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Effects of different dietary DHA:EPA ratios on gonadal steroidogenesis in the marine teleost, tongue sole (*Cynoglossus semilaevis*)

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Abstract

The present study was conducted to investigate the effects of dietary DHA and EPA on gonadal steroidogenesis in mature females and males, with a feeding trial on tongue sole, a typical marine teleost with sexual dimorphism. Three experimental diets differing basically in DHA:EPA ratio, that is, 0·68 (diet D:E-0·68), 1·09 (D:E-1·09) and 2·05 (D:E-2·05), were randomly assigned to nine tanks of 3-year-old tongue sole (ten females and fifteen males in each tank). The feeding trail lasted for 90 d before and during the spawning season. Fish were reared in a flowing seawater system and fed to apparent satiation twice daily. Compared with diet D:E-0·68, diet D:E-1·09 significantly enhanced the oestradiol production in females, whereas diet D:E-2·05 significantly enhanced the testosterone production in males. In ovaries, diet D:E-1·09 induced highest mRNA expression of follicle-stimulating hormone receptor (*FSHR*), steroidogenic acute regulatory protein, 17α-hydroxylase (*P450c17*) and 3β-hydroxysteroid dehydrogenase (*3β-HSD*). In testes, diet 2·05 resulted in highest mRNA expression of *FSHR*, cholesterol side-chain cleavage enzyme, *P450c17* and *3β-HSD*. Fatty acid profiles in fish tissues reflected closely those of diets. Female fish had more gonadal EPA content but less DHA content than male fish, whereas there was a reverse observation in liver. In conclusion, the dietary DHA:EPA ratio, possibly combined with the dietary EPA:arachidonic acid ratio, differentially regulated sex steroid hormone synthesis in mature female and male tongue soles. Females seemed to require more EPA but less DHA for the gonadal steroidogenesis than males. The results are beneficial to sex-specific nutritive strategies in domestic teleost.

Key words: ***Cynoglossus semilaevis*: Diets: DHA: EPA: Sex steroid hormone synthesis**

DHA (22 : 6*n*-3) and EPA (20 : 5*n*-3), the so-called *n*-3 long-chain PUFA (LC-PUFA), have been reported to play important roles in reproductive performances of animals[1,2], especially marine fish[3–13], which require LC-PUFA as essential fatty acids[14]. However, despite the wide studies on the effects of *n*-3 LC-PUFA on animal reproduction, little information has been published about the different effects between DHA and EPA. The present study was aimed at investigating the different efficacy between DHA and EPA in regulating reproductive processes, in a typical marine teleost, tongue sole (*Cynoglossus semilaevis*).

In animal reproduction, synthesis of sex steroid hormones is one of the most primary processes[15]. Steroid hormones activate the gonadal development and play important regulating roles all through the reproductive period, even including ovulation[16]. Compared with spawning performance and offspring quality, the regulation of steroid hormones by fatty acids has been relatively neglected, although a few studies have investigated the interactions between body fatty acid accumulation and steroid production[17,18]. Therefore, the present study focused on the regulatory effects of DHA and EPA on steroid hormone synthesis. In both terrestrial animals and fish species, less information has been available about the modulation of steroidogenesis by DHA and EPA[19–22]. Moreover, contradictory results have been reported in fish studies[23–26]. The present study will provide new insight into the regulatory effects of DHA and EPA on animal gonadal steroidogenesis. The regulation on key proteins involved in the key processes of sex steroid synthesis, that is, the response to gonadotrophins, the delivery of cholesterol substrate and biosynthetic reactions, in terms of gene expressions, was also investigated in this study, in order to elucidate the involved mechanisms.

In a previous study of our laboratory with tongue sole (*C. semilaevis*), we have studied the response of gonadal steroidogenesis to arachidonic acid (ARA, C20 : 4*n*-6), the most

Abbreviations: *3β-HSD*, 3β-hydroxysteroid dehydrogenase; *FSHR*, follicle-stimulating hormone receptor; LC PUFA, long-chain PUFA; OA, oleic acid; *P450c17*, 17α-hydroxylase; *StAR*, steroidogenic acute regulatory protein; TFA, total fatty acids.

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important *n*-6 LC-PUFA[27]. We found that dietary ARA was differentially accumulated in gonads and differentially regulated the gonadal steroidogenesis depending on fish sex and maturation stage. Interestingly, in this study, we found that DHA and EPA were also differentially accumulated between ovaries and testis. Therefore, as a follow-up study, the present study investigated the different effects of DHA:EPA on gonadal steroidogenesis between females and males. Tongue sole is a typical fish with sexual dimorphism. The females are several times bigger than males, making it a perfect species to study the sex-based difference in animal nutrition. This study will provide useful information for sex-specific nutritive strategies in domestic teleost, and these nutritive strategies may also be inspiring for other animals.

