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Dietary Olive and Perilla Oils Affect Liver Mitochondrial DNA Methylation in Large Yellow Croakers[1–3]

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Abstract

Background: Substantial progress has been made in nutritional epigenetics, but little is known regarding whether mitochondrial DNA (mtDNA) methylation is involved in this process.

Objective: The objective of this study was to determine whether dietary lipid sources [various fatty acids (FAs)] modify mtDNA methylation.

Methods: A total of 600 large yellow croakers (*Larimichthys crocea*) with an average initial weight of 151 ± 4 g were fed 1 of 5 diets (3 replicate cages/treatment) containing either fish oil (FO) (control), palmitic acid, olive oil (OO), sunflower oil, or perilla oil (PO) as the dietary lipid source (12% dry weight of the diet) for 70 d. Pyrosequencing was used to determine the effects of dietary lipid sources (FAs) on mtDNA methylation.

Results: Mitochondrial arginine transfer RNA and NAD(H) dehydrogenase 4L encoding region methylation in the liver was higher in the OO (9.5% ± 0.52%; $P < 0.05$) and PO (7.3% ± 0.33%; $P < 0.05$) groups than in the FO (5.9% ± 0.42%) group, whereas 12S ribosomal RNA (rRNA) methylation in the liver was lower in the OO group (2.7% ± 0.22%) than in the FO group (4.2% ± 0.73%) ($P < 0.05$). Additionally, fish fed the OO diet had lower liver mRNA levels of *ND3* ($P < 0.05$), *ND4L* ($P < 0.05$), *ND6* ($P < 0.05$), 12S rRNA ($P < 0.05$), and 16S rRNA ($P < 0.05$) than those fed the FO diet, whereas fish fed the PO diet had lower liver mRNA levels of 16S rRNA than those fed the FO diet ($P < 0.05$). Moreover, fish fed the OO ($P < 0.05$) or PO ($P < 0.05$) diet had lower liver mitochondrial complex I activity than did those fed the FO diet.

Conclusions: These findings provide the first evidence, to our knowledge, that dietary lipid sources influence mitochondrial function through mtDNA methylation in large yellow croakers. *J Nutr* 2015;145:2479–85.

Keywords: fatty acid compositions, mitochondrial DNA, DNA methylation, mitochondrial dysfunction, fish quality, large yellow croaker (*Larimichthys crocea*)

Introduction

A limited supply of fish oil (FO)[4] as feed for cultured fish has driven an interest in the use of alternative terrestrial oil sources. However, because of differences in FA composition between FO and terrestrial oils, the extensive use of terrestrial oils in aquaculture usually leads to changes in fish lipid metabolism, such as increased lipid accumulation in the liver and decreased long-chain PUFA concentrations in the liver and muscle, compromising fish health and product quality (1–3). Clearly, any reduction in quality, in terms of the health of the farmed fish, would decrease the health benefits of fish as a food source for humans. As a result, understanding the mechanisms by which terrestrial oils alter fish lipid metabolism may help improve fish flesh quality for human food (1–3).

Changes in mitochondrial function caused by various dietary FA compositions have been identified as a critical factor that

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[3] Supplemental Tables 1–5 and Supplemental Figures 1–5 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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[4] Abbreviations used: *ATPAF2*, ATP synthase mitochondrial F1 complex assembly factor 2; *COX*, cytochrome c oxidase; *CPT1*, carnitine palmitoyltransferase 1; *CYTB*, cytochrome b; *D-loop*, displacement loop; DNMT, DNA methyltransferase; FO, fish oil; HIF1α, hypoxia-inducible factor 1α; *LRPPRC*, leucine-rich PPR motif–containing protein; mtDNA, mitochondrial DNA; *ND*, NAD(H) dehydrogenase; *NDUFS1*, NAD(H) dehydrogenase Fe-S protein 1; *ND4L*, NAD(H) dehydrogenase 4L; *NRF1*, nuclear respiratory factor 1; OO, olive oil; PA, palmitic acid; *PGC1α*, peroxisome proliferator–activated receptor γ coactivator 1α; PO, perilla oil; *RNR1*, 12S ribosomal RNA; ROS, reactive oxygen species; rRNA, ribosomal RNA; *SDHB*, succinate dehydrogenase complex, subunit B; SO, sunflower oil; *TR*, arginine transfer RNA; tRNA, transfer RNA.

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affects fish lipid metabolism (4–6). Mitochondrial function is tightly related to the expression of genes encoded by mitochondrial DNA (mtDNA) (7). MtDNA encodes 13 proteins, 22 transfer RNAs (tRNAs), and 2 ribosomal RNAs (rRNAs) and is transcriptionally controlled by nuclear genes (7). Recent studies have revealed that mtDNA methylation is implicated in the modulation of mtDNA transcription (8) and mitochondrial function (8, 9) and could be easily modified by external factors, such as aging (10), airborne pollutants (9), drug exposure (11), lifestyle (8), and cancer (12). In addition, one implication of dietary FA regulation of nuclear gene expression is epigenetic modification through DNA methylation (13, 14). However, whether dietary lipid sources (various FAs) modify the mtDNA methylation pattern, alter mitochondrial function, and subsequently influence lipid metabolism remains unknown.

