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Effects of long-term starvation and refeeding on fatty acid metabolism-related gene expressions in the liver of zebrafish, *Danio rerio*

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Abstract: A 70-day starvation was conducted to determine the effects of long-term starvation on whole-body polyunsaturated fatty acid (PUFA) composition and fatty acid metabolism-related gene expression in the liver of zebrafish, *Danio rerio*. The starvation period was followed by a 15-day refeeding period to test recovery of both PUFA composition and gene expression levels. It was found that *n*-3 PUFA and arachidonic acid levels increased during starvation and returned to normal values after refeeding ($P < 0.05$). Expression of seven (*elovl5*, *fads2*, *cpt1-β*, *acox1*, *acadvl*, *fabp1a*, and *fabp7a*) of eight total genes downregulated significantly towards the end of the starvation. As a result of refeeding, gene expression of *elovl5* and *ppar-α* returned to the normal levels. Therefore, it was concluded that, in general, mRNA expression of genes involved in fatty acid metabolism was negatively influenced by long-term starvation (70 days); there was no direct relationship between whole-body fatty acid composition and hepatic gene expressions; and 15-day refeeding was insufficient for recovery of starvation-based metabolic losses in zebrafish.

Key words: Lipid metabolism, starvation, mRNA transcription, qPCR, zebrafish

1. Introduction

Fish are often exposed to long- or short-term starvation periods in their natural environments because of various reasons such as environmental factors, temperature, and breeding. Fish farmers also use starvation as a routine aquaculture procedure in order to decrease feed costs and increase profitability and product quality [especially n-3 polyunsaturated fatty acid (n-3 PUFA) content]. During starvation, stored energy resources are used to meet metabolic energy requirements, and this usually results in weight loss (1). Energy requirements of fish under insufficient feed conditions are met by catabolism of lipids, carbohydrates, and amino acids. Lipids are especially important to meet the energy requirements of vital metabolic functions through lipid oxidation. As lipid reservoirs become depleted, free amino acids become a more significant source of energy within body tissue. Moreover, liver glycogen is generally the first consumed substrate in food deprivation and as a metabolic response to increased gluconeogenesis in liver, tissue glycogen and plasma glucose levels are conserved (2,3).

Zebrafish (*Danio rerio*) serves the scientific world as a model organism in a wide range of research areas because of their many crucial features such as embryological characteristics, ease of breading, short lifespan, small size, low husbandry costs, and whole genomic sequence availability. However, a limited number of studies examined the relationship between starvation and fatty acid metabolism in zebrafish, especially at the molecular level. Drew et al. (4) reported that expression of elongation of very long chain fatty acids protein 5 gene (*elovl5*) in the brain and liver of adult female zebrafish decreased with starvation. Enyu and Shu-Chien (5) suggested that zebrafish utilized noncarbohydrate resources for energy during starvation.

Although many aspects of poikilothermic and mammalian physiology are quite different, their basic metabolic pathways such as fatty acid and amino acid metabolism are similar. Starvation studies, however, help researchers to better understand fish physiology and nutrient metabolism (6). Determination of the effects of long-term starvation on the expression of genes involved in fatty acid metabolism in zebrafish would significantly add to the current understanding of the process of fatty acid metabolism in poikilothermic or homoeothermic organisms. Therefore, the aims of the present study were: 1) to determine the effects of long-term starvation

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(70 days) on hepatic mRNA expression of several genes associated with fatty acid metabolism in zebrafish; 2) to learn whether a 15-day refeeding period is sufficient for recovery of PUFA content and gene expressions and; 3) to better understand fatty acid metabolism of zebrafish at the molecular level.

2. Materials and methods

2.1. Genes

Eight genes having critical roles in the elongation, desaturation, mitochondrial/peroxisomal β-oxidation, and transportation of fatty acid metabolism were selected based on previous studies. Names, metabolic functions, and IDs of genes are presented in Table 1.

