grass carp ACOX1 transcripts from our

transcriptomic database (Tian et al. 2015). All the ESTs were assembled *in silico* into a consensus sequence containing the complete open reading frame (ORF) using the SeqMan program of DNASTAR software (Burland 2000). To amplify and confirm the full length of ACOX1 cDNAs, gene-specific primers were designed (Suppl. Table 1).

For gene cloning, total RNA was isolated from grass carp liver using TRIzol Reagent (TaKaRa, Otsu, Shiga, Japan) according to the instructions of the manufacturer. The purity and the concentration of total RNA were measured by a spectrophotometer at 260 and 280 nm. The integrity was tested by electrophoresis in formaldehyde agarose gels. One microgram of total RNA and 0.1 µg of random primers were used to synthesize first-strand cDNA by reverse transcription using the M-MLV reverse transcriptase in a 10-µL reaction volume (Promega, Madison, WI, USA). The resulting product was used as a template for PCR amplification. PCR amplification was made with 2 µL of cDNA, 0.2 µM primers, and 1.25 U of PrimeStar HS DNA polymerase (Takara, Otsu, Shiga, Japan) in a total volume of 50 µL. The primers for each amplification were given in Suppl. Table 1. PCR was performed for 35 cycles at temperatures of 98 °C for 10 s, 55 °C for 5 s, and 72 °C for 2 min. Products of the PCR were electrophoresed on 1.5 % agarose gel staining with Goldview. Bands of the expected size were purified with the PCR purification kit (Tiangen, Beijing, China). The purified fragments were then cloned into a PMD18-T vector following the manufacturer's instructions (TaKaRa, Otsu, Shiga, Japan) and transformed into *E. coli* DH5α. Recombinant bacteria were identified by blue/white screening and confirmed by PCR. Plasmids containing inserts of an expected size were purified and the sequence analysis was carried out by Sangon (Shanghai, China).

#### Sequence characterization and gene organization

The predicted protein sequence was analyzed by ORF Finder (Hancock and Bishop 2004). The presence of conserved domains was analyzed by using the CDART (Geer et al. 2002) and InterProScan (Quevillon et al. 2005) programs. The exon-intron boundary of grass carp ACOX1 was characterized by aligning the cloned cDNA sequence with the genomic sequence (<http://www.ncgr.ac.cn/grasscarp/>).

#### Adipocyte isolation and fasting treatment

The grass carp pre-adipocytes were cultured as described by Liu et al. (2015) with minor modifications. Briefly, the adipose tissue (~ 180 g) was isolated by sterile dissection from the abdominal cavity of 4–5 fishes. The tissue was washed three times with phosphate-buffered saline (PBS, pH 7.4) and minced in 0.1% type I collagenase (Sigma, USA) with 2% BSA (Sigma) at room temperature for 30 min. The cell suspension was filtered through a 200-µm nylon filter and centrifuged at 590 g for 10 min. The sedimented cell pellet was incubated in erythrocyte lysing buffer for 10 min at room temperature, washed twice, and resuspended in growth medium (GM), composed of Dulbecco's modified Eagle's medium (DMEM), 10 % FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. The resuspended cells were seeded in gelatin pre-coated plates at a density of approximately 10 g tissue/25 cm<sup>2</sup>. To reach 80–90% confluency, the cells were incubated at 28 °C with 5% CO<sub>2</sub> for 1 week. The differentiation was induced in adipogenic medium (AM) containing GM supplemented with 10 µg/mL insulin (bovine) (Yangling Xin Yi Biology Technology Co., Ltd.), 10 nmol/L triiodothyronine, 1 µmol/L dexamethasone, and 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX). The medium was changed every 2 days. Cells were harvested at days 0, 2, 4, 6, and 8 after differentiation to determine ACOX1 gene expression during adipogenesis. At differentiation day 8, we changed the medium with free-FBS and extracted RNA after 6 h and 12 h. The gene expressions in cells were also evaluated at the same time. Control and experimental treatments were conducted in triplicate.

