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Molecular cloning and characterization of unfolded protein response genes from large yellow croaker (*Larimichthys crocea*) and their expression in response to dietary fatty acids

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ABSTRACT

The unfolded protein response (UPR) is a mechanism to cope with perturbed endoplasmic reticulum (ER) functions or accumulation of unfolded protein in the ER in eukaryotic cells. Furthermore, the UPR also participates in a number of physiological and pathological processes, such as nutrient sensing, lipid synthesis, and inflammatory response. In this study, four UPR-related genes (*GRP78/BiP*, *ATF6α*, *XBP1* and *CHO*) were isolated characterized from large yellow croaker (*Larimichthys crocea*), and their expression in response to dietary lipid sources (various fatty acids) such as fish oil (FO), palmic acid (PA), olive oil (OO), sunflower oil (SO), and perilla oil (PO), were examined following feeding. The results showed that the four UPR-related proteins contained highly conserved functional domains and had the closest phylogenetic relationships with other fishes. Additionally, these genes were ubiquitously expressed in large yellow croaker, as in zebrafish and medaka. Moreover, *GRP78*, *ATF6α* and spliced *XBP1* (*XBP1s*) mRNA levels in the liver, not in adipose tissue, were significantly increased in the SO group compared to the other groups ($P < 0.05$). These results indicated that dietary SO activated UPR, and the activation of UPR might provide a mechanism to improve ER function, but probably stimulated lipid synthesis and caused inflammatory response in the liver of large yellow croaker.

1. Introduction

The endoplasmic reticulum (ER) is responsible for protein folding and maturation (Ghaemmaghami et al., 2003). Unfortunately, a significant number of genetic or environmental changes perturb ER homeostasis, resulting in the accumulation of unfolded proteins in ER lumen. To limit further accumulation of unfolded proteins in the ER, the unfolded protein response (UPR) is triggered (Kaufman et al., 2002).

In mammals, the UPR is mainly mediated by three ER-localized proteins: inositol-requiring transmembrane kinase and endonuclease 1α (IRE1α), double-stranded RNA (dsRNA)-dependent protein kinase-like ER kinase (PERK), and activating transcription factor-6 (ATF6) (Mori, 2009; Hollien, 2013). Under nonstressed conditions, all three transmembrane proteins are bound by the major ER protein chaperone – immunoglobulin heavy chain binding protein/glucose-regulated protein 78 (BiP/GRP78), and maintained inactive. Perturbations that alter ER homeostasis, particularly an increase in newly synthesized, unfolded proteins in the ER lumen, increase the binding of GRP78 to luminal misfolded proteins, resulting in the release of PERK, IRE1α, and ATF6, and leading to their activation. On activation of the UPR, ATF6 translocates to the Golgi apparatus where it is cleaved by site 1 and site 2 proteases to generate a fragment that can migrate to the nucleus and act as a transcription factor. Activation of IRE1α unmasks its endoribonuclease activity that cleaves a 26 base-pair segment from the mRNA of the X-box binding protein-1 (*XBP1*), encoding the active (or spliced) form of the transcription factor (XBP1s). XBP1s, alone or in synergy with ATF6α, upregulates the transcription of genes encoding ER chaperones, ER biogenesis, phospholipid synthesis, and ER-associated protein degradation (ERAD). PERK activation phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α), subsequently leading to attenuation of translation initiation. Paradoxically, phosphorylation of eIF2α by PERK facilitates translation of

Abbreviations: ATF6, activating transcription factor-6; ATF4, activating transcription factor 4; BiP/GRP78, immunoglobulin heavy chain binding protein/glucose-regulated protein 78; CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; eIF2α, the α subunit of eukaryotic translation initiation factor 2; FO, fish oil; IL, interleukin; IRE1α, inositol-requiring transmembrane kinase and endonuclease 1α; LC-PUFA, long-chain PUFA; NF-κB, nucleus factor-κB; OO, olive oil; PA, palmic acid; PO, perilla oil; PERK, double-stranded RNA (dsRNA)-dependent protein kinase-like ER kinase; SERCA, sarco/endoplasmic reticulum calcium ATPase; SO, sunflower oil; TRB3, tribbles homolog 3; UPR, unfolded protein response; XBP1, X-box binding protein-1.

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Table 1
Ingredient composition of the experimental diets.[a]

Ingredients (g/1000 g)	Diets				
	FO	PA	OO	SO	PO
Defatted fish meal[b]	300	300	300	300	300
Soybean meal[b]	240	240	240	240	240
Wheat meal[b]	318	318	318	318	318
Fish oil[b]	80				
Palmic acid[b]		80			
Olive oil[b]			80		
Sunflower oil[b]				80	
Perilla oil[b]					80
Soybean lecithin[b]	20	20	20	20	20
Vitamin premix[c]	20	20	20	20	20
Mineral premix[d]	20	20	20	20	20
Attractant[e]	1	1	1	1	1
Mold inhibitor[f]	1	1	1	1	1
Proximate composition (%)					
Crude protein	43.5	43.8	43.1	43.8	43.3
Crude lipid	12.5	11.6	12.5	12.1	11.8

[a] Referred to Liao et al. (2015).
[b] All of these ingredients were supplied by Great Seven Biotechnology Co., Ltd., China.
[c] Vitamin premix (mg or g/kg diet): cholecalciferol, 5 mg; retinol acetate, 32 mg; thiamin 25 mg; vitamin B_{12} (1%), 10 mg; riboflavin, 45 mg; pyridoxine HCl, 20 mg; ascorbic acid, 2000 mg; alpha-tocopherol (50%), 240 mg; vitamin K_3, 10 mg; pantothenic acid, 60 mg; inositol, 800 mg; niacin acid, 200 mg; folic acid, 20 mg; biotin (2%), 60 mg; choline chloride (50%), 4000 mg; microcrystalline cellulose, 12.47 g.
[d] Mineral premix (mg or g/kg diet): $CuSO_4 \cdot 5H_2O$, 10 mg; Ca $(IO_3)_2 \cdot 6H_2O$ (1%), 60 mg; $CoCl_2 \cdot 6H_2O$ (1%), 50 mg; $FeSO_4 \cdot H_2O$, 80 mg; $MgSO_4 \cdot 7H_2O$, 1200 mg; $MnSO_4 \cdot H_2O$, 45 mg; $NaSeSO_3 \cdot 5H_2O$ (1%), 20 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $CaH_2PO_4 \cdot H_2O$, 10 g; zeolite, 8.485 g.
[e] Attractants: glycine and betaine.
[f] Mold inhibitor: contained 50% calcium propionic acid and 50% fumaric acid.

activating transcription factor 4 (*ATF4*), which induces transcription of genes involved in apoptosis and amino acids synthesis, such as C/EBP homologous protein (*CHOP*) and tribbles homolog 3 (*TRB3*).

Growing evidence indicates that, apart from limiting unfolded protein stress in the ER, the UPR also provides an essential mechanism to sense dietary fatty acids and to regulate lipid metabolism (Kaufman et al., 2002; Nivala et al., 2013; Takamura, 2014; Volmer and Ron, 2015). Dietary saturated fatty acids exerts a stronger effect on UPR activation than unsaturated fatty acids, and fatty acid-mediated UPR plays an essential role in the progress of fatty liver in chronic disease models or in humans (Fu et al., 2012; Nivala et al., 2013; Vacaru et al., 2014). Changes in fatty acid composition could disrupt ER calcium homeostasis through inhibiting the activity of sarco/endoplasmic reticulum calcium ATPase (SERCA), indirectly activating UPR transducers (Fu et al., 2011; Volmer and Ron, 2015). Alternatively, changes in fatty acid composition could directly activate IRE1 and PERK independently of unfolded proteins through modulating the ER lipid bilayer (Volmer et al., 2013).

Current data from zebrafish and medaka indicates that the three pathways of the UPR appear to be well conserved throughout fish (Hu et al., 2007; Ishikawa et al., 2011; Hollien, 2013). Although these proteins are evolutionarily conserved across broad taxa, the impact of diet is particularly interesting to study in species that have become targets of aquaculture, where the artificial diet may differ enough from the natural diet to create unexpected consequences in nutrient sensitive pathways.

