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Dietary lipid concentration affects liver mitochondrial DNA copy number, gene expression and DNA methylation in large yellow croaker (*Larimichthys crocea*)

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ABSTRACT

In response to changes in energy demand and nutrient supply, the organism regulates mitochondrial metabolic status to coordinate ATP production. To survey mitochondrial metabolic adaptation in response to dietary lipid concentration, citrate synthase (EC 2.3.3.1, CS) activity, the expression of several mitochondrial transcription factors, mitochondrial DNA (mtDNA) copy number, mitochondrial gene expression, mtDNA methylation, and oxidative stress parameters were analyzed in the liver of large yellow croaker fed one of three diets with a low (6%), moderate (12%, the control diet) or high (18%) crude lipid content for 70 d. MtDNA copy number was significantly increased in the low- and high-lipid groups compared to the control. The transcription of cytochrome *c* oxidase 1 (*COX1*), *COX2*, *COX3*, ATP synthase 6 (*ATPase 6*), *12S rRNA* and *16S rRNA* was also significantly increased in the low-lipid group compared with the control, while the transcription of these genes in the high-lipid group was unchanged. Moreover, *D-loop* (displacement loop) methylation in the high-lipid group was significantly higher than the control. The increase in mtDNA copy number and mitochondrial transcription might be a compensatory mechanism that matches ATP supply to demand under a low-lipid diet, while the increase of mtDNA copy number with unchanged mitochondrial transcription in the high-lipid group probably came from the increase of *D-loop* methylation.

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1. Introduction

Physiological, developmental, or environmental factors change the status of energy demand and supply. In response to changes in energy demand and supply, the organism regulates mitochondrial metabolic status to coordinate ATP production (Bremer et al., 2012). In fish, mitochondrial metabolic adaptation and its mechanisms in response to temperature (Battersby and Moyes, 1998; Hardewig et al., 1999; Lucassen et al., 2003; LeMoine et al., 2008; O'Brien, 2011; Bremer et al., 2012; Dos Santos et al., 2012), photoperiod (Martin et al., 2009) and exercise (McClelland et al., 2006; LeMoine et al., 2010) were widely studied. Mitochondrial metabolic adaptation in response to these stimuli can be achieved by changes in mitochondrial volume density (Urschel and O'Brien, 2008), mitochondrial membrane phospholipid fatty acid composition (Kraffe et al., 2007), mitochondrial enzyme activities (Hardewig et al., 1999; Lucassen et al., 2006; Duggan et al., 2011), mitochondrial oxidative capacity (Dos Santos et al., 2012), mitochondrial transcription factors expression (Bremer et al., 2012), mitochondrial DNA (mtDNA) copy number (Battersby and Moyes, 1998; Hardewig et al., 1999) and mitochondrial mRNA abundance (Battersby and Moyes, 1998). Dependent of these changes, oxygen and ROS balance may also be altered (Heise et al., 2007; Grim et al., 2010; Kammer et al., 2011). Although there are a myriad of pathways to coordinate mitochondrial metabolic adaptation in response to stimuli, the pathway appears versatile in species, tissue and stimuli type (O'Brien, 2011). For instance, two subspecies of killifish have different ability to increase citrate synthase activity and mitochondrial volume and surface densities at colder temperatures (Dhillon and Schulte, 2011). The activities of citrate synthase (EC 2.3.3.1, CS) and cytochrome-c oxidase (EC 1.9.3.1, COX) increased in both liver and muscle of threespine sticklebacks in response to cold acclimation, but increased mitochondrial volume density only occurred in muscle (Orczewska et al., 2010). The activities of CS and COX increased in response to both temperature and exercise in zebrafish, yet only cold acclimatization increased β-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and pyruvate kinase

Abbreviations: ATPase 6, ATP synthase 6; COX, Cytochrome c oxidase; CS, Citrate synthase; CYTB, Cytochrome b; D-loop, Displacement loop; HIF1α, Hypoxia-inducible factor 1α; mtDNA, Mitochondrial DNA; MDA, Malondialdehyde; ND6, NADH dehydrogenase subunit 6; ND2, NADH dehydrogenase subunit 2; ND4L, NADH dehydrogenase 4 L; NRF1, Nuclear respiratory factor 1; PGC1α, Peroxisome proliferator-activated receptor gamma coactivator-1-α; PPAR, Peroxisome proliferator-activated receptor; rRNA, Ribosomal RNA; SOD, Superoxide dismutase activity; 5mC, 5-Methylcytosine; 8-OHdG, 8-Hydroxydeoxyguanosine.

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

Ingredients (g/100 g)	Dietary lipid concentration (%)		
	Low (6)	Moderate (12)	High (18)
Fish meal[b]	39	39	39
Soybean meal[b]	20	20	20
Wheat meal[b]	23.3	23.3	23.3
Wheat starch[b]	12	6	0
Fish oil[b]	0	6	12
Soybean lecithin[b]	1.5	1.5	1.5
Vitamin premix[c]	2	2	2
Mineral premix[d]	2	2	2
Attractant[e]	0.1	0.1	0.1
Mold inhibitor[f]	0.1	0.1	0.1
Proximate composition (g/100 g)			
Moisture	9.5	9.4	9.2
Crude protein	43.1	42.6	43.2
Crude lipid	6.1	11.5	17.8

[a] Referred to Yan et al. (Yan 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%), and 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; $CaH_2PO_4 \cdot H_2O$, and 10 g; zeolite, 8.485 g.
[e] Attractants: glycine and betaine.
[f] Mold inhibitor: contained 50% calcium propionic acid and 50% fumaric acid.