2.2. Fish and experiment

A total of 120 mature zebrafish (~0.96 g and 1 year old) were divided into six aquariums (30 × 15 × 20 cm) with 20 fish/aquarium for an acclimatization period of 15 days at the Aquaculture Research Laboratory of Almus Vocational Collage, Gaziosmanpaşa University, Almus, Turkey. No dead fish were recorded during the acclimatization period and fish were fed ad libitum with a commercial zebrafish diet (Ahm Marina Fish Tropical) composed of 40% protein and 7% crude lipid 3 times a day at 0900, 1230, and 1630 hours. The fish in the first treatment group were starved until deaths began (for 70 days). The fish in the second group served as the control. Once deaths occurred in the starved group (more than two fish/day), they were also fed for 15 days (refeeding period). The above diet with the same feeding strategy and schedule was used for feeding of the control group during the trial and all aquariums (control + starved) for the refeeding period. The experiment was carried out in triplicate (3 experimental tanks and their respective controls). Aquariums were cleaned with external canister filters (Atman, AT - 3338) throughout the study and thermostat control heaters were used to maintain a constant water temperature of 28.5 °C, which is the optimal temperature for zebrafish growth (7).

2.3. Sampling

Following starvation and refeeding periods, six fish were randomly selected from both the starvation and control groups (two fishes from each aquarium). The fish were euthanized using ice-cold water and samples were taken for quantitative polymerase chain reaction (qPCR). The livers of the fish were then removed using sterilized equipment and magnifying glasses. Six more fish from each group were sampled for fatty acid analyses and whole fish were immediately transferred to a deep freezer (–86 °C) until analyses. Research was performed in accordance with the directives of the Atatürk University Local Ethics Committee for Animal Experiments.

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

The total RNA from the liver was extracted using TRIzol reagent by following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Quality of the extracted RNAs was evaluated with agarose gel electrophoresis. Total RNA concentration was determined with spectrometric measurements carried out at 260 nm, and then complementary DNA (cDNA) was synthesized from total RNA using the Omniscript Reverse Transcription kit (QIAGEN, Düsseldorf, Germany) as per the manufacturer's instructions. Primers were designed using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Metabion GmbH, Germany (Table 1). Primers were designed using an "Exon-Exon Junction" model to prevent heterogenic RNA or genomic DNA from mixing with the PCR product. The following conditions were used in the RT-PCR method: initial incubation (37 °C for 1 h), initial denaturation (95 °C for 120 s), denaturation (95 °C for 20 s and 35 cycles), incubation at optimum annealing temperature of each

Table 1. Ensembl gene ID and functions of lipid metabolism-related genes in zebrafish.

Gene name	Symbol	Ensembl ID	Function
Elongation of very long chain fatty acids protein 5	*elovl5*	ENSDARG00000004979	Elongation
Fatty acid desaturase 2	*fads2*	ENSDARG00000019532	Desaturation
Acyl-coenzyme oxidase 1 dehydrogenase very long chain	*acadvl*	ENSDARG00000016687	Mitochondrial β-oxidation
Carnitine palmitoyltransferase	*cpt1-β*	ENSDARG00000058285	Mitochondrial β-oxidation
Acyl-coenzyme oxidase 1	*acox1*	ENSDARG00000014727	Peroxisomal β-oxidation
Peroxisome proliferator-activated receptor-α	*ppar-α*	ENSDARG00000031777	Peroxisomal β-oxidation
Fatty acid binding protein 1a	*fabp1a*	ENSDARG00000019357	Transport
Fatty acid binding protein 7a	*fabp7a*	ENSDARG0000007697	Transport

gene (30 s) (Table 2), elongation (72 °C for 60 s), and final elongation (72 °C for 5 min).

2.5. Detection of gene expression of lipid metabolism-related genes by quantitative polymerase chain reaction (qPCR)

The mRNA transcription levels of each gene were determined according to the method described by Bustin et al. (8) using a Rotor-Gene 6000 thermal cycler system (QIAGEN) and the QuantiTect SYBR Green PCR kit (QIAGEN). The reaction conditions of qPCR were as follows: 95 °C for 15 min (initial denaturation), followed by a denaturation at 95 °C for 20 s and the optimum annealing temperature of each gene for 30 s, followed by elongation at 72 °C for 30 s (40 cycles). The genes that were equally expressed in all tissues [ribosomal protein large subunit 7 (*rpl7*) and ribosomal protein large subunit 13 alpha (*rpl13*α)] were selected as reference genes (9). For calculation of the transcription level of each gene, data were normalized by dividing the copy numbers of each gene by the copy numbers of reference genes and the averages were considered.