The large yellow croaker, with its high level of production and critical role in human food or health (15), is widely cultured and studied in China. Moreover, the use of terrestrial oils as a substitute for FO has become more common in large yellow croaker commercial diets because of the decrease in global FO production. The substitution of FO by terrestrial oils changes the lipid metabolism of the large yellow croaker, with decreased EPA (20:5n–3) and DHA (22:6n–3) concentrations in muscle and liver and abnormally increased lipid accumulation in the liver (16). Furthermore, our previous studies indicated that the FA metabolism and lipid deposition of the large yellow croaker are strikingly similar to those of other fish species and mammals (17–19), which suggests that the large yellow croaker is a useful model for studying lipid metabolism. Thus, the large yellow croaker is an appropriate fish species to use to investigate the mechanisms of how terrestrial oils affect lipid metabolism.

As such, the present study investigated the effect of 4 terrestrial oils on mtDNA methylation in the large yellow croaker, discussed the potential impact of mtDNA methylation on mitochondrial function and lipid metabolism in the liver, and proposed that the substitution of FO with terrestrial oils may alter mitochondrial function, lipid metabolism, and fish quality through mtDNA methylation.

Methods

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).

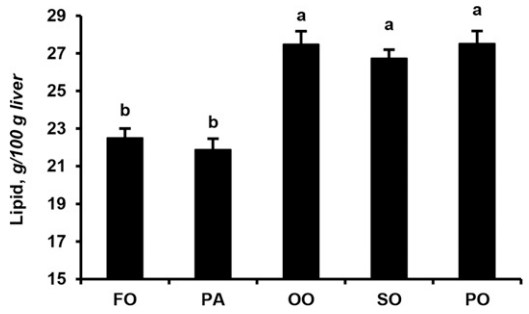

FIGURE 1 Liver lipid concentrations of large yellow croakers (*Larimichthys crocea*) fed diets with varying lipid sources for 70 d. Values are means ± SEMs (n = 3). Significance was evaluated by 1-factor ANOVA followed by a Duncan's multiple range test. Labeled means without a common letter differ, $P < 0.05$. FO, fish oil; OO, olive oil; PA, palmitic acid; PO, perilla oil; SO, sunflower oil.

Diets. Five isoproteic and isolipidic experimental diets were formulated and manufactured to contain ~43% protein and 12% lipids and vary only in dietary lipid source (**Supplemental Table 1**). The lipid sources were added at 80 g/kg and included the following: *1*) FO as a source of n–3 long-chain PUFAs (the control diet); *2*) palmitic 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. The FA composition of the 5 diets is shown in **Supplemental Table 2**.

Fish rearing and sampling. Large yellow croakers were sourced from a commercial farm in Xiangshan Bay, Ningbo, China. The fish were housed in floating sea cages (3 × 3 × 3 m) and acclimated to the experimental environments and diets before the start of the experiment. The large yellow croakers (initial weight 151 ± 4 g) were weighed and randomly distributed into 15 sea cages (1 × 1 × 1.5 m; 40 fish/cage). The fish were assigned 1 of the 5 dietary treatments (3 replicate cages/treatment). The fish were hand-fed 2 times/d to apparent satiation at 0500 and 1700 for 70 d.

The fish were deprived of food for 24 h before harvest. Then, the fish in each cage were subjected to mild anesthesia (1:10,000; Shanghai Reagent, Shanghai, China) and weighed. The fish were then killed by a blow to the head. Immediately, the liver samples were dissected out and frozen in liquid nitrogen. The livers from 10 fish in each cage were pooled into 1.5-mL tubes (RNAase-free; Axygen), frozen in liquid