Methods

Experimental diets

Three isonitrogenous and isolipidic experimental diets were formulated to contain different levels of DHA:EPA ratio (Table 1). The basal diet was formulated using fishmeal, casein and wheat meal as protein sources, and soya lecithin, soyabean oil and olive oil as lipid sources. Different levels of EPA-enriched oil (containing 11·2 % DHA and 52·0 % EPA, in the form of TAG; Xi'an Renbang Biological Science and Technology Co., Ltd) and DHA-enriched oil (containing 69·5 % DHA and 6·6 % EPA, in the form of TAG; Xi'an Renbang Biological Science and Technology Co., Ltd) were added to the basal diet to obtain different DHA:EPA ratios. The diets were made, packed and stored according to the standard procedures in our laboratory[28]. The DHA:EPA ratio in the three experimental diets was 0·68, 1·09 and 2·05, respectively (Table 2). The corresponding diets were designated as D:E-0·68, D:E-1·09 and D:E-2·05, respectively.

Experimental fish and feeding procedure

Tongue sole *C. semilaevis* broodstock, 3-years old, which have been reared with formulated feeds from the early juvenile stage, were used in the present study. The average initial body weight of females and males was 3206 and 253 g, respectively. Before the start of the feeding trial, experimental fish were reared in concrete tanks (25 m^3) and fed the control diet for 7 d to acclimate to the experimental conditions. At the onset of the feeding trial, experimental fish were distributed into nine polyethylene tanks (diameter: 230 cm, height: 100 cm) and each diet was randomly assigned to triplicate tanks. Each tank had twenty-five fish (ten females and fifteen males) and the tanks were supplied with flowing filtered seawater at a rate of 50 litre/min. Fish were hand-fed to apparent satiation twice daily. The feeding trial lasted for 90 d, from July to October, which is the natural spawning season of tongue sole. Fish were reared under the natural photoperiod and ambient temperature of Haiyang, Shandong, China (N36°41', E121°07'). During the experiment, the water temperature ranged from 20 to 26°C; salinity was 30–32; pH was 7·4–8·5; and dissolved O_2 was 5–7 mg/l. The ponds were cleaned daily by siphoning out residual feed and faeces.

Table 1. Formulation and proximate composition of the experimental diets (g/kg DM)

Ingredients	D:E-0·68	D:E-1·09	D:E-2·05
Fishmeal	580·0	580·0	580·0
Casein	140·0	140·0	140·0
Wheat meal	144·5	144·5	144·5
Vitamin premix*	10·0	10·0	10·0
Mineral premix†	10·0	10·0	10·0
Monocalcium phosphate	10·0	10·0	10·0
Choline chloride	10·0	10·0	10·0
L-Ascorbyl-2-polyphosphate	5·0	5·0	5·0
Ethoxyquin	0·5	0·5	0·5
Soya lecithin	20·0	20·0	20·0
Soyabean oil	15·0	15·0	15·0
ARA-enriched oil‡	6·0	6·0	6·0
Olive oil	19·1	20·7	22·3
EPA-enriched oil§	27·6	17·7	7·8
DHA-enriched oil‖	2·3	10·6	18·9
Proximate composition			
Crude protein	576·2	579·0	578·9
Crude lipid	125·5	125·8	125·0
Ash	141·4	135·8	141·5

ARA, arachidonic acid.
* Vitamin premix (mg or g/kg diet): thiamin, 25 mg; riboflavin, 45 mg; pyridoxine HCl, 20 mg; vitamin B_{12}, 0·1 mg; vitamin K_3, 10 mg; inositol, 800 mg; pantothenic acid, 60 mg; niacin, 200 mg; folic acid, 20 mg; biotin, 1·2 mg; retinol acetate, 32 mg; cholecalciferol, 5 mg; *a*-tocopherol, 120 mg; wheat middling, 13·67 g.
† Mineral premix (mg or g/kg diet): $MgSO_4.7H_2O$, 1200 mg; $CuSO_4.5H_2O$, 10 mg; $ZnSO_4.H_2O$, 50 mg; $FeSO_4.H_2O$, 80 mg; $MnSO_4.H_2O$, 45 mg; $CoCl_2.6H_2O$ (1 %), 50 mg; $NaSeSO_3.5H_2O$ (1 %), 20 mg; $Ca(IO_3)_2.6H_2O$ (1 %), 60 mg; zoelite, 13·485 g.
‡ ARA-enriched oil: containing 41·0 % ARA (of total fatty acids), in the form of TAG; Jiangsu Tiankai Biotechnology Co., Ltd.
§ EPA-enriched oil: containing 11·2 % DHA and 52·0 % EPA (of total fatty acids), in the form of TAG; Xi'an Renbang Biological Science and Technology Co., Ltd.
‖ DHA-enriched oil: containing 69·5 % DHA and 6·6 % EPA (of total fatty acids), in the form of TAG; Xi'an Renbang Biological Science and Technology Co., Ltd.