(EC 2.7.1.40) (McClelland et al., 2006). Although mitochondrial metabolic adaptation and its versatility in response to temperature, exercise and photoperiod have been widely examined, few studies have examined mitochondrial metabolic adaptation in response to diet in fish (LeMoine et al., 2008).

Changes in diet have a pronounced effect on the tissue-specific metabolic strategy and mitochondrial phenotypes in most vertebrate species (Blasco et al., 1992; Ojano-Dirain et al., 2005; Chanseaume et al., 2007). In mammals, mitochondrial proliferation is triggered by dietary restrictions (Civitarese et al., 2007). Meanwhile, several studies on mammals indicated that high-lipid diet increases mitochondrial content to maintain normal respiratory function as a possible response to an increased lipid overload (Hancock et al., 2008; Carabelli et al., 2011; Ruggiero et al., 2011). In fish, dietary nutrient density was associated with mitochondrial function such as mitochondrial gene expression and mitochondrial respiratory chain enzyme activities (Eya et al., 2011, 2012). Further study on the mitochondrial metabolic adaptation of goldfish to dietary lipid has suggested that increases in aerobic metabolic capacity (CS) may not always coincide with mitochondrial biogenesis (COX) in the liver, and nuclear respiratory factor 1 (*NRF1*) and peroxisome proliferator-activated receptors (*PPARs*) were involved in the regulation of mitochondrial gene expression and fatty acid oxidation gene expression (LeMoine et al., 2008). However, it is unclear how dietary lipid concentration affects qualitative aspects of mitochondrial metabolism, such as mtDNA copy number and mitochondrial gene expression. In addition, whether changes in ROS balance are involved in metabolic adaptation in response to dietary lipid concentration in fish remains unknown. Current evidence suggests that mitochondria are susceptible to ROS (Kujoth et al., 2005), a mediator of DNA methylation (Franco et al., 2008), but no studies have investigated whether changes in ROS metabolism affect mtDNA such as mtDNA methylation in response to dietary lipid concentration.

The goals for this study were to determine: (1) how large yellow croaker *Larimichthys croceus*, one of the most important mariculture fish species in China, coordinate mtDNA copy number and mitochondrial gene expression in responses to changes of dietary lipid concentration; (2) whether changes in ROS balance are involved in metabolic adaptation in response to changes of dietary lipid concentration; and

Table 2
Primers used in this study[a].

Accession	Gene	Forward	Reverse
Quantitative real-time PCR primer			
KM593915	*HIF1α*	GGAAGGTGCTCCACTGCT	TATGGCGGCTGAGGAAG
KM593916	*NRF1*	GTGCCGTCTCAAACTGTGG	GTGCCAACCTGGATGAGC
KM593914	*PGC1α*	CTGCTCAGTATGGCAACGA	GGTCACTGGCATTGGTCAC
KF998577	*PPARα*	GTCAAGCAGATCCACGAAGCC	TGGTCTTTCCAGTGAGTATGAGCC
GU584189	*β-Actin*	CTACGAGGGTTATGCCCTGCC	TGAAGGAGTAACCGCGCTCTGT
XM_010751754	*Ubiquitin*	TGGAGGATGGACGCACACTG	GCAGACGGGCATAGCACTTG
GQ168793	*β-ActinNDA*	CCCAACTTGAGCCTAACAT	TACCTCCAGACAGCACGG
EU339149	*12S r RNA*	ACAACCAACCATAGCCCACA	GTGGCTGGCACGAGTTTGA
EU339149	*ND4L*	CTTCTCCGCAGCCTTCATT	GCGATGAATAGGGAAAGCA
EU339149	*D-loop*	CTGAGGTTGGTGGAGTGC	GGGTTGCTCCCACTTATGT
EU339149	*16S r RNA*	TATGAATGGCAAGACGAGG	TAGGACAGGGCTCAGTTAGTT
EU339149	*ND2*	GACCTCATTACAGGACTTATCAT	TGTAGGACGAGGATTATTCAG
EU339149	*ND3*	CTATGAGTGCGGCTTTGAC	AAGGTAAGGAGAAGCAGGAC
EU339149	*ND6*	ATGTTGGTGGTGTTTGCG	CCTCGCAATACAGATAACTCC
EU339149	*COX1*	CCTGCTGCTCTCACTACCTG	CCGAAGAATCAGAATAGGTGTT
EU339149	*COX2*	GAGTGCTAATCTCCGCTGAAG	TGGGACTGCTTCAACTACGAT
EU339149	*COX3*	ACTTCCACTCTACAATCCTCCTAT	AGAAGACCTCTGATGTGATGAAT
EU339149	*ATPase6*	ATTAGCGATTGCTCTCATACT	CGAGTATTAGGGCTCAGTTAT
EU339149	*CYTB*	GCCTCTACTATGGCTCCTATCTT	AGGCACTGCTGACAAGAGGT
Bisulfite-pyrosequencing primer			
EU339149	*D-loop*-F	GGGATATTGATTGATAATTATTTGG	
	D-loop-R(bio)	ACGACRACCTTATACCTAAATACCTC	
	D-loop-sp	GGTATTTTTTTATTTTGATTG	
EU339149	*12S*-F	ATGAGTTGAATAGGCGATTAGTTTA	
	12S-R(bio)	TACTAAACCTACTAATCCTAAAATAAAAACTAC	
	12S-sp	ATTTGATTTYGGTTTAAAAG	
EU339149	*ND6*-F	TTATTAATATTAGTTTTGATAATTTTAGTGTT	
	ND6-R(bio)	AATATTAATAATTAATACTTAAATATTACTTTTAAC	
	ND6-sp	GATAATTTTAGTGTTYGTTTTTTAA	