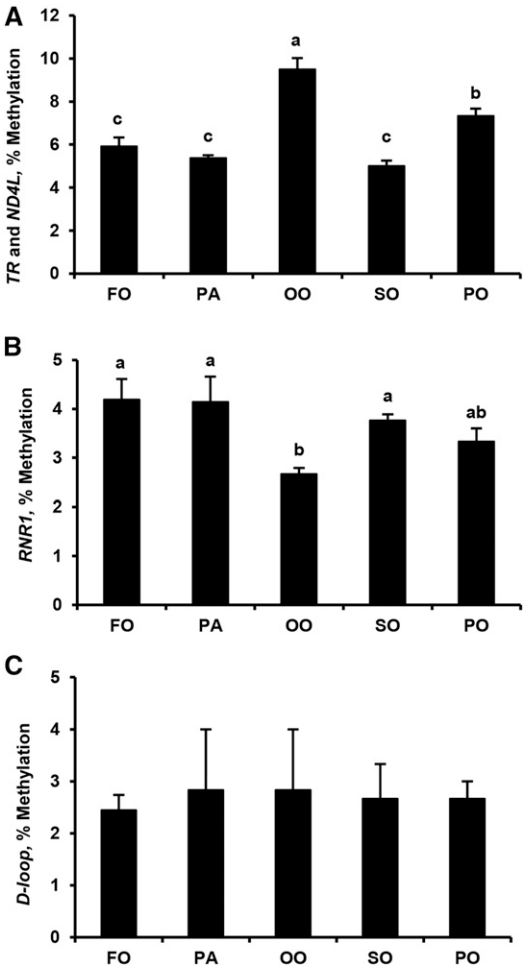

FIGURE 2 Methylation status of *TR* and *ND4L* (A), *RNR1* (B), and *D-loop* (C) in the liver of large yellow croakers (*Larimichthys crocea*) fed diets with varying lipid sources for 70 d. Values are means ± SEMs (n = 3). Significance was evaluated by 1-factor ANOVA followed by a Duncan's multiple range test. Labeled means without a common letter differ, $P < 0.05$. *D-loop*, displacement loop; FO, fish oil; *ND4L*, NAD(H) dehydrogenase 4L; OO, olive oil; PA, palmitic acid; PO, perilla oil; *RNR1*, 12S ribosomal RNA; SO, sunflower oil; *TR*, arginine transfer RNA.

nitrogen, and stored at −80°C for later analysis of gene expression and DNA methylation. The livers from another 10 fish in each cage were dissected out, weighed, and frozen in liquid nitrogen, then stored at −80°C for assays of enzyme activity, FA composition, and crude lipids.

Biochemical analysis. Liver lipid concentration was quantified with the use of chloroform methanol extraction following a modification of the method by Bligh and Dyer (20). The procedures for analysis of the FA profiles were based on the method described by Metcalfe et al. (21), with some modification (22).

DNA methylation analysis. DNA methylation analysis was performed with the use of highly quantitative bisulfite-PCR pyrosequencing (a schematic of quantitative bisulfite–PCR pyrosequencing is shown in **Supplemental Figure 1**), as described by Byun et al. (9) and Wong et al. (23). An assay was designed for the arginine transfer RNA (*TR*) and NAD(H) dehydrogenase 4L (*ND4L*) encoding region; the 12S ribosomal RNA (*RNR1*) gene; the displacement loop (*D-loop*) region; NAD(H) dehydrogenase Fe-S protein 1 (*NDUFS1*); succinate dehydrogenase complex, subunit B (*SDHB*); leucine-rich PPR motif–containing protein (*LRPPRC*); and ATP synthase mitochondrial F1 complex assembly factor 2 (*ATPAF2*) (the primer sequences are shown in **Supplemental Table 3**). The data were validated by internal controls and are presented as percentage 5-methylcytosine. Multiple cytosine poly-guanines within each region of interest were investigated, and the average across all of the cytosine poly-guanines was calculated.

Oxidative stress assays. The protein concentration of liver homogenate was determined with the use of the method from Lowry et al. (24). Malondialdehyde was assayed by a thiobarbituric acid assay kit (Nanjing Jiancheng Bioengineering Institute) (19). Liver 8-hydroxydeoxyguanosine was assessed by an ELISA kit (IBL International) following the manufacturer's instructions. Superoxide dismutase activity was measured based on the procedures described by Zuo et al. (19). Activities of superoxide radical scavenging and hydroxyl radical scavenging were detected by biochemical methods following the instructions for the reagent kits (Nanjing Jiancheng Bioengineering Institute) with some modification (25). Activities of catalase and glutathione peroxidase were measured based on the methods described by Bell et al. (26) and Puangkaew et al. (27). α-Tocopherol was assayed by reversed-phase HPLC with the use of a C-l8 column (Waters) as described previously (28).

Real-time qPCR. Real-time qPCR and cDNA preparation were based on the procedures described by Zuo et al. (19). Specific primers were designed for each target gene (Supplemental Table 3). The gene expression levels were studied with the use of the qRT-PCR: $2^{-\Delta\Delta CT}$ method (29). Gene expression was normalized to β-actin and ubiquitin expression; the data are reported as the fold of the normalized values obtained for fish fed the FO diet.

Western blot. Total protein was extracted with the use of a tissue or cell total protein extraction kit (Sangon Biotech). Mitochondrial protein was extracted with the use of a cytoplasmic and mitochondrial protein extraction kit (Sangon Biotech), and the concentrations were quantified with the use of a noninterference protein assay kit (Sangon Biotech). Protein was separated by denaturing SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with either DNA methyltransferases (DNMTs) (DNMT1, DNMT3A, and DNMT3B) or hypoxia-inducible factor 1α (HIF1α) antibodies (Cell Signaling Technology). GAPDH was used for data normalization with the use of a specific primary GAPDH antibody (Beyotime Institute of Biotechnology).

Biochemical analysis of mitochondrial complex activity. Citrate synthase activity was determined by the method used by Haas et al. (30). Mitochondria complex I activity was determined by the method used by Ragan (31). Mitochondria complex II activity was measured based on the method of Sottocasa et al. (32). Mitochondria complex III activity was measured by the method from Ragan et al. (33). Mitochondria complex IV activity was determined with the use of the method from Wharton and Tzagotoff (34).