Large yellow croaker (*Larimichthys crocea*), with its high production and critical role in human food or health, is widely cultured and studied in China (Wu et al., 2014). In practice, the use of terrestrial oils as a substitute for fish oil (FO) has become more common in large yellow croaker commercial diets because of the decrease of global FO production. However, due to differences in fatty acid composition between FO and terrestrial oils, the extensive use of terrestrial oils in large yellow croaker diets usually leads to changes in fish lipid metabolism, such as increased lipid accumulation in the liver and decreased long-chain PUFA (LC-PUFA) concentrations in liver and muscle, compromising fish health and product quality (Wang et al., 2012; Zuo et al., 2013). Our previous studies indicated that dietary fatty acids could influence mitochondrial function through mtDNA methylation in this species (Liao et al., 2015). However, the molecular signals and mechanisms of large yellow croaker to dietary fatty acids remain largely unknown.

In the present study, four UPR-related genes (*GRP78*, *ATF6α*, *XBP1* and *CHO*) were cloned, and their tissue distributions were determined. To better understand the potential role of UPR in fatty acid sensing, the

Table 2
Fatty acid composition of the experimental diets (of total fatty acid).[1]

Fatty acids	Diets				
	FO	PA	OO	SO	PO
14:0	5.4	1.4	0.93	0.90	0.91
16:0	18.6	52.9	13.5	10.0	10.8
18:0	2.4	3.7	1.8	2.5	1.7
20:0	2.7	1.1	nd	nd	nd
∑SFA	29.2	59.1	16.2	13.5	13.5
14:1	0.56	nd	nd	nd	nd
16:1	9.1	nd	2.8	1.9	1.9
18:1	20.8	20.9	51.1	25.9	22.1
22:1n−9	1.9	nd	nd	0.50	0.69
∑MUFA	32.3	20.9	53.9	28.3	24.7
18:2n−6	17.7	14.8	25.5	54.7	20.6
18:3n−6	nd	0.65	nd	nd	nd
20:4n−6	0.50	0.78	nd	nd	nd
∑n−6PUFA	18.2	16.2	25.5	54.7	20.6
18:3n−3	3.3	1.2	1.2	0.9	39
18:4n−3	1.3	nd	nd	nd	nd
20:5n−3	6.1	1.6	1.4	1.4	1.4
22:6n−3	6.8	1.0	0.91	0.68	0.76
∑n−3PUFA	17.6	3.76	3.52	2.98	41.0
n−3/n−6PUFA	0.97	0.23	0.14	0.054	2.0
∑n−3HUFA	12.9	2.58	2.29	2.06	2.18
DHA/EPA	1.1	0.66	0.66	0.50	0.54

[1] Referred to Liao et al. (2015). DHA, docosahexaenoic acids; EPA, eicosapentaenoic acids; FO, fish oil; HUFA, highly unsaturated fatty acids; MUFA, monounsaturated fatty acids; OO, olive oil; PA, palmic acid; PO, perilla oil; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acids; SO, sunflower oil; nd, not detected, <0.5%.

Table 3
Primers used in this study.[a]

Primer set	Forward (5′–3′)	Reverse (5′–3′)
RT primer		
GRP78	ACCTCTAACCCCGAGAACAC	TGTCAGGCGGTTCTGGTCGT
ATF6α	GGACATCAGTTTGTTTGATGAG	CACCTGACAGTCAATCTGCATC
XBP1	AACAGAGTGGCAGCTCAGAC	TCAAAGTGGATCAGTTCATTAA
CHOP	TGGAGGCGTGGTATGAAGAC	TCTCCCAGCCTTTCGATCTC
Semi-qPCR primer		
XBP1s	CTACTGACAGAAAATGAGGAAC	GCAAATCAGACTCACTGTCTG
qPCR primer		
GRP78	GGTGGCGATGACAAGCAAAC	CTGAGAACAGCAGCAACAAGC
ATF6α	CAGATAATAAGGAGGCTGAGAGTGC	CGTAGGTATGATGAGGTGCGTAGT
XBP1	CCTCAGCGGTCTGGATAAG	CGGGCTCAGTGGTGTTCT
CHOP	TCTGGATGTTCTGGAGAGTTGTTC	AGGATGATGATGAGGTGTGATGC
β-actin	CTACGAGGGTTATGCCCTGCC	TGAAGGAGTAACCGCGCTCTGT
3′RACE primer		
GRP78	ACCAGCCCACTGTCACCATT	AGGCACAGGCAACAAGAA
ATF6α	CACTGATGGCGGTGAAGGA	TGCCACCAACCACAACAA
XBP1	TTGTCCACTGGGAATGAAGC	ATTTGCTACTGGGCATTCTG
CHOP	TAATGTCCAACCTGCGTCTC	CAGTGCCTTGTCCTCTTTCG
5′RACE primer		
GRP78	ACCGTTGTCAATGGTCAGGAGG	CGGGAGCAAATGTCTTCATCGTG
ATF6α	CACGCTCACCTCGCCATCTGT	GCGTTCCACGGCGGTATGTC
XBP1	GGATGTCCAGAATGCCCAGTAGC	ATCCCCAAACCTGCTTCATTCC
CHOP	GAGGCGAAAGAGGACAAGGCAC	CCGTGTCGGAGCCCAGAAT

[a] GRP78, glucose-regulated protein 78; ATF6, activating transcription factor 6; XBP1, X-box binding protein 1; CHOP CCAAT/enhancer-binding protein homologous protein.