[a] *ATPase6*, ATP synthase; *COX*, cytochrome *c* oxidase; *CYTB*, cytochrome b; *HIF1α*, hypoxia-inducible factor 1α; *ND*, NADH dehydrogenase; *NRF1*, nuclear respiratory factor 1; *PGC1α*, peroxisome proliferator-activated receptor gamma coactivator 1α; and *PPAR*, peroxisome proliferator-activated receptor. All sequences available from the GenBank database (www.ncbi.nlm.nih.gov).

(3) whether changes in ROS metabolism affect mtDNA methylation in response to changes of dietary lipid concentration.

2. Materials and methods

2.1. Ethics statement

The protocols for animal care and handling used in this study were approved by the Institutional Animal Care and Use Committee of Ocean University of China (Permit Number: 20001001).

2.2. Animals and liver sampling

The feeding experiment was conducted in Xihu Bay, Ningbo, China as described thoroughly by Yan et al. (2015). Specifically, three isonitrogenous diets (42% crude protein) were formulated to contain low (6%), moderate (12%, the control diet) and high (18%) crude lipid levels (described in Table 1). Increasing lipid levels were obtained by increasing fish oil inclusion and lowering the amounts of wheat starch. The analyzed dietary lipid contents were 6.1, 11.5 and 17.8%, respectively. The fatty acid composition of the experimental diets was similar and could be obtained from a supplementary material (Yan et al., 2015, doi: 10.1371/journal.pone.0129937). Large yellow croaker were fed one of three diets for 70 d. At the end of the feeding trial, five fish from each cage (3 biological replicates per experimental diet) were sacrificed, and liver samples were dissected. After the dissection, the liver samples were immediately frozen in liquid nitrogen and stored at −80 °C for later analysis.

2.3. Enzyme analysis

CS activity was determined by the method of Haas et al. (1995). Briefly, the liver was homogenized (1:10, w/v) in SETH buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4). Thirty microliters of liver homogenate was added in triplicates for each sample to a 96-well plate with each well containing 170 mL of reaction mixture (100 mM Tris, 30 mM acetyl CoA, 10 mM 5.5′-dithiobis[2-nitrobenzoic acid]). Then, the reaction was initiated by the addition of 10 mM oxaloacetic acid and monitored at 412 nm for 3 min at 25 °C.

2.4. Measurement of mtDNA copy number

Liver mtDNA copy number was determined as described previously (Hartmann et al., 2011). Relative mtDNA copy number was measured by quantitative real time PCR with primers for the mitochondrial *D-loop* (displacement loop) and *16S rRNA* region and with primers for the nuclear *β-actin* gene, which was analyzed in parallel. The primers used for quantitative real time PCR were depicted in Table 2. Total DNA was extracted from the liver tissue using a TaKaRa MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa, Japan). Quantitative real time PCR was performed as described below.

2.5. Quantitative real-time PCR

Total RNA was extracted from the liver tissue using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The RNA was treated with DNase I (Takara, Japan) to remove DNA and reverse transcribed to complementary DNA (cDNA) by a PrimeScript™ RT Reagent Kit (Takara, Japan) following the manufacturer's instructions. Specific primers were designed for each target gene (Table 2). Quantitative real-time PCR was based on the procedures described by Zuo et al. (2013). The gene expression levels were studied by qRT-PCR method: $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Gene expression was normalized using *β-actin* and *Ubiquitin* expression; data are reported as fold of the normalized values obtained for fish fed the control diet. Statistical analysis was conducted using ANOVA followed by Dunnett's test.