Table 2. Fatty acid compositions of the experimental diets (% total fatty acids)

Fatty acids	D:E-0·68	D:E-1·09	D:E-2·05
C14 : 0	2·1	2·4	2·1
C16 : 0	14·6	16·5	15·5
C18 : 0	4·4	4·7	4·5
∑SFA	21·0	23·6	22·1
C16 : 1*n*-7	2·1	2·4	2·2
C18 : 1*n*-9	20·4	21·8	22·5
C18 : 1*n*-7	2·4	2·4	2·2
∑MUFA	24·9	26·6	26·9
C18 : 2*n*-6	15·6	15·7	15·9
C20 : 4*n*-6	3·8	3·4	3·2
∑*n*-6 PUFA	19·3	19·1	19·1
C18 : 3*n*-3	1·9	1·7	1·8
C20 : 5*n*-3	14·5	10·7	7·5
C22 : 5*n*-3	1·5	1·8	2·4
C22 : 6*n*-3	9·9	11·7	15·3
∑*n*-3 PUFA	27·7	26·0	26·9
DHA:EPA	0·7	1·1	2·1
∑*n*-3:∑*n*-6	1·4	1·4	1·4

Sampling

At the end of the feeding trial, serum, liver, muscle and gonad samples from five mature females and five mature males per tank were collected. The maturity of female and male fish was confirmed by spontaneous ovulation and the release of milt

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when handled, respectively. After being anaesthetised with eugenol (1:10 000), blood was drawn from all fish via the caudal vein to collect serum samples. Fish were then dissected to collect liver, muscle and gonad samples. All samples were frozen with liquid N_2 immediately, and then stored at −80°C before analysis. All sampling protocols, as well as fish rearing practices, were reviewed and approved by the animal care and use committee of the Yellow Sea Fisheries Research Institute.

Proximate composition analysis and fatty acid analysis

The proximate composition analyses of experimental diets were performed in accordance with the standard methods of the Association of Official Analytical Chemists (AOAC). The fatty acid compositions of diet and fish tissue lipids were analysed via a gas chromatograph, using a flame ionisation detector. Fatty acids in freeze-dried samples were esterified first with KOH–methanol and then with HCl–methanol, on a 72°C water bath. Fatty acid methyl esters were extracted with hexane and then separated via gas chromatography (HP6890; Agilent Technologies Inc.) with a fused silica capillary column (007-CW; Hewlett Packard). The column temperature was programmed to rise from 150°C up to 200°C at a rate of 15°C/min, and then from 200°C to 250°C at a rate of 2°C/min. Both the injector and detector temperatures were 250°C. Results were expressed as the percentage of each fatty acid with respect to total fatty acids (TFA).

Quantitative real-time PCR analysis and the analysis of oestradiol and testosterone in serum

Total RNA in gonads was extracted using RNAiso Plus (TaKaRa Biotechnology (Dalian) Co., Ltd) and reverse-transcribed with PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa Biotechnology (Dalian) Co., Ltd) according to the user manual.

Specific primers for key proteins in steroidogenesis and the reference gene *β-actin* were designed on the basis of the sequences available in the GenBank database and synthesised by Sangon Biotech (Table 3). The real-time PCR was carried out with SYBR Green Real-time PCR Master Mix (TaKaRa Biotechnology (Dalian) Co., Ltd) in a quantitative thermal cycler (Mastercycler-eprealplex; Eppendorf). The detailed program was similar to that of Xu *et al.*[29]. The mRNA expression levels were studied by quantitative real-time PCR method: $2^{-\Delta\Delta C_t}$[30].