#### NEFA content measurement

At day 8 of differentiation, adipocytes cultured in 24-well plates were treated 6 h and 12 h. After the treatment, medium in each well was collected for measurement of NEFA content concentration using a commercial kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocol. Briefly, a mixture of 4 µL medium and 250 µL determination working liquid was added to the 96-well plate, which was then incubated at 37 °C for 15 min, after which the OD value was read in a microplate spectrophotometer at 546 nm.

## Quantitative real-time PCR

Total RNA was extracted as described above. The extracted and purified total RNA was treated with RNase-free DNase to prevent genomic DNA contamination. One microgram of total RNA was used for reverse transcription in a 10- $\mu$ L reaction volume with First Strand cDNA Synthesis Kit (ToYoBo, Tokyo, Japan). Real-time PCR (RT-PCR) was performed using a CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The amplification was performed in a final volume of 20  $\mu$ L containing 0.6  $\mu$ L of each primer (0.5  $\mu$ M), 1  $\mu$ L of the diluted cDNA (tenfold), 10  $\mu$ L of 29 SYBR®Premix Ex TaqTMII (TaKaRa, Dalian, Liaoning, China), and 7.8  $\mu$ L of sterilized double-distilled water. The real-time PCR contained an initial activation step at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 15 s. The standard curve of each gene expression was plotted using the results of the serial dilution standards. The concentrations of all cDNA samples in this study were within this standard range. Gene expression levels were normalized to  $\beta$ -actin mRNA levels by calculating delta cycle thresholds ( $\Delta$ Ct) ( $\Delta$ Ct = Ct of the target gene (ACOX1) – Ct of  $\beta$ -actin). Relative mRNA expression for each gene was normalized to control group by using  $2^{-\Delta\Delta$ Ct method (Pfaffl, 2001).

## Statistical analysis

Statistical analyses were performed with SPSS 20.0 software (SPSS, Chicago, IL, USA). Data are expressed as mean  $\pm$  SEM and were analyzed using either Student's *t* test or ANOVA test (Tukey's multiple comparisons test). Differences were considered to be significant if  $P < 0.05$ .

## Results

### Identification of ACOX1 and gene structure in grass carp

We obtained one ACOX1 gene from grass carp by in silico screening and alternative exon usage results in two isoforms, named ACOX1a and ACOX1b. Grass carp ACOX1a and ACOX1b both encoded a predicted protein of 660 amino acids. ACOX1a and ACOX1b contained three major structural domains: Fatty acyl-

CoA oxidase domain “KWWPGG,” FAD-binding motif “CGGHGY,” and peroxisomal targeting signal “SKL.” These domains are highly conserved between species (Suppl Fig.1).

By using CDS sequences of different ACOX1 transcripts to search grass carp genomic database, the sizes and organizations of two ACOX1 isoforms were analyzed. As shown in Suppl Fig. 2, grass carp ACOX1a and ACOX1b genes are composed of 14 exons, and the locations of splice donor/acceptor sites in all introns follow the consensus “GT/AG” rule (data not shown). The only different region for these two isoforms was the third exon, resulting in the production of two ACOX1 proteins by alternative splicing in grass carp.

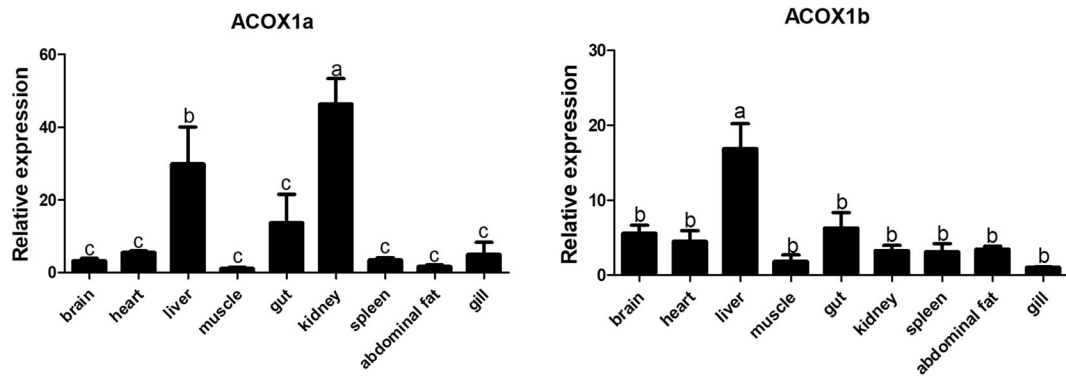
### Tissue distribution of ACOX1s in grass carp and gene expression during grass carp preadipocyte differentiation