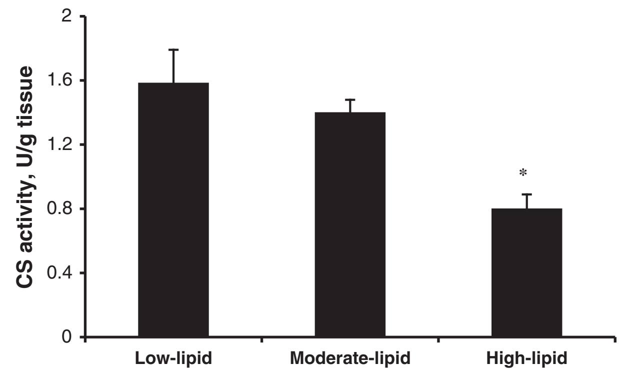

Fig. 1. Liver citrate synthase activity of large yellow croaker (*Larimichthys crocea*) fed diets with varying lipid concentration for 70 d. Values are expressed as the means ± SEMs (n = 3). Statistical significance was evaluated by one-way ANOVA, followed by the Dunnett's test. *$P < 0.05$ versus the moderate-lipid group. CS, citrate synthase.

2.6. DNA methylation analysis

The mitochondria were isolated using a Mitochondrial Isolation Kit for Tissues (Thermo Scientific, Hudson, NH, USA) following the manufacturer's instructions. MtDNA was extracted from the mitochondrial pellet using a TaKaRa MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa, Japan). DNA methylation analysis was performed using highly quantitative bisulfite-PCR pyrosequencing, an accurate method for determining mtDNA 5-methylcytosine (5mC) content (Byun et al., 2013). MtDNA was treated with the EZ DNA methylation Gold kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. Purified converted DNA (10 ng) was then PCR amplified, and 25 μL of product was sequenced using a PyroMark Q96 ID (conditions supplied by Qiagen using the Pyromark software). The assay for the NADH dehydrogenase subunit 6 encoding region (*ND6*), *12S rRNA* gene, and *D-loop* region (primer sequences are shown in Table 2) was designed. Run conditions were as follows: 95 °C for 15 min, 45 × (95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s), 72 °C for 5 min. The data was validated by internal controls and presented as percent 5mC. Multiple CpGs within each region of interest were interrogated, and the average across all the CpGs was calculated.

2.7. Oxidative stress assays

The protein concentration of liver homogenate was determined using the method of Lowry et al. (1951). Malondialdehyde (MDA) was assayed by a thiobarbituric acid (TBA) assay kit (Nanjing Jiancheng

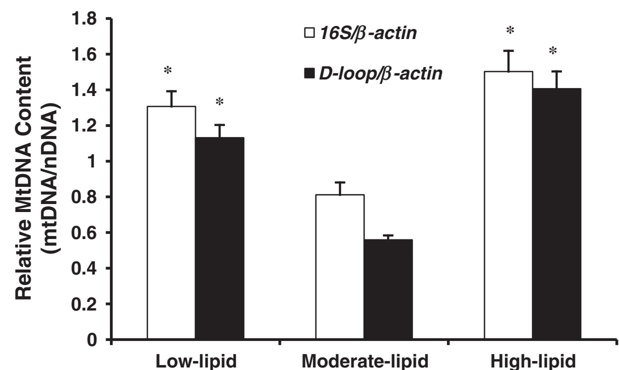

Fig. 2. Relative mitochondrial DNA copy number in the liver of large yellow croaker (*Larimichthys crocea*) fed diets with varying lipid concentration for 70 d. Values are expressed as the means ± SEMs (n = 3). Statistical significance was evaluated by one-way ANOVA, followed by the Dunnett's test. *$P < 0.05$ versus the moderate-lipid group.

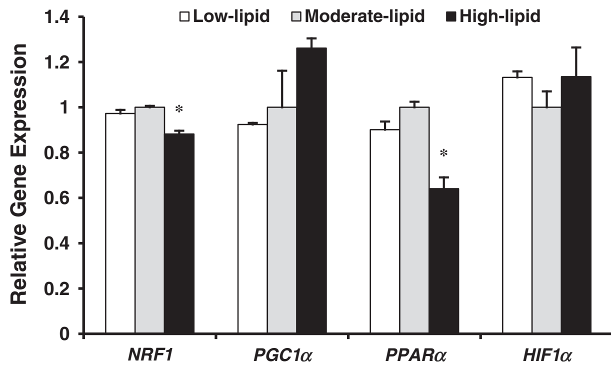

Fig. 3. Relative mRNA abundance of several transcription factors in liver of large yellow croaker (*Larimichthys crocea*) fed diets with varying lipid concentration for 70 d. Values are expressed as the means ± SEMs (n = 3). Statistical significance was evaluated by one-way ANOVA, followed by the Dunnett's test. *$P < 0.05$ versus the moderate-lipid group. *HIF1α*, hypoxia-inducible factor 1α; *NRF1*, nuclear respiratory factor 1; *PGC1α*, peroxisome proliferator-activated receptor gamma coactivator 1α; and *PPAR*, peroxisome proliferator-activated receptor.

Bioengineering Institute, Nanjing, China). MDA reacts with TBA to produce a TBA–MDA complex, which can be measured by a spectrophotometric procedure (Zuo et al., 2013). Liver 8-hydroxydeoxyguanosine (8-OHdG) was assessed by ELISA kit (IBL International GmbH, Hamburg, Germany) following the manufacturer's instructions. Results were expressed as ng per g of protein. Superoxide dismutase activity (SOD) was measured spectrophotochemically by the ferricytochrome *c* method using xanthine/xanthine oxidase as the source of superoxide radicals (Zuo et al., 2013).