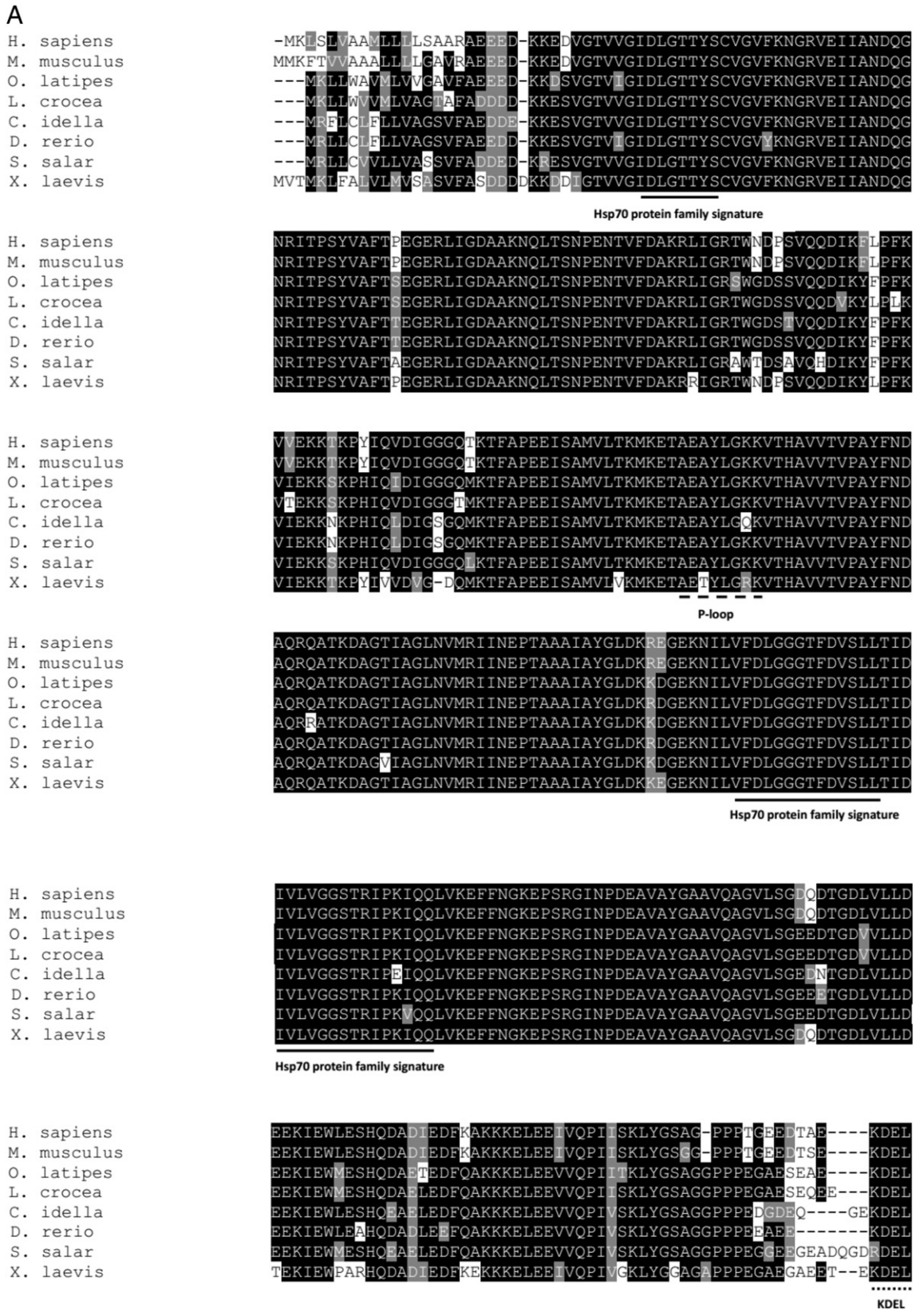

Fig. 1. Comparison of the deduced amino acid sequences of GRP78 (A), ATF6α (B), XBP1 (C) and CHOP (D) from large yellow croaker (*Larmichthys crocea*) and several other species. Parts of aligned sequences have been shown. The three highly conserved *hsp70* protein family signatures and conserved basic-leucine zipper (bZIP) domain are solid underlined, the ATP/GTP binding site motif A (P-loop) is dash underlined. The endoplasmic reticulum retention sequence (KDEL) is dot-underlined. The variable region between XBP1s and XBP1u is arrowed. GRP78, glucose-regulated protein 78; ATF6, activating transcription factor 6; XBP1, X-box binding protein 1; CHOP CCAAT/enhancer-binding protein homologous protein.

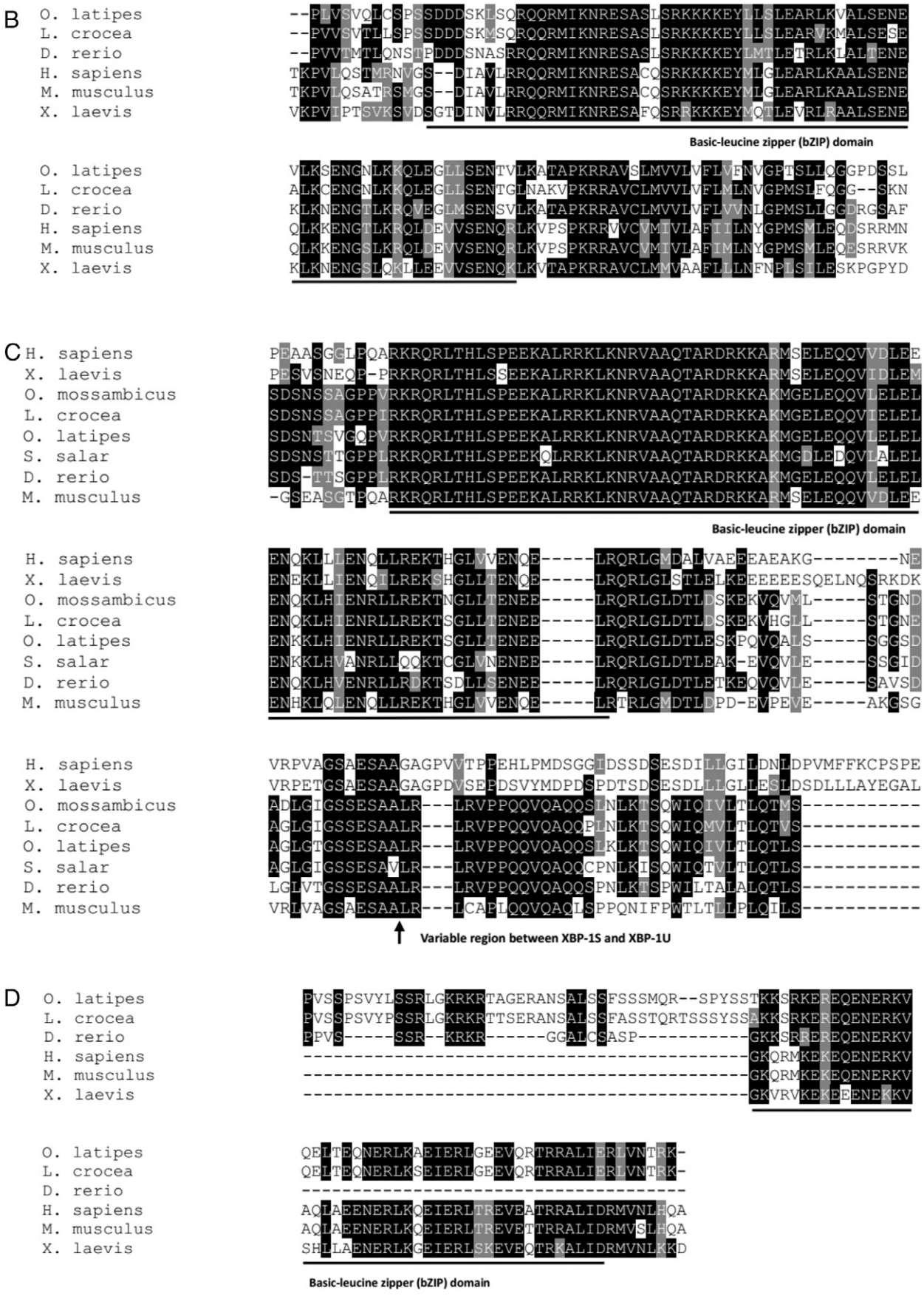

Fig. 1 (*continued*).

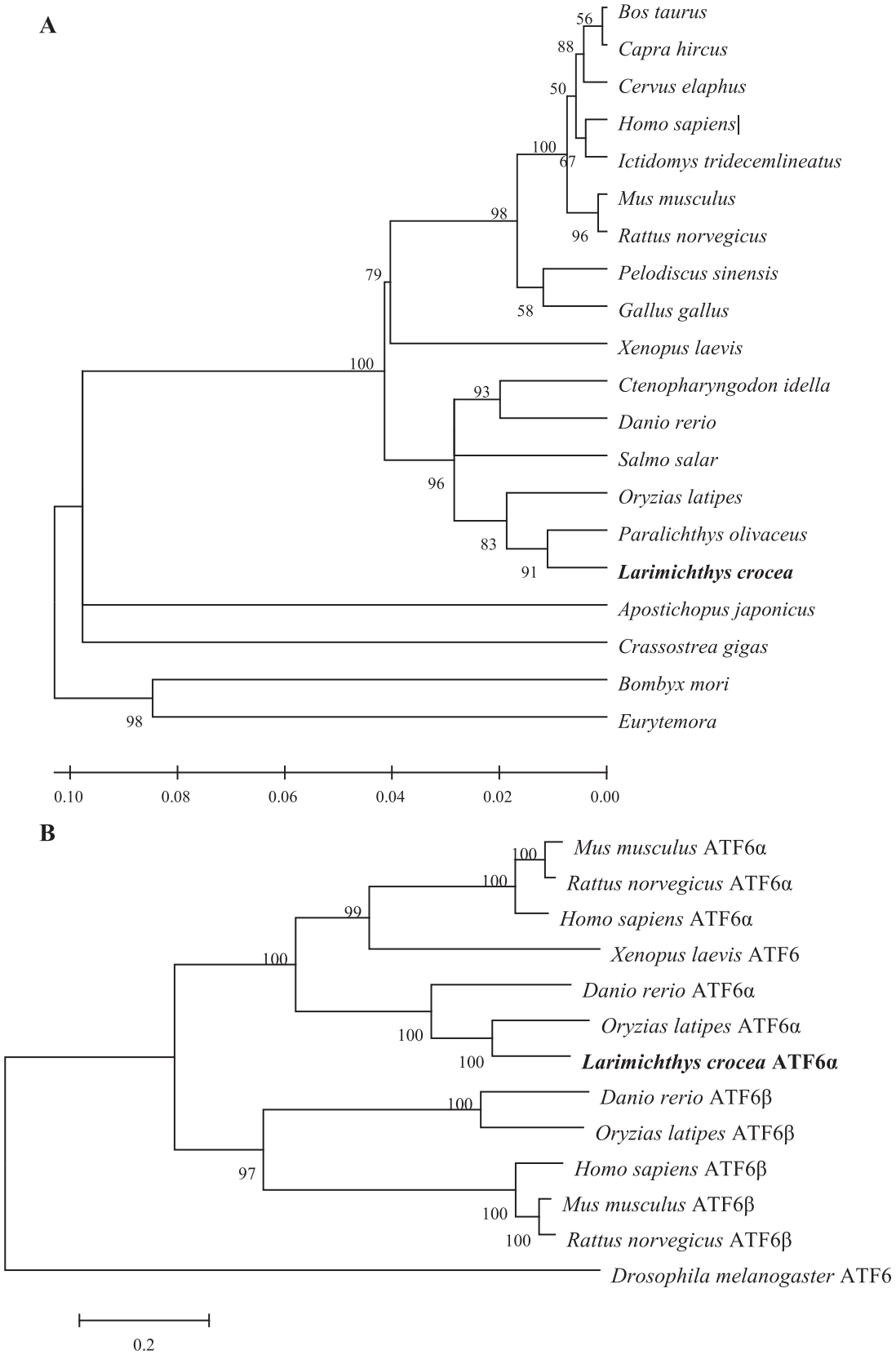

Fig. 2. Phylogenetic tree of GRP78 (A), ATF6α (B), XBP1 (C) and CHOP (D). The horizontal branch length is proportional amino acid substitution rate per site. The numbers represent the frequencies with which the tree topology presented here was replicated after 1000 bootstrap iterations. GRP78, glucose-regulated protein 78; ATF6, activating transcription factor 6; XBP1, X-box binding protein 1; CHOP CCAAT/enhancer-binding protein homologous protein.