The mRNA tissue expression pattern of the two ACOX1 gene isoforms in grass carp was detected by real-time PCR in the liver, adipose tissue, muscle, gill, spleen, heart, brain, gut, and kidney. Real-time PCR showed that both ACOX1a and ACOX1b were broadly distributed in various tissues; while the abundance of each gene mRNA varied among tissues (Fig. 1). The highest mRNA levels of grass carp ACOX1a were observed in the kidney, followed by the liver. The grass carp ACOX1b mRNA levels were the highest in the liver.

ACOX1a expression reached maximum levels by day 2 ( $P < 0.05$ ) and then decreased gradually from day 4 to day 8. ACOX1b expression increased and reached maximum levels by day 8 ( $P < 0.05$ ) (Fig. 2).

### The effect of fasting on grass carp adipocyte lipolysis and gene expression

The upregulation of ATGL mRNA levels (Fig. 3) indicated that adipocyte lipolysis was induced. Interestingly, the NEFA content was not significantly different at 6 h. Fatty acids hydrolyzed from lipolysis fuel energy production via mitochondrial  $\beta$ -oxidation (Ducharme and Bickel 2008; Lass et al. 2011). The expression levels of CPT1b, PPAR $\alpha$ , and Aco2 mRNAs were significantly upregulated, indicating that FFAs released from lipolysis were used for fatty acid  $\beta$ -oxidation in grass carp adipocytes at the early state of fasting. The expression of ACOX1a gene was higher at 12 h after fasting.

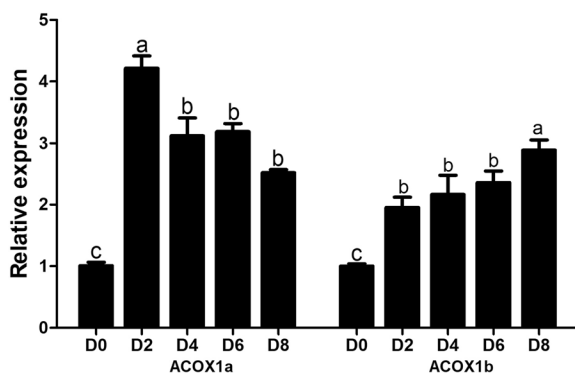


**Fig. 1** Tissue distribution of ACOX1 mRNA in grass carp. Data (mean  $\pm$  SEM,  $n = 4$ ) were normalized to housekeeping gene ( $\beta$ -actin). For a given ACOX1 subtype, groups with different letters were significantly different ( $P < 0.05$ )

However, ACOX1b gene expression did not show any significant change during 12 h of fasting (Fig. 3).

## Discussion

ACOX1 is the rate-limiting step in peroxisomal  $\beta$ -oxidation (Inestrosa et al. 1979; Tugwood et al. 1992). Total  $\beta$ -oxidation in cell is controlled by peroxisomal  $\beta$ -oxidation and mitochondrial  $\beta$ -oxidation. Although mitochondrial and peroxisomal  $\beta$ -oxidation show different capacities in liver and muscle of fish (Frøyland et al. 2000), little is known about their difference in adipocytes. In the present study, two ACOX1 isoforms were for the first time isolated and characterized in grass carp. Grass carp ACOX1a and ACOX1b were differentially expressed in tissues and primary cultured preadipocytes



**Fig. 2** ACOX1a and ACOX1b expression during adipogenesis. ACOX1a and ACOX1b expression were determined by quantitative real-time PCR on days 0, 2, 4, 6, and 8 of differentiation. Gene expression was normalized to  $\beta$ -actin and was referred to the expression of ACOX1a and ACOX1b in day 0. Data are means  $\pm$  SEM,  $n = 4$ . Different letters indicate significant differences at  $P < 0.05$

during differentiation. Their mRNA expression levels and CPT1b mRNA expression were also determined in adipocytes lipolysis regulated by fasting in vitro. These findings will contribute toward our understanding on the genomic structure and physiological function of ACOX1 gene in fish.