2.6. Lipid and fatty acid analysis

Lipids were extracted from whole-body zebrafish samples according to the method reported by Folch et al. (10). Two fish from each aquarium (six fish per treatment) were crushed and merged for homogenization. Approximately 1 g of homogenized sample was transferred into a 15 mL-tube and 20 mL of 2:1 chloroform/methanol mixture (containing 0.01% butylated hydroxyl toluene to act as an antioxidant) was added. The mixture was then homogenized at 20–22 °C for 1 min. After that, the samples were filtered using Whatman filter paper (No. 1) under vacuum conditions and the lipid content was determined gravimetrically. The method described by Metcalfe and Schmitz (11) was used for the preparation of methyl esters of fatty acids (FAME). Prior to trans-methylation, 8 mg of nonadecanoate acid (C19:0) per 50 mg of lipid was incorporated into the samples as an internal standard.

Crude lipid extracts were saponified with 2 M NaOH and FAMEs were prepared with trans-methylation using 25% boron trifluoride in methanol. Fatty acid (FA) content of lipids was determined by gas chromatography (GC, Hewlett Packard, 6890 N) equipped with a capillary column (60 m, 0.25 mm i.d., and 0.25 μm). Oven temperature was set to 190 °C for 35 min and then increased by 30 °C/min until the temperature reached 220 °C, and this temperature was maintained for 5 min. The carrier gas (H_2) flow was adjusted to 2 mL/min and the split ratio was set to 30:1. Each FA was determined by comparing them with a standard FA mixture (Supelco 37 component FAME mix, Cat No. 47885-U) and their concentrations were calculated according to the peak area of the C19:0 internal standard.

2.7. Statistical analyses

The statistical analysis of FA levels was performed with SPSS 10.0 (12). A one-way analysis of variance (ANOVA) test was performed for data analyses and Duncan's multiple comparison tests were used to compare significant means of fatty acid compositions of experimental groups ($P < 0.05$; $n = 6$).

3. Results

3.1. Fatty acid metabolism-related genes

3.1.1. Elongation and desaturation-related genes

Both the elongation of very long chain fatty acids protein 5 gene (*elovl5*) and the fatty acid desaturase 2 gene (*fads2*) were downregulated as a result of starvation (5.4- and 12.5-fold, respectively). While a 15-day refeeding period was not sufficient for recovery of *elovl5* gene expression, it was enough for that of *elovl5* mRNA expression (Figure).

Table 2. Primers used in RT-qPCR and optimum annealing temperatures (Tm).

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Tm (°C)
elovl5	CTAAAGGTTGAAGATGGAGACG	CGTCCAGCAGAAACCATCCT	60.6
fads2	CCCGTGGAGTATGGCGTTA	TGTGGCTCATCTGTGTGACC	58.1
acadvl	GCAGTCGTTAGTGTGGAATCA	GACCAAAGGCTCCCATCTCC	60.2
cpt1-ß	GTCCTGAGACGGATTCTTTCC	TGACTGCTAACTGGTGCTGG	62.6
acox1	CCAGGTGGATTGGGTAAAAC	CGAATGGGTGTGATGAAAGCA	60.6
ppar-α	CTCCGCCCTTCGTCATTCA	CGTATTTCAGCAGCGTCACC	62.6
fabp7a	GCTACCAGGCAAGTAGGCAA	CCAAGGATACAGTAGACTTCACA	60.6
fabp1a	GAAGGCAGTCGGTGTGC	CCGTCACAGACTCAATCCCC	63.4
rpl13α	AGCAAGTGCTGTTGGGCCAC	GTGTGGCGGTGATGGCCTGG	63.1
rpl7	CAGAGGTATCAATGGTGTCAGCCC	TTCGGAGCATGTTGATGGAGGC	65.6