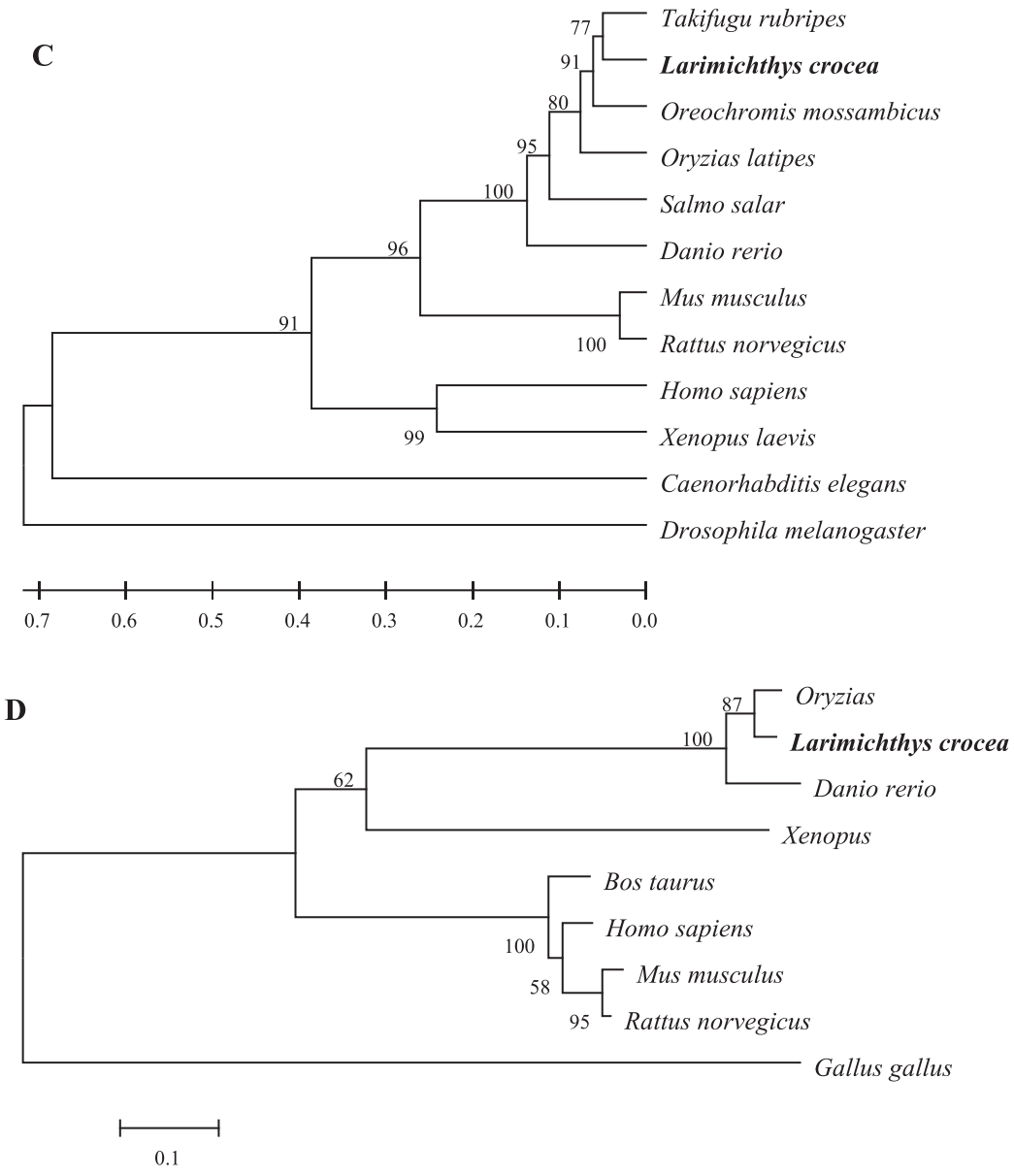

Fig. 2 (*continued*).

effect of dietary fatty acids on the expression of these genes in the liver and adipose tissue of large yellow croaker was evaluated.

2. Materials and methods

2.1. Experimental design and sampling

All animals used in this study were handled according to the Institutional Animal Care and Use Committee of Ocean University of China (Permit Number: 20001001). The experimental design and sampling were conducted as described thoroughly by Liao et al. (2015). Specifically, five isoproteic and isolipidic experimental diets were formulated and manufactured to contain approximately 43% protein and 12% lipids and vary only in dietary lipid source (Tables 1 and 2). The lipid sources were added at 80 g/kg and included the following: 1) FO as a source of n − 3 LC-PUFAs (the control diet), 2) palmic acid (PA) as a source of 16:0, 3) olive oil (OO) as a source of 18:1n − 9, 4) sunflower oil (SO) as a source of 18:2n − 6, and 5) perilla oil (PO) as a source of 18:3n − 3. Large yellow croaker were sourced from a commercial farm in Xiangshan Bay, Ningbo, China. Fish were housed in floating sea cages (3 × 3 × 3 m) and acclimatized to the experimental environments and diets prior to the start of the experiment. Large yellow croaker (initial weight 151 ± 4 g) were weighed and randomly distributed into fifteen sea cages (1 × 1 × 1.5 m, 40 fish/cage). Fish were assigned 1 of the 5 dietary treatments (3 replicate cages/treatment). Fish were hand-fed twice daily to apparent satiation at 05:00 h and 17:00 h for 70 d. At the end of the experiment, fish were deprived of food for 24 h before harvest. After that, fish in each cage were killed under anesthesia (1:10,000; Shanghai Reagent, Shanghai, China). Livers and adipose tissue from ten fish in each cage were pooled into 1.5 ml tubes (RNAase-Free; Axygen, Union City, CA, USA), frozen in liquid nitrogen, and then stored at − 80 °C for later analysis of gene expression.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted from liver and adipose tissue using Trizol reagents (Invitrogen, USA) following the instructions of the manufacturer and then electrophoresed on a 1.5% agarose gel to test the

integrity. The first cDNA was synthesized from 1 μg of RNA without DNA contamination using PrimeScript RT reagent kit (Takara, Japan) according to the manufacturer's protocol.

2.3. Molecular cloning of GRP78, ATF6α, XBP1 and CHOP

The full-length cDNAs of *GRP78*, *ATF6α*, *XBP1* and *CHOP* were isolated using gene specific primers for reverse transcription (RT)-PCR and rapid amplification of cDNA ends (RACE)-PCR reactions. Four specific primer pairs (Table 3) were designed based on highly conserved regions from the genes of other fish available in the GenBank database and synthesized by Biosune (Shanghai, China). Liver cDNA were used as the template for amplification. PCR amplifications using the primers and Taq DNA Polymerase (Takara, Japan) were performed with an initial denaturation at 95 °C for 3 min and 35 cycles of "95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min", followed by a final extension at 72 °C for 10 min. Eight gene-specific primers of each gene were designed based on the known sequences of the internal fragments of *GRP78*, *ATF6α*, *XBP1* and *CHOP* cDNA singlet to clone the 3′- and 5′-end by rapid amplification of cDNA ends (RACE) using the SMARTerTM RACE cDNA Amplification Kit (Clontech, California, USA) (Table 3). For 3′ RACE and 5′ RACE, the first and nested PCR rounds were performed according to the user's manual. All PCR products were run on a 1.5% agarose gel, and then purified by SanPrep PCR Purification Kit (Sangon Biotech, Shanghai, China). PCR products were cloned into pEASY-T1 simple cloning vector (TransGen, Beijing, China) and sequenced in BioSune (Shanghai, China).