TABLE 1 Relative mRNA concentrations of DNA methyltransferases and mitochondria-related genes in the liver of large yellow croakers (*Larimichthys crocea*) fed diets with varying lipid sources for 70 d[1]

	Fold of FO				
Gene	FO	PA	OO	SO	PO
DNMT1	1.0 ± 0.0082^{a}	0.49 ± 0.046^{b}	0.53 ± 0.067^{b}	0.85 ± 0.030^{a}	0.92 ± 0.053^{a}
DNMT3A	1.0 ± 0.033^{c}	1.1 ± 0.034^{b,c}	1.8 ± 0.098^{a}	1.2 ± 0.051^{b,c}	1.3 ± 0.14^{b}
DNMT3B	1.0 ± 0.05^{a}	0.54 ± 0.011^{b}	0.45 ± 0.031^{b}	0.47 ± 0.0046^{b}	0.46 ± 0.094^{b}
HIF1α	1.0 ± 0.028^{c}	1.2 ± 0.0090^{b,c}	1.9 ± 0.11^{a}	1.4 ± 0.12^{b}	1.9 ± 0. 21^{a}
ND2	1.0 ± 0.036^{a}	0.48 ± 0.0035^{c,d}	0.38 ± 0.036^{d}	0.78 ± 0.089^{b}	0.61 ± 0.053^{c}
ND3	1.0 ± 0.026^{a}	0.67 ± 0.020^{b}	0.46 ± 0.025^{c}	1.1 ± 0.088^{a}	0.58 ± 0.066^{b,c}
ND4L	1.0 ± 0.14^{b,c}	1.2 ± 0.042^{a,b}	0.70 ± 0.013^{d}	1.3 ± 0.017^{a}	0.85 ± 0.0083^{c,d}
ND6	1.0 ± 0.14^{a}	0.78 ± 0.00053^{a,b}	0.49 ± 0.10^{c}	0.87 ± 0.00058^{a,b}	0.63 ± 0.022^{b,c}
CYTB	1.0 ± 0.017^{a}	0.50 ± 0.031^{b,c}	0.42 ± 0.067^{c}	0.47 ± 0.022^{b,c}	0.61 ± 0.082^{b}
COX1	1.0 ± 0.11^{a}	0.61 ± 0.094^{b}	0.35 ± 0.062^{c}	0.56 ± 0.025^{b,c}	0.53 ± 0.018^{b,c}
COX2	1.0 ± 0.084^{a}	0.58 ± 0.042^{b,c}	0.37 ± 0.030^{d}	0.51 ± 0.065^{c,d}	0.71 ± 0.049^{b}
COX3	1.0 ± 0.041^{a}	0.62 ± 0.11^{b}	0.50 ± 0.053^{b}	0.55 ± 0.073^{b}	0.51 ± 0.014^{b}
ATPase6	1.0 ± 0.14^{a}	0.53 ± 0.049^{b}	0.56 ± 0.050^{b}	0.62 ± 0.034^{b}	0.88 ± 0.055^{a}
12S rRNA	1.0 ± 0.12^{a}	1.0 ± 0.046^{a}	0.44 ± 0.050^{b}	0.89 ± 0.096^{a}	0.80 ± 0.020^{a}
16S rRNA	1.0 ± 0.020^{a}	1.1 ± 0.0064^{a}	0.45 ± 0.072^{c}	1.04 ± 0.069^{a}	0.80 ± 0.028^{b}
PPARA	1.0 ± 0.18^{a}	0.93 ± 0.056^{a,b}	0.58 ± 0.066^{c}	0.88 ± 0.091^{a,b,c}	0.66 ± 0.022^{b,c}
CPT1	1.0 ± 0.061^{b}	5.1 ± 0.63^{a}	2.5 ± 0.26^{b}	5.0 ± 0.50^{a}	1.7 ± 0.38^{b}
PGC1α	1.0 ± 0.11^{b,c}	2.2 ± 0.22^{a}	1.4 ± 0.25^{b}	0.71 ± 0.093^{c}	1.1 ± 0.23^{b,c}
NRF1	1.0 ± 0.018^{d}	1.2 ± 0.026^{b}	1.3 ± 0.040^{b}	1.1 ± 0.012^{c}	1.4 ± 0.028^{a}

[1] Values are means ± SEMs, n = 3. Labeled means in a row without a common letter differ, $P < 0.05$. Significance was evaluated by 1-factor ANOVA followed by a Duncan's multiple range test. *COX*, cytochrome c oxidase; *CPT1*, carnitine palmitoyltransferase 1; *CYTB*, cytochrome b; *DNMT*, DNA methyltransferase; FO, fish oil; *HIF1α*, hypoxia-inducible factor 1α; *ND*, NAD(H) dehydrogenase; *NRF1*, nuclear respiratory factor 1; OO, olive oil; PA, palmitic acid; *PGC1α*, peroxisome proliferator–activated receptor gamma coactivator 1α; PO, perilla oil; rRNA, ribosomal RNA; SO, sunflower oil.