Serum oestradiol and testosterone concentrations were assayed in collaboration with the affiliated hospital of Qingdao University, using an electrochemiluminescence method. COBAS-6000 (e 602 module) automatic electrochemiluminescence immunoassay analyzer (Roche Diagnostics) and affiliated commercial kits (Elecsys Estradiol III and Testosterone II) supplied by Roche were used in this assay. The standard curve for oestradiol was between 18·4 and 11 010 pmol/l, and for testosterone it was between 0·087 and 52 nmol/l. To validate the assays for tongue sole, an assay of serial dilutions of various tongue sole plasma samples was conducted. The dilutions were found to be parallel to the standard assay curve. The validation of the recovery was also conducted by the assay of added steroids in tongue sole plasma. The recovery of oestradiol and testosterone was 90·5 % and 92·6 %, respectively. The steroid values were corrected for recovery losses. The inter- and intra-assay CV for the oestradiol assay were 9·3 (*n* 7) and 4·3 % (*n* 9), respectively, and those for the testosterone assay were 9·6 (*n* 7) and 5·2 % (*n* 9), respectively.

Table 3. Sequences of the primers used in this work

Primers	Sequence (5′-3′)	GenBank reference
FSHR-F	AAGATCAAGGGAAAACGCTA	EU_661784·2
FSHR-R	CTCAGATGGTTGGAGGAAAG	
StAR-F	ACCTCGTGGGTGACCATCGTGT	NM_001294220·1
StAR-R	AGGACGGCTGGACCACTGAAAT	
P450ssc-F	TTCTGTGCTGTATGGCGAAC	GH_232539·1
P450ssc-R	CTTTTGACCCAATCCGTCTC	
P450c17-F	GCCCACTCGCTCCCTACATACT	EU_580533·2
P450c17-R	GTCTTTCCCATCTCGGGTCAG	
3β-HSD-F	CACCACTGGGTAAGCACTATC	XM_008328505·1
3β-HSD-R	AGGTTATCGCAAACAGCATT	
17β-HSD-F	AATGTGCAGGCTCTAACTGCTTC	XM_008330027·1
17β-HSD-R	AGGTTCCTCATGGTGGCGTA	
Aromatase-F	TGCGATTTCAGCCCGT	EF_134716·1
Aromatase-R	TGCGACCCGTGTTCAGA	
β-Actin-F	TTGGCTCGTGTTCGTCGTTC	KP_033459·1
β-Actin-R	TCAGGGTGTTGGGCTTGTTG	

FSHR, follicle-stimulating hormone receptor; *StAR*, steroidogenic acute regulatory protein; *P450ssc*, cholesterol side-chain cleavage enzyme; *P450c17*, 17*α*-hydroxylase; *3β-HSD*, 3*β*-hydroxysteroid dehydrogenase; *17β-HSD*, 17*β*-hydroxysteroid dehydrogenase.

Statistical methods

All data were subjected to one-way ANOVA in SPSS 16.0 for Windows. All percentage data were arcsine transformed before analysis. Significant differences between the means were detected by Tukey's multiple-range test. The level of significance was chosen at $P < 0{\cdot}05$. The results are presented as means of triplicate groups.

Results

Concentrations of oestradiol and testosterone in serum

In female fish, the oestradiol concentration in group D:E-0·68 was significantly ($P < 0{\cdot}05$) lower than that in group D:E-1·09, whereas no significant difference was observed either between groups D:E-0·68 and D:E-2·05 or between groups D:E-1·09 and D:E-2·05 (Fig. 1(A)). In male fish, the testosterone concentration in group D:E-0·68 was significantly ($P < 0{\cdot}05$) lower than that in group D:E-2·05, whereas no significant difference was observed either between groups D:E-0·68 and D:E-1·09 or between groups D:E-1·09 and D:E-2·05 (Fig. 1(B)).

Gonadal mRNA expressions of sex steroid-synthesising proteins

In mature ovaries (Fig. 2(A)), the mRNA expressions of follicle-stimulating hormone receptor (*FSHR*) and 17*α*-hydroxylase (*P450c17*) were significantly higher ($P < 0{\cdot}05$) in group D:E-1·09 than in the other two groups, whereas no significant difference was observed between the other two groups. The mRNA