2.4. Bioinformatic analysis

The *GRP78*, *ATF6α*, *XBP1* and *CHOP* mRNA sequences were analyzed using the NCBI ORF Finder computational tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). SMART program (http://smart.emblheidelberg.de/) and PROSITE program (http://kr.expasy.org/prosite/) were used to predict the functional sites or domains in the amino acid sequence. Multiple alignments were performed with some of the available GRP78, ATF6α, XBP1 and CHOP proteins from NCBI using ClustalW. The accession numbers of those proteins selected were AAF42836.1 (*Homo sapiens* GRP78), NP_071705.3 (*Mus musculus* GRP78), NP_998223.1 (*Danio rerio* GRP78), AAB41582.1 (*Xenopus laevis* GRP78), BAL14281.1 (*Oryzias latipes* GRP78), NP_001135114.1 (*Salmo salar* GRP78), ACJ65009.1 (*Ctenopharyngodon idella* GRP78), NP_031374.2 (*Homo sapiens* ATF6α), NP_001074773.1 (*Mus musculus* ATF6α), NP_001103989.1 (*Danio rerio* ATF6α), NP_001088791.1 (*Xenopus laevis* ATF6), BAL14276.1 (*Oryzias latipes* ATF6α), BAB82982.1 (*Homo sapiens* XBP1), AAB81862.2 (*Mus musculus* XBP1), NP_571949.1 (*Danio rerio* XBP1), NP_001080523.1 (*Xenopus laevis* XBP1), BAL14283.1 (*Oryzias latipes* XBP1), NP_001239281.1 (*Salmo salar* XBP1), AAQ08006.1 (*Oreochromis mossambicus* XBP1), NP_001181982.1 (*Homo sapiens* CHOP), NP_031863.3 (*Mus musculus* CHOP), NP_001076294.1 (*Danio rerio* CHOP), NP_001082635.1 (*Xenopus laevis* CHOP), BAL14286.1 (*Oryzias latipes* CHOP).

Phylogenetic analysis was carried out based on amino acid sequences using the Molecular Evolutionary Genetics Analysis package MEGA 7 software. The phylogenetic dendrogram was created with the Maximum-Likelihood method using 1000 bootstrap replicates. The accession numbers of the sequences were: AAF42836.1 (*Homo sapiens* GRP78), NP_071705.3 (*Mus musculus* GRP78), NP_037215.1 (*Rattus norvegicus* GRP78), NP_990822.1 (*Gallus gallus* GRP78), NP_001068616.1 (*Bos taurus* GRP78), NP_998223.1 (*Danio rerio* GRP78), AAB41582.1 (*Xenopus laevis* GRP78), BAL14281.1 (*Oryzias latipes* GRP78), AGA84579.1 (*Bombyx mori* GRP78), ACT46911.1 (*Cervus elaphus* GRP78), NP_001269183.1 (*Ictidomys tridecemlineatus* GRP78), ADW08701.1 (*Pelodiscus sinensis* GRP78), NP_001135114.1 (*Salmo salar* GRP78), AEV42204.1 (*Eurytemora affinis* GRP78), BAD15288.1 (*Crassostrea gigas* GRP78), AGG79327.1 (*Capra hircus* GRP78), ACJ65009.1 (*Ctenopharyngodon idella* GRP78), ABG56392.1 (*Paralichthys olivaceus* GRP78), AKN63086.1 (*Apostichopus japonicus* GRP78), NP_031374.2 (*Homo sapiens* ATF6α), NP_001129625.1 (*Homo sapiens* ATF6β), NP_001074773.1 (*Mus musculus* ATF6α), NP_059102.2 (*Mus musculus* ATF6β), NP_001100666.1 (*Rattus norvegicus* ATF6α), NP_001002809.2 (*Rattus norvegicus* ATF6β), NP_610159.1 (*Drosophila melanogaster* ATF6), NP_001103989.1 (*Danio rerio* ATF6α), NP_001071188.1 (*Danio rerio* ATF6β), NP_001088791.1

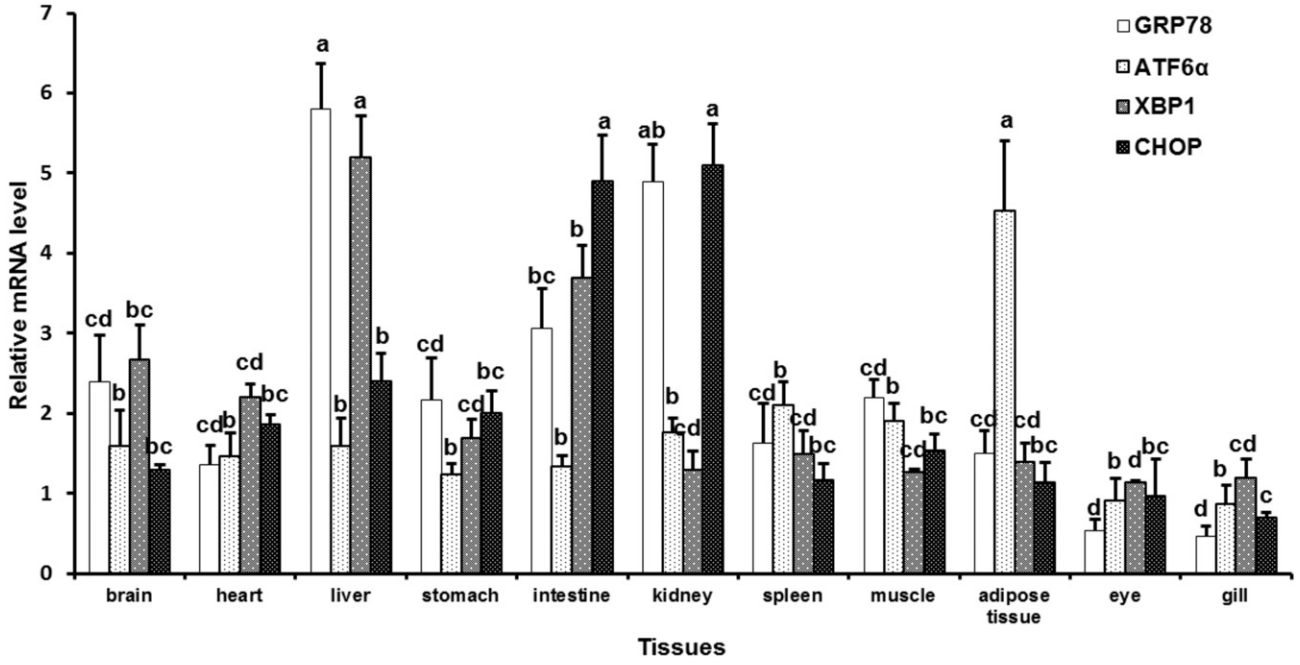

Fig. 3. Tissue distribution of *GRP78*, *ATF6α*, *XBP1* and *CHOP* in large yellow croaker (*Larmichthys crocea*). The values are expressed as the mean ± SEM ($n = 3$). Significance was evaluated by one-way ANOVA followed by the Tukey's multiple range test. The labeled means without a common letter differ, $P < 0.05$. GRP78, glucose-regulated protein 78; ATF6, activating transcription factor 6; XBP1, X-box binding protein 1; CHOP CCAAT/enhancer-binding protein homologous protein.

(*Xenopus laevis* ATF6), BAL14276.1 (*Oryzias latipes* ATF6α), BAL14277.1 (*Oryzias latipes* ATF6β), BAB82982.1 (*Homo sapiens* XBP1), AAB81862.2 (*Mus musculus* XBP1), AAH79450.1 (*Rattus norvegicus* XBP1), NP_571949.1 (*Danio rerio* XBP1), NP_001080523.1 (*Xenopus laevis* XBP1), BAL14283.1 (*Oryzias latipes* XBP1), NP_524722.2 (*Drosophila melanogaster* XBP1), BAE45318.1 (*Takifugu rubripes* XBP1), AAQ08006.1 (*Oreochromis mossambicus* XBP1), NP_001239281.1 (*Salmo salar* XBP1), AAL60200.1 (*Caenorhabditis elegans* XBP1), NP_001181982.1 (*Homo sapiens* CHOP), NP_031863.3 (*Mus musculus* CHOP), NP_001103456.1 (*Rattus norvegicus* CHOP), AJA72779.1 (*Gallus gallus* CHOP), NP_001071631.1 (*Bos taurus* CHOP), NP_001076294.1 (*Danio rerio* CHOP), NP_001082635.1 (*Xenopus laevis* CHOP), BAL14286.1 (*Oryzias latipes* CHOP).