Measurement of mtDNA content. Liver mtDNA content was determined as described previously (35). The primers used for real time qPCR are shown in Supplemental Table 3.

Statistical analyses. Differences due to diet were determined by 1-factor ANOVA with the use of SPSS 16.0 for Windows, and a Duncan's multiple range test was used to inspect differences between groups. If unequal variance was determined by Levene's test, the data were log-transformed before statistical analysis. Statistical analysis for liver weight (g/100 g body weight) and visceral weight (g/100 g body weight) were conducted on data after arcsine transformation. The average percentage of 5-methylcytosine in each region was log-transformed before statistical analysis because the data were not normally distributed. Densitometry after Western blotting was quantified by Quantity One (Bio-Rad) and then normalized by GAPDH. The correlation between HIF1α and *TR* and *ND4L* methylation was determined with the use of a Spearman rank correlation test. Data are expressed as means ± SEMs, and P values of <0.05 were considered significant.

Results

Animal characteristics and liver lipid concentration. The body weights of the large yellow croakers were not different between those fed diets that contained FO, PA, OO, SO, or PO ($P = 0.09$; **Supplemental Table 4**). Relative liver weight was higher in fish fed diets with OO and SO than in those fed diets with FO and PA ($P < 0.05$) (Supplemental Table 4). The SO group had a greater relative visceral weight than the PA group ($P = 0.002$) (Supplemental Table 4). The liver lipid concentrations in the OO, SO, and PO groups were higher than those in the FO and PA groups ($P < 0.01$) (**Figure 1**).

DNA methylation of mitochondrial genes in liver, muscle, and adipose tissue. *TR* and *ND4L* methylation in the liver of fish fed diets with OO (9.5% ± 0.52%) and PO (7.3% ± 0.33%) was higher than in those fed the FO (5.9% ± 0.42%), PA, (5.4% ± 0.13%) and SO (5.0% ± 0.25%) diets ($P < 0.05$) (**Figure 2**A). MtDNA methylation of the *RNR1* gene in the OO (2.7% ± 0.22%) group was lower than that in the FO (4.2% ± 0.73%), PA (4.1% ± 0.89%), and SO (3.8% ± 0.22%) groups ($P < 0.05$) (Figure 2B). *D-loop* methylation in the liver was independent of dietary treatments ($P = 0.3$) (Figure 2C). Promoter methylation of nuclear-encoded mitochondrial genes (*NDUFS1*, *SDHB*, *LRPPRC*, and *ATPAF2*) in the liver was not influenced by dietary lipid sources (**Supplemental Figure 2**). *TR* and *ND4L*, *RNR1*, and *D-loop* methylation in muscle and adipose tissue was unaffected by dietary lipid sources (**Supplemental Figures 3** and **4**).

Expression of DNMT1, DNMT3A, DNMT3B, *and* HIF1α *in liver.* The expression of *DNMT1* in the PA and OO groups was lower than that in the FO, SO, and PO groups ($P < 0.05$) (**Table 1**). Correspondingly, mitochondrial DNMT1 protein concentrations in the PA and OO groups were lower than those in the FO and PO groups ($P < 0.05$) (**Figure 3**A). *DNMT3A* mRNA ($P < 0.01$) and protein ($P < 0.01$) levels in the OO group were higher than in the other groups (Table 1, Figure 3B). *DNMT3B* expression was higher in the FO group than in the other groups ($P < 0.05$) (Table 1), whereas DNMT3B protein remained unaffected in liver mitochondria between dietary treatments ($P = 0.1$) (Figure 3C). In addition, the liver *HIF1α* mRNA levels in the OO and PO groups were higher than those in the FO, PA, and SO groups ($P < 0.05$) (Table 1). The liver HIF1α protein concentration was greater in the OO and PO groups than in the other groups ($P < 0.05$) (Figure 3D).