Superoxide radical scavenging activity was detected by biochemical methods following the instructions for the reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, superoxide radical generated by the hypoxanthine and xanthine oxidase system reacts with nitroblue tetrazolium to form a colored formazan compound, which is proportional to the concentration of superoxide radical (Yang et al., 2005).

The hydroxyl radical scavenging activity was measured with a commercial detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions (Yang et al., 2005). The hydroxyl radical generated by the Fenton reaction is treated with nitroblue tetrazolium to form a stable colored substance, which is direct in proportion to amount of hydroxyl radical and spectrophotometrically detected at 550 nm.

2.8. Statistical analysis

Differences were determined by one way ANOVA using SPSS 16.0 for Windows (SPSS Inc.), and Dunnett's test was used to inspect differences between the control group and the treatment groups. If unequal variance was determined by Levene's test, data were log-transformed before statistical analysis. The average %5mC content of each region was log-transformed prior to statistical analysis because they were not normally distributed. Data were expressed as means ± SEM., and *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Mitochondrial enzyme activity

Compared with the control group, the activity of CS in the liver decreased about 50% in the high-lipid group (Fig. 1).

3.2. MtDNA copy number

The liver mtDNA copy number was significantly higher in the low- and high-lipid groups compared to the control group (Fig. 2), as evidenced by an increase in the ration of *D-loop* or *16S rRNA* to *β-actin* DNA.

3.3. Transcription factor coordinates mitochondrial metabolic adaptation

The liver *NRF1* and *PPARα* mRNA levels were significantly lower in the high-lipid group relative to the control group (Fig. 3). Peroxisome proliferator-activated receptor gamma coactivator 1α (*PGC1α*) and hypoxia-inducible factor 1α (*HIF1α*) mRNA levels in the liver did not significantly differ between the control group and the treatment groups (Fig. 3).

3.4. Mitochondrial-encoded gene transcription

The relative mRNA abundances of NADH dehydrogenase subunit 3 (*ND3*), *ND4L* and *ND6* in the low- and high-lipid groups were significantly higher than the control group (Fig. 4). The *ND2* mRNA level was significantly higher in the high-lipid group compared with the control group (Fig. 4). There were no significant differences in cytochrome b (*CYTB*) mRNA abundance between the control group and the treatment groups (Fig. 4). The relative transcript abundance of cytochrome *c* oxidase 1 (*COX1*), *COX2*, *COX3*, ATP synthase 6 (*ATPase 6*), *12S rRNA* and *16S rRNA* in the low-lipid group was significantly higher than that in the control group (Fig. 4).

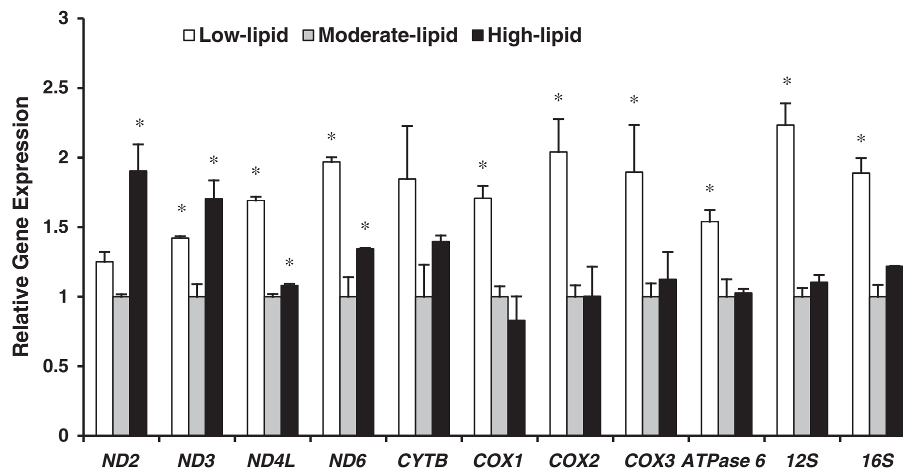

Fig. 4. Relative transcripts of mitochondria-related genes in liver of large yellow croaker (*Larimichthys crocea*) fed diets with varying lipid concentration for 70 d. Values are expressed as the means ± SEMs (n = 3). Statistical significance was evaluated by one-way ANOVA, followed by the Dunnett's test. *$P < 0.05$ versus the moderate-lipid group. *ATPase 6*, ATP synthase 6; *COX*, cytochrome *c* oxidase; *CYTB*, cytochrome b; and *ND*, NADH dehydrogenase.

3.5. MtDNA methylation

D-loop methylation in the liver from fish fed the high-lipid diet was significantly higher compared with the control group (Fig. 5A). MtDNA methylation of the *12S rRNA* gene in the control group was significantly higher compared with the low-lipid group and was not significantly different from the high-lipid group (Fig. 5B). Changes in dietary lipid concentration had little impact on liver *ND6* methylation (Fig. 5C).