2.5. Quantitative real-time PCR analysis and semi-quantitative RT-PCR analysis

First strand cDNA was synthesized as described previously in Section 2.2 and then diluted by 4 times using sterilized double-distilled water. Specific primers were designed for each target gene (Table 3). The real-time PCR was carried out in a quantitative thermal cycler (Mastercyclerep realplex, Eppendorf, Germany) in a final volume of 25 μl containing 2 × TransStartTM Green qPCR SuperMix (TransGen, Beijing, China), primer pairs, and cDNA. The program was 95 °C for 2 min followed by 40 cycles of "95 °C for 10 s, 58 °C for 10 s, and 72 °C for 20 s". Melting curve (1.85 °C increment/min from 58 °C to 95 °C) was performed after the amplification phase for confirmation. Each sample was run in triplicate. The gene expression levels of putative *GRP78*, *ATF6α*, *XBP1* and *CHOP* were studied by RT-qPCR method: $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). Gene expression was normalized using *β-actin* (GenBank accession No. GU584189) expression; data were reported as fold of the normalized values obtained for fish fed the diet with FO.

Semi-qPCR was also used to assay the mRNA expression of *XBP1s* and *β-actin*. The primer pairs used for *β-actin* were the same as quantitative real-time PCR analysis (Table 3). Specific primers for *XBP1s* were designed based on the spliced site of *XBP1* mRNA by IRE1 (Table 3). Semi-qPCR was performed with 1 μl of the first-strand cDNA and 12.5 μl Takara Ex Taq mix (Takara, Japan). Semi-qPCR was carried out with an initial denaturation at 94 °C for 3 min, followed by 28 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. PCR products were visualized on 1% agarose gel. The expression of the housekeeping gene *β-actin* (GenBank accession No. GU584189) was used as internal

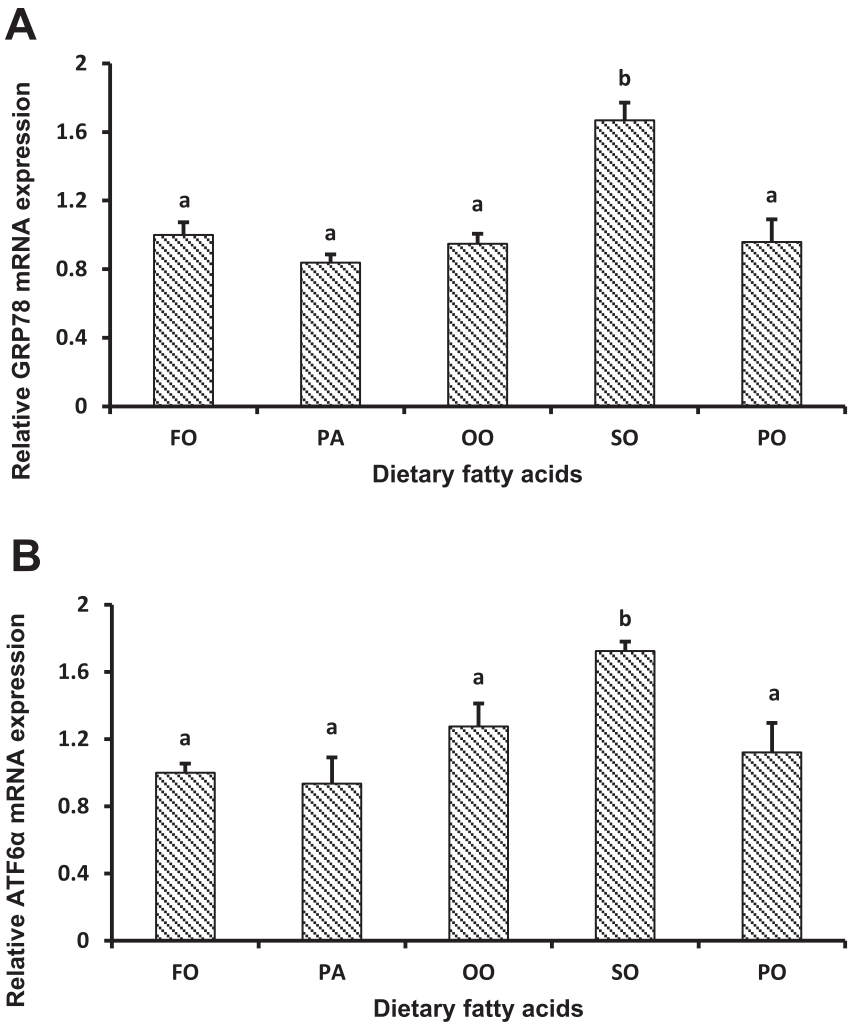

Fig. 4. Relative mRNA concentrations of *GRP78* (A), *ATF6α* (B), *CHOP* (C) and *XBP1s* (D) in the liver of the large yellow croaker (*Larimichthys crocea*) fed diets with varying lipid sources for 70 d. The values are expressed as the mean ± SEM ($n = 3$). Significance was evaluated by one-way ANOVA followed by the Tukey's multiple range test. The labeled means without a common letter differ, $P < 0.05$. FO, fish oil; OO, olive oil; PA, palmic acid; PO, perilla oil; SO, sunflower oil; GRP78, glucose-regulated protein 78; ATF6, activating transcription factor 6; XBP1, X-box binding protein 1; CHOP CCAAT/enhancer-binding protein homologous protein.

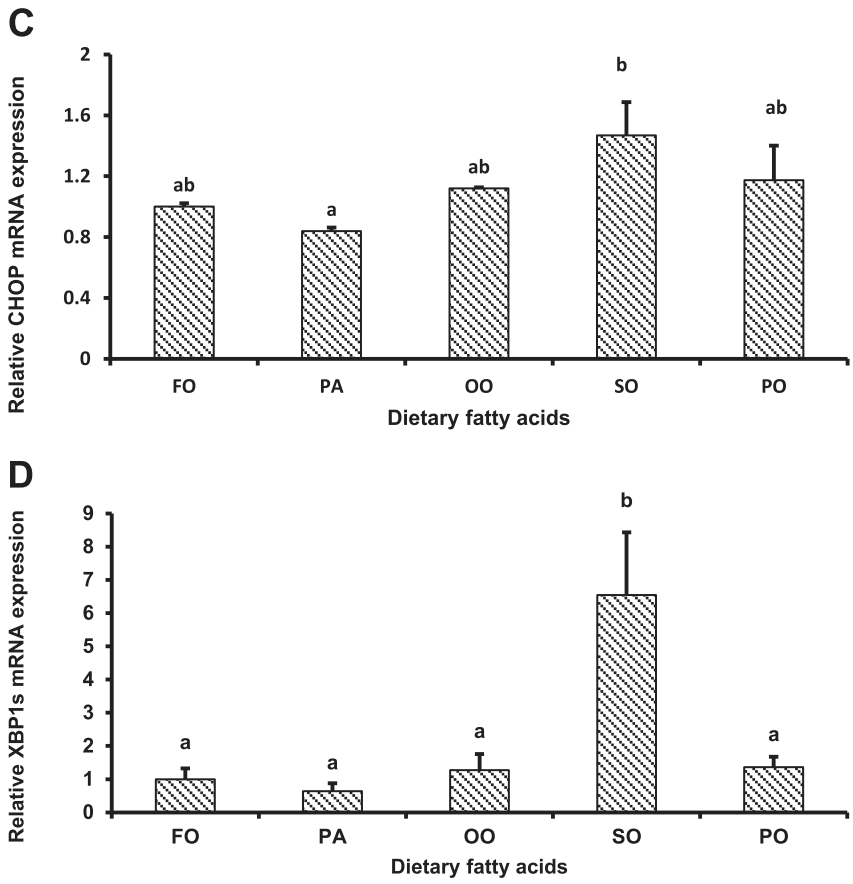

Fig. 4 (*continued*).

control to check the efficiency of cDNA synthesis and cDNA integrity. The results repeated 3 times followed by densitometric and statistical analysis.