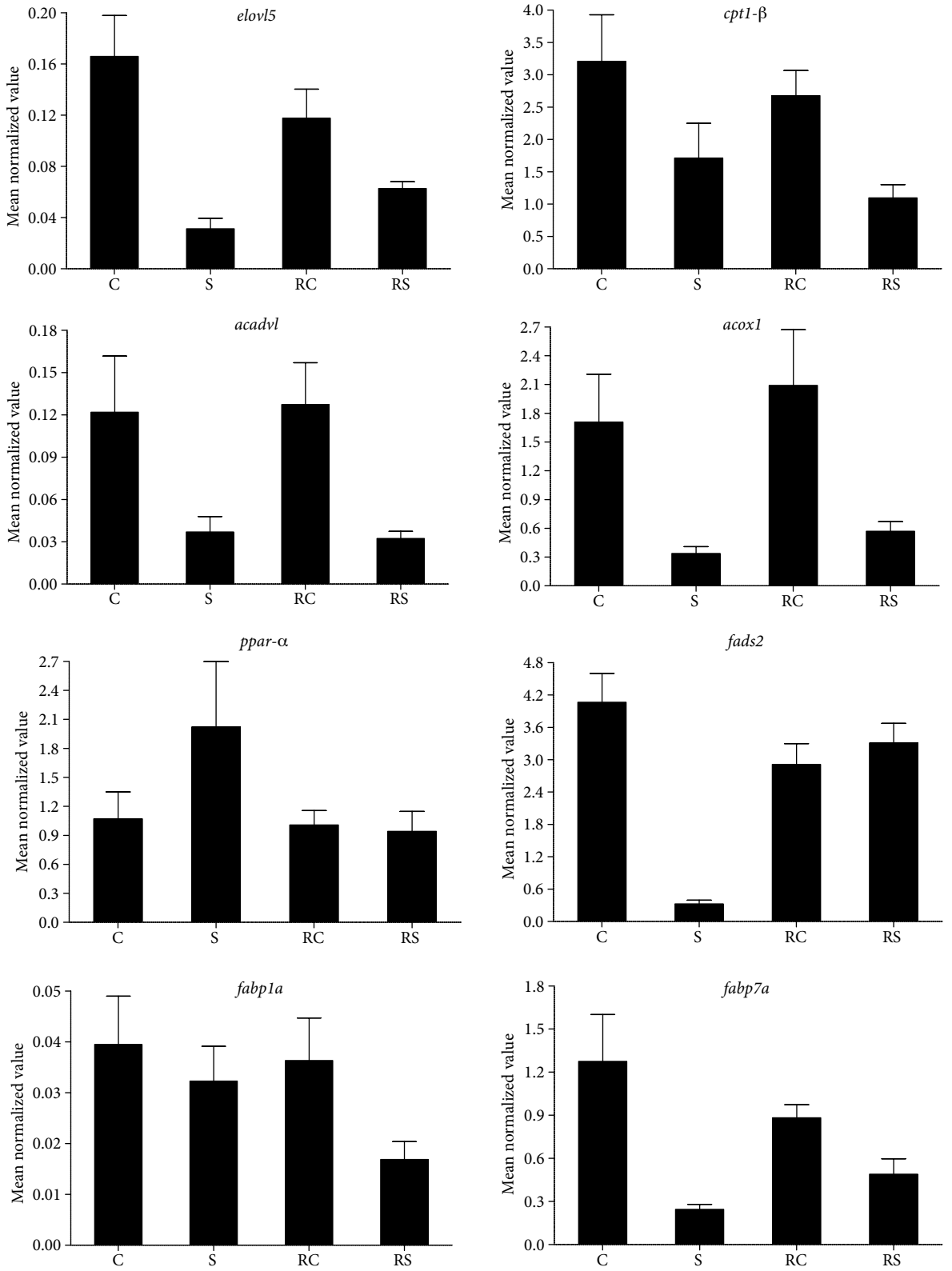

Figure. Expression level of lipid metabolism-based genes in the liver of zebrafish exposed to long-term starvation and refeeding (C: control; S: starved; RC: refeeding, control; RS: refeeding, starved).

3.1.2. Mitochondrial and peroxisomal β-oxidation-related genes

Starvation resulted in 4- and 1.9-fold decreases in mRNA expression of acyl-CoA dehydrogenase very long chain (*acadvl*) and carnitine palmitoyltransferase 1-β (*cpt1-β*), respectively. After the refeeding, no significant difference was observed in *acadvl* expression between starved and fed fish; however, refeeding could not stop the decrease in mRNA expression of *cpt1-β* (Figure).