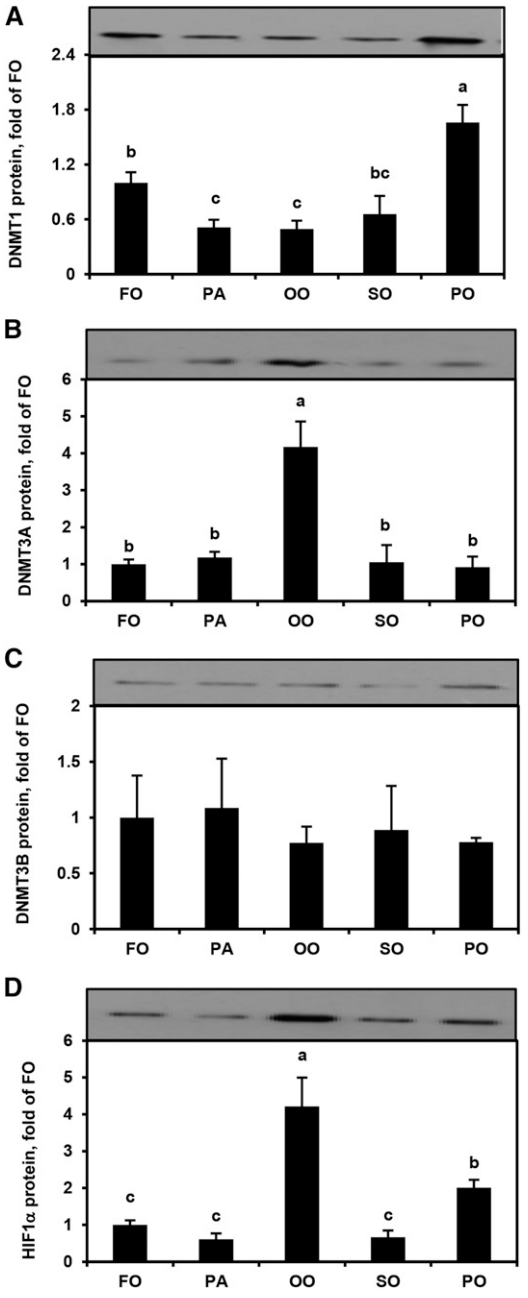

FIGURE 3 Protein concentrations of DNMT1 (A), DNMT3A (B), DNMT3B (C), and HIF1α (D) in the liver of large yellow croakers (*Larimichthys crocea*) fed diets with varying lipid sources for 70 d. Liver mitochondrial protein was used for DNMT Western blot analysis; liver total protein was used for HIF1α Western blot analysis. Densitometry after Western blotting was quantified by Quantity One (Bio-Rad) and then normalized to GAPDH. Values are means ± SEMs ($n = 3$). Significance was evaluated by 1-factor ANOVA followed by a Duncan's multiple range test. Labeled means without a common letter differ, $P < 0.05$. DNMT, DNA methyltransferase; HIF1α, hypoxia-inducible factor 1α; FO, fish oil; OO, olive oil; PA, palmitic acid; PO, perilla oil; SO, sunflower oil.

Oxidative stress-related variables in the liver. The concentrations of malondialdehyde and 8-hydroxydeoxyguanosine were higher in fish fed the diet with SO than those in the other groups ($P < 0.05$; **Table 2**). Superoxide dismutase activity in the liver was higher in the FO group than in the PA and PO groups ($P < 0.05$; Table 2). The activity of scavenging superoxide radicals in the OO and PO groups was higher than in the PA

TABLE 2 Oxidative stress variables in the liver of large yellow croakers (*Larimichthys crocea*) fed diets with varying lipid sources for 70 d[1]

Item	FO	PA	OO	SO	PO
Malondialdehyde, nmol/mg protein	1.1 ± 0.11[b]	0.80 ± 0.054[b]	0.54 ± 0.039[b]	3.5 ± 0.31[a]	0.91 ± 0.14[b]
8-OHdG, ng/g protein	25 ± 2.1[b]	26 ± 1.4[b]	27 ± 2.9[b]	32 ± 2.0[a]	24 ± 2.5[b]
SOD activity, U/mg protein	66 ± 1.4[a]	51 ± 0.4[c]	62 ± 1.7[a,b]	64 ± 2.2[a,b]	59 ± 0.52[b]
Superoxide radical scavenging activity, U/mg protein	261 ± 14.0[a,b]	231 ± 13.2[b]	288 ± 12.3[a]	276 ± 11.8[a,b]	289 ± 9.28[a]
Hydroxyl radical scavenging activity, U/mg protein	270 ± 21.6[b,c]	251 ± 11.0[c]	424 ± 26.9[a]	341 ± 14.5[a,b]	282 ± 11.1[b,c]
Catalase, U/mg protein	38 ± 3.0[b]	30 ± 4.5[b]	79 ± 12[a]	41 ± 2.4[b]	66 ± 9.2[a]
Glutathione peroxidase, U/mg protein	64.2 ± 7.91[b]	54.5 ± 3.45[b]	101 ± 11.9[a]	65.0 ± 8.59[b]	94.6 ± 8.73[a]
α-tocopherol, μg/g	66 ± 8.5[a,b]	45 ± 4.1[b]	88 ± 14[a]	83 ± 14[a]	66 ± 4.3[a,b]

[1] Values are means ± SEMs (n = 3). Labeled means in a row without a common letter differ, $P < 0.05$. Significance was evaluated by 1-factor ANOVA followed by a Duncan's multiple range test. FO, fish oil; OO, olive oil; PA, palmitic acid; PO, perilla oil; SO, sunflower oil; SOD, superoxide dismutase; 8-OHdG, 8-hydroxydeoxyguanosine.

group ($P < 0.05$) (Table 2). Liver scavenging hydroxyl radical activity was higher in the OO group than in the the FO, PA, and PO groups ($P < 0.05$) (Table 2). Catalase and glutathione peroxidase activities were increased in the fish fed the diets with OO and PO than in the FO, PA, and SO groups ($P < 0.05$) (Table 2). Liver α-tocopherol concentrations were lower in the PA group than in the OO and SO groups ($P < 0.05$) (Table 2).