3.6. Oxidative stress parameters

The concentrations of MDA and 8-OHdG were significantly higher in the high-lipid group compared with the control group, and were not significantly different between the low-lipid and control group (Fig. 6A and Fig. 6B). The activity of SOD in the liver did not differ between the control group and the treatment groups (Fig. 6C). The activity of scavenging superoxide radical was not significantly different between the control group and the treatment groups (Fig. 6D). Fish fed the control diet displayed a significant increase in scavenging hydroxyl radical capacity relative to fish fed diets with low- and high-lipid (Fig. 6E).

In addition, specific growth rate, feed intake and liver weight can be obtained from a supplementary material (Yan et al., 2015, doi: 10.1371/journal.pone.0129937). Briefly, there were no significant differences in specific growth rate and feed intake between the control group and the treatment groups. The liver weight was higher in the high-lipid group compared with the control group.

4. Discussion

Different stimuli cause different mitochondrial adaptive responses in individual tissues (Battersby and Moyes, 1998; McClelland et al., 2006; LeMoine et al., 2008, 2010). For example, temperature mainly affected the muscles (Battersby and Moyes, 1998), while diet exerts its main effects on liver in fish (Blasco et al., 1992; LeMoine et al., 2008; Eya et al., 2011; Lu et al., 2014b). Moreover, some previous studies have indicated that the liver of large yellow croaker is the most sensitive tissue to dietary lipid (Zuo et al., 2012, 2013). In the present study, therefore the effects on the liver were emphasized.

In the present study, the activity of CS in the liver was significantly higher in fish fed the control diet compared to fish fed the high-lipid diet for 70 d. However, goldfish fed the diet with 20% lipid for 21 d displayed no significant changes in CS activity compared with fish fed the normal diet (LeMoine et al., 2008). This inconsistent result probably accounts for the fact that mitochondrial metabolic adaptation is time-dependent, as was shown in mice that high-lipid diet induced an initial increase in mitochondrial bioenergetics and then decreased mitochondrial function (Ruggiero et al., 2011; Begriche et al., 2013). Previous studies have showed that the expression of CS was correlated with *NRF1* mRNA level in zebrafish muscle (McClelland et al., 2006; LeMoine et al., 2010), goldfish muscle (LeMoine et al., 2008; Bremer et al., 2012) and liver (LeMoine et al., 2008), and threespine sticklebacks muscle and liver (Orczewska et al., 2010). Although little relationship between CS enzyme activity and *CS* mRNA level has been suggested in the liver of cod (Lucassen et al., 2006), in the present study, *NRF1* mRNA and CS activity all significantly reduced in the liver of fish fed high-lipid diet compared with the control group. Thus, there may be a possible regulatory relationship between NRF1 and CS activity in the liver of large yellow croaker. In addition to NRF1, PGC1α was a master regulator coordinating mitochondrial metabolism in mammals (Fernandez-Marcos and Auwerx, 2011). In the present study, *PGC1α* mRNA was unaffected by dietary lipid concentration, probably indicating that, as already showed in other species (LeMoine et al., 2008, 2010; Bremer and Moyes, 2011; Windisch et al., 2011; Bremer et al., 2012), the PGC1α did not play a direct regulatory role in these adaptations in fish. Moreover, there was a regulatory relationship between *PPARα* and mitochondrial fatty acid oxidation gene expression in response to dietary lipid concentration (LeMoine et al., 2008; Lu et al., 2014b). In the present study, *PPARα* mRNA was significantly lower in the high-lipid group compared with the control group, probably suggesting that high-lipid diet induced a reduction in mitochondrial fatty acid oxidation gene expression and subsequently decreased mitochondrial aerobic metabolic capacity. Taken together, these results suggested that liver mitochondria tend to decrease its aerobic metabolic capacity in response to high-lipid diet in large yellow croaker.

MtDNA transcription is closely related to mtDNA replication, and several transcription factors (mtTFA, mtTF1 and TFAM) regulate both mitochondrial transcription initiation and mtDNA copy number (Scarpulla, 2008). In mammals, the expression of mitochondrial genes is regulated by their copy number dosage (Williams, 1986). In the present study, mtDNA copy number concomitant with the transcription of *ND3*, *ND4L*, *ND6*, *COX1*, *COX2*, *COX3*, *ATPase 6*, *12S rRNA* and *16S rRNA* was significantly higher in the low-lipid group compared with the