Hepatic expression of acyl-coenzyme oxidase 1 (*acox1*), which has crucial functions in peroxisomal β-oxidation, in starved fish decreased by 5.1-fold. It was also found that 15 days was not enough for recovery of *acox1* (Figure). As an exception, mRNA transcription of peroxisome proliferator-activated receptor-α (*ppar-α*) was upregulated by 1.2-fold compared to the control group and returned to normal values after refeeding (Figure).

3.1.3. Transport genes

While mRNA expression of fatty acid binding protein 7a (*fabp7a*) was dramatically reduced by starvation at 5.2-fold, fasting did not affect fatty acid binding protein 1a (*fabp1a*) expression (Figure). Partial increase was observed in mRNA expression of *fabp7a* after refeeding. Unexpectedly, mRNA expression of *fabp1a* decreased with refeeding (Figure).

3.2. Fatty acid content

As seen in Table 3, the *n*-3 PUFA, eicosapentaenoic acid (EPA; 20:5 *n*-3), docosahexaenoic acid (DHA; 22:6 *n*-3), and *n*-3 PUFA/*n*-6 PUFA levels increased significantly to 25.19 ± 0.25%, 3.26 ± 0.04%, 17.09 ± 0.23%, and 2.16 ± 0.02%, respectively ($P < 0.05$). The *n*-6 PUFA and linoleic acid (LA; 18:2 *n*-6) levels were decreased by starvation, while arachidonic acid (ARA; 20:4 *n*-6) levels increased during this period (Table 3, $P < 0.05$). After the refeeding period, all fatty acid levels normalized to the control group's levels (Table 3).

4. Discussion

4.1. Fatty acid composition

In general, *n*-3 PUFA levels increased in response to the starvation treatment whereas *n*-6 PUFA levels decreased (except for ARA). These results were in agreement with previous studies of gilthead seabream (*Sparus aurata*) and European perch (*Perca fluviatilis*) (13,14). Henderson and Tocher (15) reported that fish species protect *n*-3 PUFA during starvation periods in order to the preserve the cell membrane. Luo et al. (16) and Silva et al. (17) reported an increase of *n*-3/*n*-6 PUFA levels in channel catfish and hybrid tilapia (*Oreochromis mossambicus* × *O. niloticus*) after long-term starvation. Similar to these observations, in our study the *n*-3/*n*-6 PUFA ratio increased 1.8 times because of high *n*-3 PUFA levels with starvation. It has been well known that PUFAs play important roles as structural components during organogenesis, act as initiators for eicosanoids and prostaglandins, and also affect several behavioral properties such as early larval learning performance (18,19). Therefore, the increase in the *n*-3 PUFA levels in starved zebrafish might be related to important physiological and metabolic functions of *n*-3 PUFA.

In fish, *n*-6 PUFAs are catabolized as an energy source during starvation (14,16); this could be the explanation for low *n*-6 PUFA in starved zebrafish. Moreover, Silva et al. (17) reported that EPA and ARA are the precursors of the eicosanoids, thromboxanes, leukotrienes, and prostaglandins, which are biologically active molecules, and they were preserved in starved hybrid tilapia because of these roles; this is a possible explanation for increased ARA level in starved zebrafish.

Table 3. Polyunsaturated fatty acid composition of experimental groups (% of fatty acids).

Fatty acid	Control	Starved	Refeeding, control	Refeeding, starved
18:3 *n*-3	3.82 ± 0.02^{c}	7.64 ± 0.01^{a}	4.32 ± 0.22^{b}	4.10 ± 0.02^{ab}
20:5 *n*-3	2.24 ± 0.02^{b}	3.26 ± 0.04^{a}	2.23 ± 0.04^{b}	2.08 ± 0.03^{c}
22:5 *n*-3	1.59 ± 0.19^{c}	2.72 ± 0.04^{a}	2.17 ± 0.34^{b}	1.55 ± 0.06^{c}
22:6 *n*-3	11.29 ± 0.28^{b}	17.09 ± 0.23^{a}	10.05 ± 1.05^{c}	9.59 ± 0.33^{c}
Σ*n*-3	19.40 ± 0.27^{b}	25.19 ± 0.25^{a}	19.33 ± 1.65^{b}	17.84 ± 0.43^{b}
18:2 *n*-6	12.31 ± 0.02^{c}	3.58 ± 0.08^{d}	14.46 ± 0.41^{a}	13.53 ± 0.05^{b}
20:4 *n*-6	2.05 ± 0.01^{b}	6.95 ± 0.06^{a}	1.91 ± 0.08^{c}	1.77 ± 0.02^{d}
Σ*n*-6	15.76 ± 0.06^{c}	11.62 ± 0.22^{d}	17.36 ± 0.32^{a}	16.30 ± 0.01^{b}
Σ*n*–3/Σ*n*-6	1.23 ± 0.01^{b}	2.16 ± 0.02^{a}	1.11 ± 0.11^{c}	1.09 ± 0.02^{c}