Expression of mitochondria-related genes, mitochondrial complex activity, and mtDNA content in the liver. The liver mRNA abundance of NAD(H) dehydrogenase (*ND*) 2, cytochrome b (*CYTB*), cytochrome c oxidase (*COX*) 1, *COX2*, and *COX3* was higher in the FO group than in the other groups ($P < 0.05$) (Table 1). The liver transcript abundance of *ND3*, *ND4L*, *ND6*, and 16S rRNA was lower in the OO group than in the FO, PA, and SO groups ($P < 0.05$) (Table 1). The expression of 12S rRNA in the OO group was lower than in the FO, PA, SO, and PO groups ($P < 0.05$), and no difference could be found between the latter groups ($P = 0.06$) (Table 1).

The liver 16S rRNA abundance was lower in the PO group than in the FO, PA, and SO groups ($P < 0.05$; Table 1). The liver ATP synthase 6 mRNA abundance was lower in the PA, OO, and SO groups than in the FO and PO groups ($P < 0.05$) (Table 1). Transcription of nuclear-encoded mitochondrial genes (*NDUFS1*, *SDHB*, *LRPPRC*, and *ATPAF2*) was unaffected by dietary lipid sources (**Supplemental Table 5**).

The liver carnitine palmitoyltransferase 1 (*CPT1*) mRNA levels were higher in the PA and SO groups than in the other groups ($P < 0.05$) (Table 1). The expression of *PPARA* was lower in the OO and PO groups than in the FO group ($P < 0.05$) (Table 1). The hepatic peroxisome proliferator–activated receptor gamma coactivator 1α (*PGC1α*) expression in the PA group was higher than in the other groups ($P < 0.05$) (Table 1). The liver mRNA abundance of nuclear respiratory factor 1 (*NRF1*) was lower in the FO group than in the other groups ($P < 0.05$) (Table 1).

Mitochondrial complex I activity was higher in the FO, PA, and SO groups than in the OO and PO groups ($P < 0.05$) (**Table 3**), whereas complex II activity was lower in the FO group than in the other groups ($P < 0.01$) (Table 3).

There were no differences in the mtDNA content of the livers of fish fed diets that contained FO, PA, OO, SO, or PO ($P = 0.07$) (**Supplemental Figure 5**).

Discussion

The liver, muscle, and adipose tissue are all major organs of lipid metabolism in fish (36). However, only the pattern of methylation of the liver mtDNA was changed in the large yellow croaker by dietary lipid sources. This tissue-specific effect on mtDNA methylation likely indicates that the liver of the large yellow croaker is more sensitive than muscle and adipose tissue to different lipid sources, as already shown in previous studies on this species (16).

In the liver, the promoter DNA methylation, as well as transcription of nuclear-encoded mitochondrial genes, was unaffected regardless of dietary lipid sources. Interestingly, *TR* and *ND4L* methylation was significantly increased in fish fed diets with OO and PO compared with those fed diets with FO, whereas *RNR1* methylation was significantly decreased in fish fed a diet with PO compared with those fed diets containing FO. Although no reports have been published regarding the associations between dietary nutrients and mtDNA methylation, fewer investigations have indicated that the alteration of the mtDNA methylation pattern is possibly linked to changes in DNMT activity (8, 23, 37, 38), the occurrence of oxidative stress (9, 37, 39),

TABLE 3 Mitochondrial respiratory chain enzyme activities in the liver of large yellow croakers (*Larimichthys crocea*) fed diets with varying lipid sources for 70 d[1]

	Enzyme activity, U/mg protein				
Item	FO	PA	OO	SO	PO
CS	19.7 ± 1.96	21.7 ± 3.63	25.9 ± 3.47	26.5 ± 1.03	24.0 ± 1.14
CI	1.5 ± 0.21[a]	1.5 ± 0.082[a]	0.58 ± 0.10[b]	1.3 ± 0.23[a]	0.73 ± 0.17[b]
CII	0.20 ± 0.0070[b]	0.72 ± 0.13[a]	0.63 ± 0.097[a]	0.77 ± 0.058[a]	0.64 ± 0.098[a]
CIII	0.42 ± 0.26	0.55 ± 0.23	0.59 ± 0.16	0.79 ± 0.078	0.51 ± 0.19
CIV	1.1 ± 0.27	1.6 ± 0.51	1.5 ± 0.40	2.0 ± 0.17	1.7 ± 0.16

[1] Values are means ± SEMs (n = 3). Significance was evaluated by 1-factor ANOVA followed by a Duncan's multiple range test. Labeled means in a row without a common letter differ, $P < 0.05$. CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CS, citrate synthase; FO, fish oil; OO, olive oil; PA, palmitic acid; PO, perilla oil; SO, sunflower oil.

and hypoxia (8). In the present study, HIF1α was significantly increased in fish fed diets with OO and PO compared with those fed diets with FO, and it was significantly correlated with *TR* and *ND4L* methylation (R: 0.90; $P < 0.05$), which likely indicated that the feeding of OO or PO disrupted oxygen balance, elicited local hypoxic stress, and activated HIF1α in the liver of the large yellow croaker. Hypoxia induces the production of reactive oxygen species (ROS) (40, 41), which are determinants of DNA methylation alterations (42). Moreover, mtDNA is more vulnerable to ROS than nuclear DNA because of the absence of protective histones and an inefficient DNA repair system (43). Therefore, the fact that mitochondrial but not nuclear DNA methylation was modified by dietary lipid sources may be associated with hypoxia. However, future studies are needed to address this question.