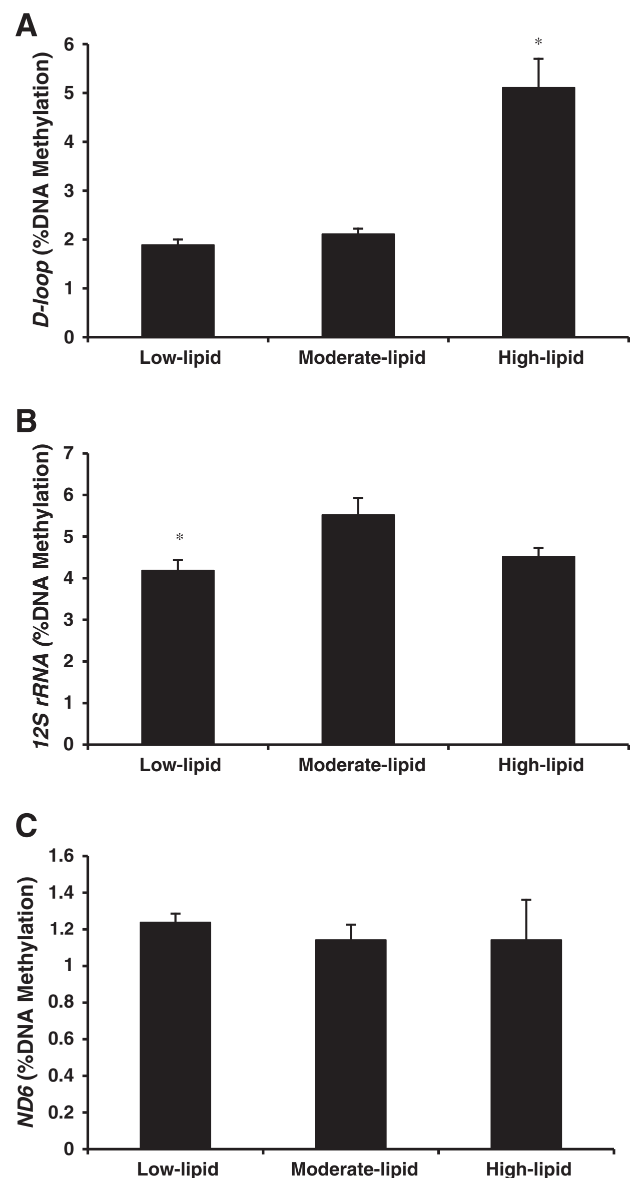

Fig. 5. The status of *D-loop* (A), *12S rRNA* (B) and *ND6* (C) methylation in the liver of large yellow croaker (*Larimichthys crocea*) fed diets with varying lipid concentration for 70 d. Values are expressed as the means ± SEMs (n = 3). Statistical significance was evaluated by one-way ANOVA, followed by the Dunnett's test. *$P < 0.05$ versus the moderate-lipid group. *D-loop*, displacement loop; *ND6*, NADH dehydrogenase 6.

control group. This probably means that the increase of mtDNA copy number and mitochondrial transcripts is a compensatory mechanism that matches ATP supply to demand under a lower energy diet. However, in contrast to changes observed with transcription, an increase in mtDNA copy number was not tightly coupled to an increase in transcription of *COX1*, *COX2*, *COX3*, *ATPase 6*, *12S rRNA* and *16S rRNA* in the high-lipid group. Actually, a flexible relationship between mitochondrial transcripts and mtDNA copy number was reported in fish (Battersby and Moyes, 1998; Leary et al., 1998). Moreover, levels of mitochondrial *COX1*, *COX2* and *16S rRNA* transcripts were increased in the white musculature of cold acclimated eelpout (Hardewig et al., 1999), yet cold acclimation of trout did not lead to significant increases of mtDNA copy number in white muscle (Battersby and Moyes, 1998). The authors ascribed increased transcript levels to either an enhanced transcription of the mitochondrial genome or a higher stability of mRNA (Hardewig et al., 1999). Therefore, an increase in mtDNA copy number with unchanged levels of *COX1*, *COX2*, *COX3*, *ATPase 6*, *12S rRNA* and *16S rRNA* in the high-lipid group probably indicated that mitochondrial transcript abundance was regulated at transcriptional and post-transcriptional levels.

Temperature or exercise acclimation typically results in restructuring of biological membranes and various metabolic changes in fish, and these physiological responses should impact the susceptibility of biological membranes to oxidative stress (Grim et al., 2010; Kammer et al., 2011). Increased enzymatic antioxidants such as SOD in oxidative muscle may be required to prevent oxidative stress brought about by increased mitochondrial density during cold acclimation of threespine stickleback (Kammer et al., 2011). In the present study, enzymatic antioxidants (such as SOD, scavenging superoxide radical activity, scavenging hydroxyl radical capacity) were not echoed by increased mtDNA copy number, probably partially resulting in increased oxidative stress parameters, such as MDA and 8-OHdG contents, in fish fed high-lipid diet. Alternatively, increased mtDNA copy number may be a result of an increase of oxidative stress caused by lipid overload (Hancock et al., 2008; Lu et al., 2014a, 2014b). Growing evidence from experimental models and human studies has shown that mtDNA methylation is susceptible to oxidative stress (Kujoth et al., 2005; Shock et al., 2011; Dzitoyeva et al., 2012; Pirola et al., 2012; Byun et al., 2013). In the present study, *D-loop* methylation was significantly higher in the high-lipid group compared to the control group, which confirmed the above statement. *D-loop* is a promoter region in mitochondrial transcription (Scarpulla, 2008). Studies on colorectal cancer suggest that demethylation of the *D-loop* plays a key role in promotion mitochondrial transcription, such as *ND2* expression (Feng et al., 2012). Collectively, these results indicate that unchanged levels of *COX1*,