a, b, c, d in a row show statistically different experimental groups. Values are given as mean ± SD (n = 6) ($P < 0.05$).

4.2. Gene expressions

elovl5 and *fads2* play essential roles in the biosynthesis of highly unsaturated fatty acid (HUFA) from the precursor molecules α-linolenic acid (ALA; 18:3 *n*-3) and LA (20,21). We found that the expression of *elovl5* and *fads2* decreased in response to long-term starvation. Similarly, Drew et al. (4) and Salem et al. (22) reported that starvation downregulated mRNA transcription of *elovl5* (approximately 2-fold) and *fads2* (9.5-fold) in zebrafish and rainbow trout, respectively. Biosynthesis of PUFA is a process with a high metabolic energy cost; 10 ATP are used in each desaturation and 2-carbon elongation (22). During starvation, metabolic activity in fish is lowered by some physiological adaptation activities such as reduction of PUFA synthesis. This allows the fish to conserve energy when resources are scarce. Therefore, we concluded that the energy-saving strategy during starvation is the reason for reduced mRNA expression in *elovl5* and *fads2*. On the contrary, in the present study, *n*-3 PUFA, EPA, and DHA levels of the whole body increased with starvation (Table 3); this may be explained by the essential role(s) of the liver in lipid homeostasis, and further study will be needed to improve our understanding of this relationship.

ACADVL is a mitochondrial membrane protein that functions during the first stage of mitochondrial β-oxidation (23). *cpt1-β*, which regulates peroxisome and mitochondrial fatty acid oxidation, also plays a role in the transport of fatty acids across the external membrane of the mitochondria (24). In this study, decreased *acadvl* and *cpt1-β* expressions in response to starvation are thought to be a result of a reduction in the transportation of fatty acids across the mitochondrial membrane. The *acox1* gene, which catalyzes the first step of peroxisomal β-oxidation, is also a limiting step of peroxisome β-oxidation (25,26). The decrease of *acox1* mRNA expression in fasted fish may be the result of the recruitment of muscle tissue for fatty acid oxidation in order to meet energy requirements. *ppar-α* can be activated by saturated fatty acids, palmitoleic acid, and PUFAs (27). In our study, mRNA transcription of *ppar-α* was upregulated with starvation. This may have occurred as a result of the activation of *ppar-α* in response to the increased levels *n*-3 PUFA caused by starvation. Similarly, Erol et al. (28) reported that starvation significantly decreased hepatic *ppar-α* expression in mice.

Expression of both transport genes (*fabp1a* and *fabp7a*) was negatively affected by the starvation stress. *fabp7a* plays a role in the transportation of fatty acids such as DHA, which are essential for proper nervous system function (29). Rapid fat metabolism occurs in the intestine, liver, adipose tissue, and muscle. Therefore, high *fabp* gene expression levels are associated with the intake and use of fatty acids in these tissues (30). Hence, the decrease in *fabp* expressions in the liver is likely due to fatty acid levels that were too low to trigger *fabp* expressions.

In conclusion, it was determined that mRNA expressions of several genes involved in fatty acid metabolism in the liver of zebrafish were downregulated (except for *ppar-α*) by long-term starvation; a 15-day refeeding period, in general, was insufficient for recovery of mRNA expressions; and there was no direct relationship between PUFA composition and fatty acid metabolism-related gene expressions. Finally, we think that more studies are needed to explain the molecular effects of starvation in teleost fishes to better understand fatty acid metabolism.

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