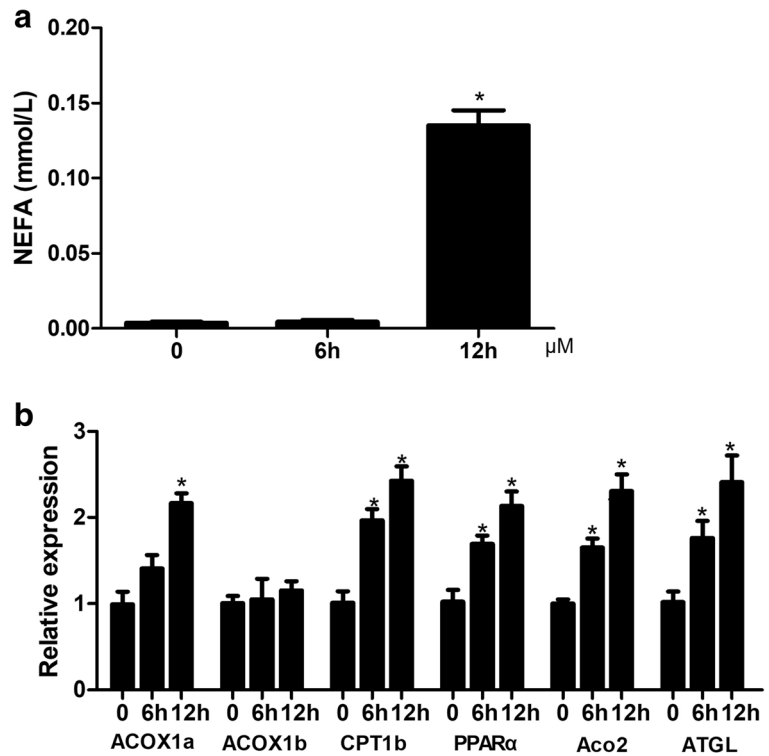
In grass carp, ACOX1 consists of 15 exons and 14 introns, with two exons 3 named 3a and 3b. Alternative splicing of the 3a and 3b leads to the synthesis of two mRNA variants which encode two ACOX1 isoforms: ACOX1a and ACOX1b. The result is in accordance with that in tilapia (He et al. 2014), brown trout (Madureira et al. 2016), zebrafish (Morais et al. 2007), rodents (Osumi et al. 1987), and human (Varanasi et al. 1994), indicating that the occurrence of alternative splicing variants of two isoforms for ACOX1 is conserved through evolution. It is noteworthy that sequence similarity between 3a is higher than that in 3b, suggesting that the third exon of ACOX1a was more conserved in vertebrates than that of ACOX1b.

The grass carp ACOX1 deduced protein shares several of the features commonly found in homologous mammalian enzymes (Suppl Fig. 1) (Okazaki et al. 1986). These conservations include the fatty acyl-CoA oxidase domain (KWWPGG) (Hooks et al. 1999), the FAD-binding motif (CGGHGY), and the Glu421 catalytic active site (Matsubara et al. 1989). In addition, like most other ACOXs, grass carp ACOX1 has one SKL peroxisomal targeting signal at its carboxy-terminal end, which is conserved in many peroxisomal matrix proteins in animals (Kunau et al. 1995). These results indicate that the gene structure and functional domains of ACOX1 are remarkably conserved.

The analysis of tissue expression pattern is useful to have a better understanding of their physiological



**Fig. 3** The effect of fasting on FFA release and gene expressions of ACOX1 and CPT1b in grass carp adipocytes. The adipocytes were fully differentiated for 8 days and then were exposed to growth medium without FBS. **a** FFA concentration in the medium. **b** mRNA expression of ACOX1, ATGL, PPAR $\alpha$ , Aco2, and CPT1b genes. The gene expression levels were determined by quantitative Real-Time PCR. Data (mean  $\pm$  SEM,  $n = 4$ ) were referred to the control treatment using  $\beta$ -actin as a control. Different letters indicate significant differences at  $P < 0.05$