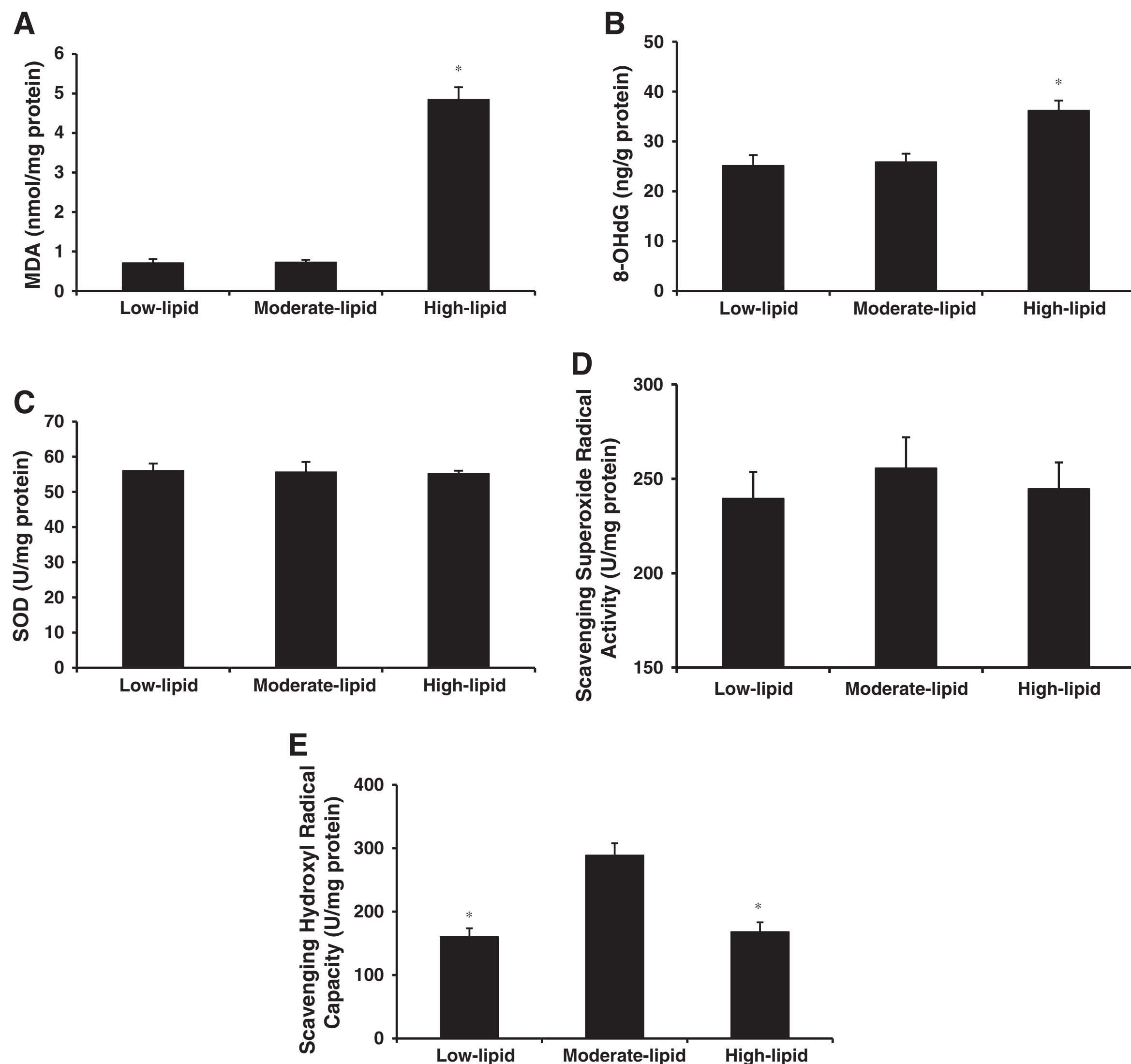

Fig. 6. Oxidative stress parameters in liver of large yellow croaker (*Larimichthys crocea*) fed diets with varying lipid concentration for 70 d. Values are expressed as the means ± SEMs (n = 3). Statistical significance was evaluated by one-way ANOVA, followed by the Dunnett's test. *$P < 0.05$ versus the moderate-lipid group. MDA, malondialdehyde; SOD, superoxide dismutase activity; and 8-OHdG, 8-hydroxydeoxyguanosine.

COX2, *COX3*, *ATPase 6*, *12S rRNA* and *16S rRNA* with an increase in mtDNA copy number in the high-lipid group may be attributable to increased methylation in its promoter. To our knowledge, this is the only evidence in any organism that mtDNA methylation was involved in the regulation of mitochondrial metabolism in responses to changes in dietary lipid concentration. Like in mammals, oxidative stress may indirectly regulate mitochondrial metabolic adaptation through mtDNA methylation in fish, and future studies will address this question.

In summary, in response to low-lipid diet, liver mitochondria regulate its metabolic status to coordinate ATP production through increasing mtDNA copy number and mitochondrial transcription. The reduction of CS activity, *NRF-1* and *PPARα* mRNA levels and the increase of oxidative stress and *D-loop* methylation occurred in parallel with mitochondrial metabolic adaptation in response to high-lipid diet.

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Appendix A

(Supplementary materials related to this article can be found online at doi: 10.1371/journal.pone.0129937)

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