MtDNA encodes 7 (*ND1*, *2*, *3*, *4*, *4L*, *5*, and *6*) of complex I, 1 (*CYTB*) of complex III, 3 (*COX1*, *COX2*, and *COX3*) of complex IV, and 2 (*ATPase 6* and *ATPase 8*) of complex V. MtDNA also encodes the 12S and 16S rRNA genes and the 22 tRNA genes that are required for mitochondrial protein synthesis (7). The alteration of mtDNA methylation would decrease the transcription of mitochondrial-encoded genes (8, 10–12, 37, 38). The reduced transcriptions of the polypeptide genes of mitochondrial complexes are known to decrease the activities of mitochondrial complexes and the synthesis rate of ATP and increase ROS generation (35). Meanwhile, the reduction of 12S and 16S rRNA decreases the efficiency of mitochondrial protein synthesis (9). In the present study, the transcription of *ND3*, *ND4L*, *ND6*, 12S rRNA, and 16S rRNA was significantly reduced in fish fed a diet with OO compared with one containing FO, and the transcription of 16S rRNA was significantly decreased in fish fed a diet with PO compared with a diet that contained FO, likely indicating an effect of *ND4L* and *RNR1* methylation on mitochondrial transcription. Furthermore, the alteration of *ND4L* and *RNR1* methylation occurred in parallel with the decrease of complex I activity in the liver of fish fed diets with OO and PO compared with diets that contained FO. These results suggest that the alteration of *ND4L* and *RNR1* methylation decreased *ND3*, *ND4L*, *ND6*, 12S rRNA, and 16S rRNA transcription and subsequently decreased complex I activity and impaired mitochondrial protein synthesis. In addition to decreasing mtDNA transcription and complex activity, the alteration of mtDNA methylation could result in mitochondrial morphology defects, such as a loss of inner membrane and larger mitochondria (8), an increase in mtDNA copy number (a biomarker of mitochondrial dysfunction) (9), and loss of mitochondria (23). Taken together, these findings suggest that the alteration of mtDNA methylation in *ND4L* or *RNR1* loci caused mitochondrial dysfunction in the liver of fish fed diets with OO and PO.

Mitochondrial dysfunction with concomitant downregulation of FA β-oxidation results in abnormal lipid accumulation in the liver of fish (44), as in mammals (45). In the liver of bluntsnout bream fed a high-fat diet, the reduced capacity for mitochondrial and peroxisomal β-oxidation after the decreased expression of genes for complex I and *PPARA* mRNA largely contributed to abnormal lipid accumulation (46, 47). Although FA β-oxidation was not assayed in the present study, gene expression of FA oxidation–related factor *PPARA* in the OO and PO groups was significantly lower than in the FO group. In addition, fish fed diets with OO, SO, and PO exhibited higher liver lipid concentrations than did fish fed the diet with FO. Therefore, it is possible that methylation modifications in mtDNA decrease the expression of genes for oxidative phosphorylation and mitochondrial protein synthesis, which leads to mitochondrial dysfunction and subsequently abnormal lipid accumulation in the liver of the large yellow croaker fed diets with OO and PO.

In addition to lipid abnormal accumulation in the liver, the long-chain PUFA concentrations in fish flesh must be carefully considered when selecting potential terrestrial oils to replace FO in the diets of fish (1, 2). Many factors are involved in the regulation of the long-chain PUFA status in fish, such as diet and genetic or sex hormones (48). Long-chain PUFA status is also compromised by increased ROS, most likely due to its sensitivity to ROS because of the high degree of unsaturation in the aliphatic tail (49). In the present study, the alteration of mtDNA methylation induced by OO and PO might lead to mitochondrial dysfunction and, therefore, likely disturb ROS homeostasis, because almost one-quarter of the cellular responses to oxidative stress are mediated through mitochondrial dysfunction (50). In support of the above statement, fish fed diets with OO and PO displayed a significant increase in the activities of catalase and glutathione peroxidase, which indicates an increase in ROS in salmon and trout (26, 27). These results raise the possibility that the alteration of mtDNA methylation induced by OO and PO compromised long-chain PUFA concentrations in the liver of the large yellow croaker.

In summary, the substitution of FO by OO and PO influences mtDNA methylation in the liver of the large yellow croaker. The alteration of mtDNA methylation patterns likely impairs mitochondrial function, changes lipid metabolism with respect to the increase in lipid abnormal accumulation and the decrease in long-chain PUFA concentrations, and subsequently reduces fish quality and its health benefits in human food.

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