2.6. Statistical analysis

Differences were determined by one way ANOVA using SPSS 16.0 for Windows (SPSS Inc.). Differences between means were tested by Tukey's multiple range test. If unequal variance was determined by Levene's test, data were log-transformed before statistical analysis.The level of significance was chosen at $P < 0.05$ and the results were presented as means ± standard error of the mean.

3. Results

3.1. Cloning of large yellow croaker GRP78, ATF6α, XBP1 and CHOP

The complete cDNA of *GRP78* was 2571 bp long and consisted of an open-reading frame (ORF) of 1962 bp (GenBank accession No. KX218379), encoding a predicted polypeptide of 654 amino acid residues, a 157 bp 5′-untranslated region (UTR) and a 452 bp 3′-UTR. The complete cDNA of *ATF6α* was 2521 bp long and consisted of an ORF of 1935 bp (GenBank accession No. KX218380), encoding a predicted polypeptide of 645 amino acid residues, a 108 bp 5′-UTR and a 478 bp 3′-UTR. The complete cDNA of *XBP1* was 1809 bp long and consisted of an ORF of 795 bp (GenBank accession No. KX218381), encoding a predicted polypeptide of 265 amino acid residues, a 117 bp 5′-UTR and a 897 bp 3′-UTR. The complete cDNA of *CHOP* was 1568 bp long and consisted of an ORF of 936 bp (GenBank accession No. KX218382), encoding a predicted polypeptide of 312 amino acid residues, a 229 bp 5′-UTR and a 403 bp 3′-UTR.

A multiple sequence alignment performed with most of the available GRP78, ATF6α, XBP1 or CHOP protein sequences showed high sequence conservation among vertebrates. Similar to other vertebrate GRP78 proteins, the mature large yellow croaker GRP78 contained three highly conserved *hsp*70 protein family signatures, an ATP/GTP binding site motif A (P-loop) and an endoplasmic reticulum retention sequence (KDEL; Fig. 1A). Additionally, large yellow croaker ATF6α, XBP1 and CHOP had a highly conserved basic-leucine zipper (bZIP) domain respectively (Fig. 1B, C and D).

3.2. Phylogenetic analysis of large yellow croaker GRP78, ATF6α, XBP1 and CHOP

Phylogenetic tree analysis indicated that large yellow croaker GRP78 was in the same subgroup with other fishes and had the closest phylogenetic relationship with *Paralichthys olivaceus* (Fig. 2A). The vertebrate ATF6 were formed two orthologous groups according to the result of phylogenetic tree performed in Fig. 2B. The obtained large yellow croaker ATF6 belonged to ATF6α group. Additionally, XBP1 or CHOP from large yellow croaker was in the same subgroup with other fishes and had close phylogenetic relationships with *Takifugu rubripes* or *Oryzias latipes* (Fig. 2C and D).

3.3. Expression analysis of GRP78, ATF6α, XBP1 and CHOP mRNA in large yellow croaker tissues

GRP78, *ATF6α*, *XBP1* and *CHOP* mRNA were ubiquitously expressed in large yellow croaker (Fig. 3). *GRP78* had the highest mRNA expression levels in the liver and kidney, relatively lower levels in intestine, and the lowest level in eye and gill (Fig. 3). The highest expression level of *ATF6α* was detected in the adipose tissue (Fig. 3). *XBP1* mRNA

expression in the liver was also higher compared to other tissues (Fig. 3). While the highest expression level of *CHOP* was observed in the intestine and kidney (Fig. 3). In contrast, *ATF6α*, *XBP1* and *CHOP* mRNA were weakly expressed in the eye and gill (Fig. 3).

3.4. Effect of dietary fatty acids on GRP78, ATF6α, XBP1s and CHOP expression

The liver *GRP78* and *ATF6α* mRNA levels were higher in the SO group than the other groups ($P < 0.05$; Fig. 4A and B). Additionally, the liver *XBP1s* mRNA level was higher in the SO group compared with the other groups (Fig. 4D). The hepatic CHOP expression in the SO group was higher than the PA group ($P < 0.05$; Fig. 4C). There were no significant differences in the expression of *GRP78*, *ATF6α*, *XBP1s* and *CHOP* in adipose tissue of fish fed diets with FO, PA, OO, SO and PO ($P > 0.05$; Fig. 5).

4. Discussion

Dietary fatty acid composition could affect energy metabolism and lipid homeostasis in large yellow croaker (Wang et al., 2012, 2013). However, the physiological mechanisms that sense dietary fatty acids in large yellow croaker still remain unclear. The UPR signalling pathways are conserved in all eukaryotes, from yeast to mammals (Mori, 2009), and function as a fatty acid sensor (Kaufman et al., 2002). In the present study, four UPR-related genes (*GRP78*, *ATF6α*, *XBP1* and *CHOP*) cDNA were characterized from large yellow croaker. Putative protein sequence analysis indicated that these four cloned genes possessed highly conserved functional domains.

There are two isoforms of ATF6, ATF6α and ATF6β, in mammals and fish (Mori, 2009; Hollien, 2013). The functional difference in these two isoforms is that ATF6α, not ATF6β, is a major inducer of ER chaperones (Yamamoto et al., 2007; Ishikawa et al., 2011). In the present study, phylogenetic analysis showed that the obtained large yellow croaker ATF6 belonged to ATF6α group, which might play a role in the induction of ER chaperones during ER stress. In addition, large yellow croaker GRP78, XBP1 and CHOP were in the same phylogenetic cluster with other fishes, indicating that the UPR pathways are evolutionarily conserved across fish.

The tissue expression analysis showed that *GRP78*, *ATF6α*, *XBP1* and *CHOP* were ubiquitously expressed in large yellow croaker. These results were consistent with previous studies in zebrafish (Thakur et al.,

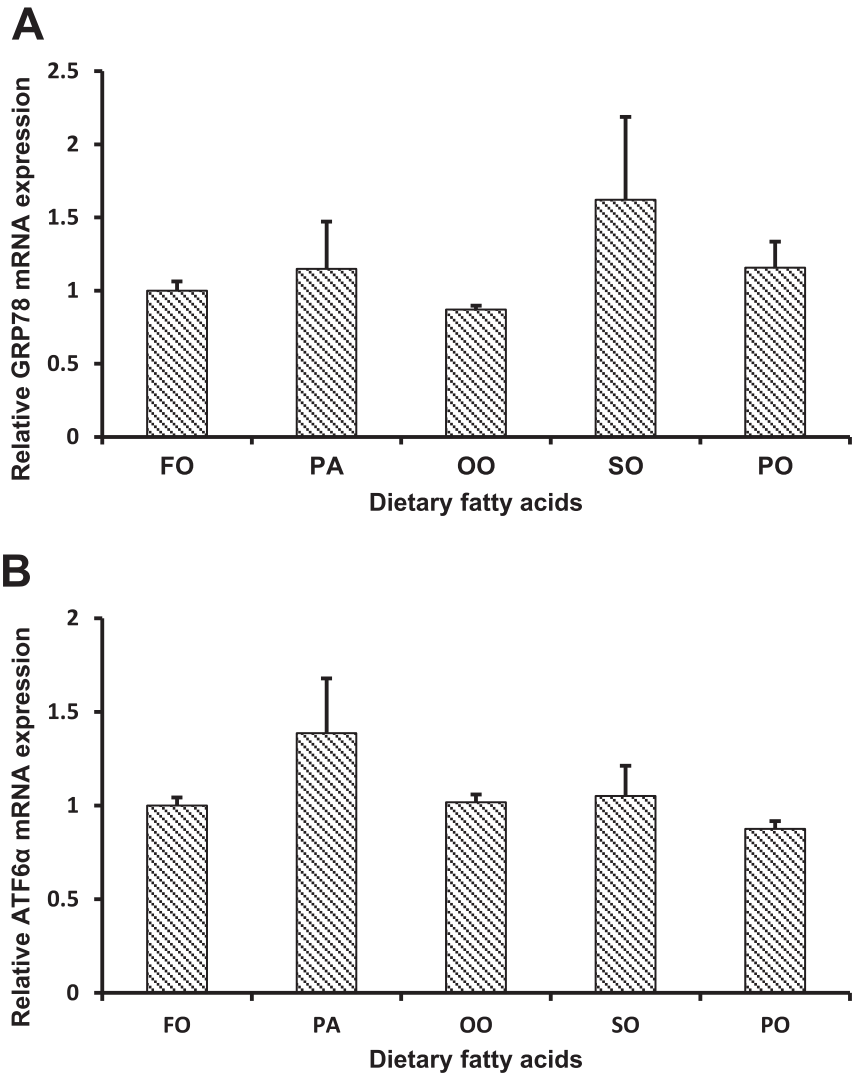

Fig. 5. Relative mRNA concentrations of *GRP78* (A), *ATF6α* (B), *CHOP* (C) and *XBP1s* (D) in adipose tissue of the large yellow croaker (*Larimichthys crocea*) fed diets with varying lipid sources for 70 d. The values are expressed as the mean ± SEM (n = 3). Significance was evaluated by one-way ANOVA followed by the Tukey's multiple range test. FO, fish oil; OO, olive oil; PA, palmic acid; PO, perilla oil; SO, sunflower oil; GRP78, glucose-regulated protein 78; ATF6, activating transcription factor 6; XBP1, X-box binding protein 1; CHOP CCAAT/enhancer-binding protein homologous protein.