roles. In this study, the divergent expression of ACOX1a and ACOX1b genes was observed in grass carp. For ACOX1a, the highest expression level was observed in the kidney followed by the liver. The highest number of ACOX1b transcripts was observed in the liver. The result that ACOX1a and ACOX1b were both abundant in the liver is similar to that in mammalian and fish studies (Cherkaoui-Malki et al. 2012; Vluggens et al. 2010; Nöhammer et al. 2000; He et al. 2014; Morais et al. 2007). However, the expression grass carp ACOX1b in the kidney is lower than that in the liver, which is different from the result in tilapia (He et al. 2014) and zebrafish (Morais et al. 2007). These different results may own to the species-specificity. In tilapia, the mRNA level of ACOX1a not ACOX1b was influenced by nutritional stage in the kidney (He et al. 2014). We speculate that the difference between ACOX1a and ACOX1b mRNA levels in the kidney may be due to their different roles in the nutritional stage and this should be investigated in the future. Thus, the different expression patterns between ACOX1a and ACOX1b provided evidence that these transcripts may function different roles in the same organs. Hence, more research is needed to reveal their roles in regulating metabolism.

Little is known about the role of ACOX1 in regulating adipocyte differentiation. In this study, the respective unique expression patterns of ACOX1a and ACOX1b at different time points of grass carp preadipocyte culture indicate that ACOX1a and ACOX1b may play a role at different times of grass carp preadipocytes differentiation (Fig. 2). The expression level of ACOX1a reached a maximum at day 2 of differentiation, suggesting that ACOX1a plays an important role during the start of differentiation, while the expression level of ACOX1b reached a maximum at day 8 of differentiation, suggesting that ACOX1b plays an important role during the stage of terminal differentiation. Samulin et al. (2008) suggested that the observed increase in ACOX1 mRNA during pig adipocyte adipogenesis is due to peroxisomal  $\beta$ -oxidation and proliferation rather than differentiation. Thus, details of the role of ACOX1a and ACOX1b in the mechanisms controlling adipocyte differentiation require further investigation.

During fasting, fatty acids released from adipocytes via lipolysis are subsequently transported to other target tissues and utilized as substrates for energy production. Interestingly, in this study, we found that FFAs released from lipolysis were used for fatty acid  $\beta$ -oxidation in grass carp at the start of fasting, indicating that adipocytes may be “egoistic” holding released FAs to satisfy their own energetic

requirements, as reported in 3T3-L1 adipocytes (Barbato et al. 2014). However, the expression levels of the ACOX1 gene have no changes at this stage, suggesting that mitochondrial  $\beta$ -oxidation rather than peroxisomal  $\beta$ -oxidation dominated at the egoistic stage of adipocytes during fasting. Compared with 6 h, ACOX1a gene and CPT-1b gene both were upregulated, indicating that peroxisomal  $\beta$ -oxidation and mitochondrial  $\beta$ -oxidation may cooperate to oxidize fatty acids derived from lipolysis at the altruistic stage of adipocytes during fasting. Previous studies found that the two types of ACOX1 showed different substrate specificities (Setoyama et al. 1995; Oaxaca-Castillo et al. 2007). Here, ACOX1a and ACOX1b showed different expression during fasting, suggesting that the two ACOX1 isoforms might have different roles in metabolic regulation in grass carp, and ACOX1a may play a relatively more important role in peroxisomal  $\beta$ -oxidation in grass carp adipocytes.

In summary, two grass carp ACOX1 isoforms named ACOX1a and ACOX1b were cloned and characterized in grass carp. Conservation of functional domain in ACOX1a and ACOX1b suggests that they may perform a similar function in grass carp as reported in mammals. The differential expression of two ACOX1 transcripts in different tissues and grass carp primary preadipocyte differentiation may have functional significance. Fasting induced the mRNA expression of ACOX1a, but not ACOX1b, suggesting two ACOX1 isoforms may serve somewhat different roles in peroxisomal  $\beta$ -oxidation of grass carp adipocytes. In addition, mitochondria and peroxisomes might possess different capacities in fasting-induced fatty acid oxidation. These results will help increase our understanding on the physiological function of ACOX1 in fish adipocytes.

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**Compliance with ethical standards** All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals and approved by the Northwest A&F University Institutional Animal Care and Use Committee.

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