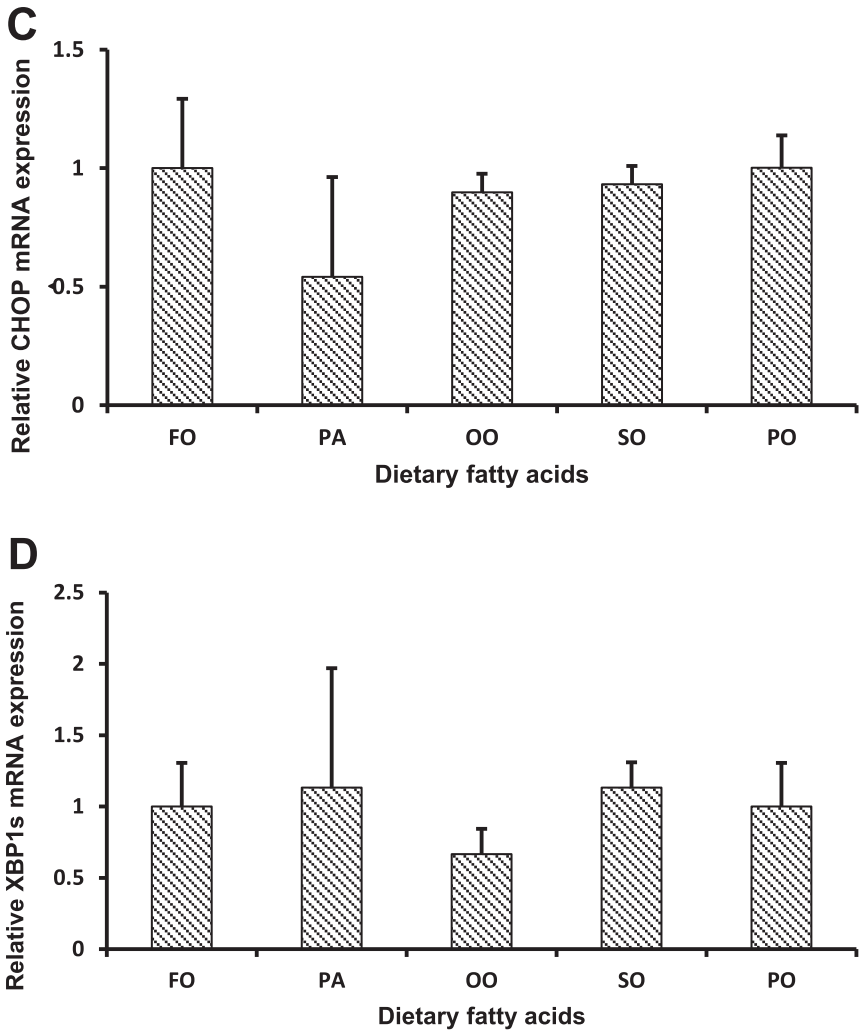

Fig. 5 (*continued*).

2011) and medaka (Ishikawa et al., 2011), as well in mammals (Mori, 2009), and implied fundamental functions of GRP78, ATF6α, XBP1 and CHOP between these species.

In the present study, *GRP78*, *ATF6α* and *XBP1s* mRNA in the liver were significantly increased in the SO group compared to the other groups, suggesting the activation of UPR in fish fed the diet with SO (18:2n−6 accounted for 54.7% of total fatty acid). This was not consistent with findings that saturated fatty acids, not 18:2n−6, activated the UPR signalling pathways in mammals *in vivo* (Nivala et al., 2013) and *in vitro* (Borradaile et al., 2006). The discrepancy may be a result of difference in fatty acids metabolism between large yellow croaker and mammals. Actually, large yellow croaker, unlike mammals, could successfully use saturated fatty acids as substrates for β-oxidation (Liao et al., 2015), but had insufficient capacity to utilize dietary 18:2n−6 (Zuo et al., 2013).

Several mechanisms have been implicated in fatty acids regulated UPR activation. Dietary fatty acids are selectively incorporated into ER membrane (Hammer and Wills, 1978). Changes in dietary fatty acid composition could alter the fatty acid composition of ER membrane, which in turn affect the membrane structural integrity, fluidity and lipid-protein interactions, and thus disrupt ER function resulting in UPR activation (Hammer and Wills, 1978; Volmer and Ron, 2015). In addition, accumulative evidence has shown that oxidative stress can trigger UPR (Yokouchi et al., 2008; Nakajima and Kitamura, 2013). One mechanism by which oxidative stress-induced UPR is related to depletion of the calcium store in the ER (Viner et al., 1996). In the other way, reactive oxygen species may impair ER function through modification of unfolded proteins, resulting in accumulation of oxidatively modified proteins in the ER and subsequently activating UPR (Nakajima and Kitamura, 2013). In our previous study, we indeed found that oxidative stress parameters, such as MDA and 8-OHdG contents, were significantly higher in the SO group compared with other groups (Liao et al., 2015). In the present study, therefore oxidative stress probable contributed to the activation of UPR and promoted the transcription of *GRP78*, *ATF6α* and *XBP1s* in the SO group.

UPR activation allows cells to adapt to the unfolded protein burden and to restore homeostasis by enhancing protein folding and quality control in the ER (Vacaru et al., 2014). However, chronic or severe UPR activation induces apoptotic death *via* the PERK^{-}eIF2α^{-}ATF4^{-}-CHOP pathway (Wang and Kaufman, 2012). In the present study, the abundance of *CHOP* mRNA in the SO group was not significantly different from the FO group (the control group). This result probably indicated that dietary SO-induced UPR was not enough to activate UPR-induced cell death signalling.

The functionality of GRP78, ATF6α and XBP1s is well clarified in mammals (Kaufman et al., 2002; Wang and Kaufman, 2012; Volmer et al., 2013; Takamura, 2014), as well as in zebrafish (Thakur et al., 2011; Vacaru et al., 2014). First, ATF6α and XBP1s, alone or in synergy with each other, enhance the expression of genes encoding ER chaperones, ER biogenesis, phospholipid synthesis, and ERAD (Wang and Kaufman, 2012). Second, ATF6α and XBP1s can promote lipid synthesis. ATF6 overexpression induces fatty liver, in part, through promotion of

lipid synthesis in zebrafish (Howarth et al., 2014). XBP1 is required for the function of normal fatty acid synthesis in mice liver (Lee et al., 2008). Third, ATF6α could activate NF-κB, while XBP1s induces production of the inflammatory cytokines IL-8 and IL-6 in mammal cell lines (Hotamisligil, 2010). Therefore, in the present study, the increased expression of *GRP78*, *ATF6α* and *XBP1s* on the one hand might provide a mechanism to assist ER homeostasis. On the other hand, UPR activation probably stimulated lipid synthesis and caused inflammatory response in the liver of large yellow croaker fed the diet with SO.

In summary, four UPR-related genes *GRP78*, *ATF6α*, *XBP1* and *CHO* from large yellow croaker were cloned and characterized. The gene expression of *GRP78*, *ATF6α* and *XBP1s* in the liver of large yellow croaker was up- regulated by dietary sunflower oil (18:2n − 6). The increased expression of *GRP78*, *ATF6α* and *XBP1s* might provide a mechanism to improve ER function, but probably stimulated lipid synthesis and caused inflammatory response in the liver.

(Supplementary materials related to this article can be found online at http://dx.doi.org/10.3945/jn.115.216481.)

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