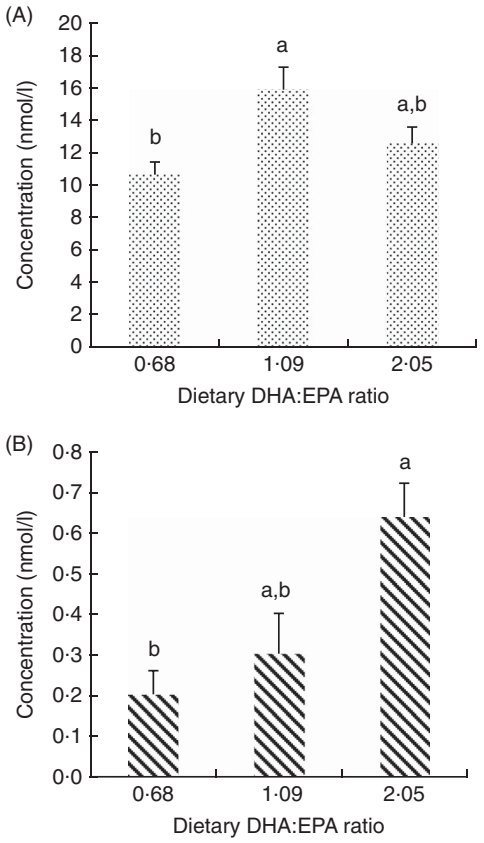

Fig. 1. Effects of dietary DHA:EPA ratios on the serum concentrations of oestradiol in females (A) and testosterone in males (B). Values are means of triplicate groups, with their standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0·05$).

expressions of steroidogenic acute regulatory protein (*StAR*) and 3*β*-hydroxysteroid dehydrogenase (*3β-HSD*) in group D:E-1·09 were significantly higher ($P<0·05$) than those in group D:E-0·68, whereas no significant difference was observed either between groups D:E-0·68 and D:E-2·05 or between groups D:E-1·09 and D:E-2·05. No significant difference was observed in mRNA expressions of cholesterol side-chain cleavage enzyme (*P450ssc*), 17*β*-hydroxysteroid dehydrogenase (*17β-HSD*) and aromatase among dietary groups.

In mature testis (Fig. 2(B)), the mRNA expression of *FSHR* was significantly higher ($P<0·05$) in group D:E-1·09 than in group D:E-0·68. The gene expression of *P450ssc* in group D:E-0·68 was significantly lower ($P<0·05$) than that in groups D:E-1·09 and D:E-0·68, whereas no significant difference was observed between the latter groups. The mRNA expression of *P450c17* was the highest in group D:E-2·05, significantly higher ($P<0·05$) than that in groups D:E-0·68 and D:E-1·09. The mRNA expression of *3β-HSD* was significantly higher in group D:E-2·05 compared with group D:E-0·68, whereas no significant difference was observed either between groups D:E-0·68 and D:E-1·09 or between groups D:E-1·09 and D:E-2·05. The gene expression of *StAR*, *17β-HSD* and aromatase showed no significant difference among dietary groups.

Tissue fatty acid profiles

In all tissues of female and male fish, except the muscle of male fish, the EPA content significantly decreased ($P<0·05$) and the DHA content significantly increased with increasing dietary DHA:EPA ratios (Tables 4–6). No significant difference was observed in DHA content in muscle of male fish (Table 6). The DHA:EPA ratios in fish tissues always significantly increased with increasing dietary DHA:EPA ratios.

Compared with ovaries, testes had lower average EPA content but higher average DHA content (Table 4). On the contrary, compared with female livers, male livers had higher average EPA content but lower average DHA content (Table 5). Testes had much lower average C18 : 1*n*-9 content but higher average C16 : 0 content than ovaries. In muscle, both EPA and DHA contents were higher in males than in females. The ARA contents were higher in all tissues of male fish compared with those of female fish.

Group D:E-0·68 had significantly lower ($P<0·05$) testis C16 : 0 content than group D:E-1·09. Females from group D:E-2·05 showed significantly higher ($P<0·05$) liver C18 : 2*n*-6 content but significantly lower ($P<0·05$) ovary C18 : 1*n*-7 content compared with group D:E-0·68.

Discussion

The present study showed that both oestradiol in females and testosterone in males had the lowest concentrations in the group with the lowest dietary DHA:EPA ratio (0·68). This indicated that a low DHA:EPA ratio in the diet may have inhibitory effects on gonadal steroidogenesis in tongue sole. This inhibitory effect could partly be attributed to the high EPA levels in diets. In *in vitro* studies with gonadal tissues from goldfish and rainbow trout, EPA has been shown to inhibit the gonadotrophin-stimulated testosterone production[19,23]. High levels of EPA could competitively inhibit the production of ARA-derived eicosanoids such as 2-series PG (PGE_2), which play important roles in gonadal steroidogenesis and maturation, and consequently inhibit the gonadal steroidogenesis and maturation[14,19,20,31–35]. In this regard, altered EPA:ARA ratios could also modulate the gonadal steroidogenesis and maturation of fish. In this study, although the dietary ARA level was relatively constant among groups, the EPA:ARA ratio varied among diets with different DHA:EPA ratios, being 3·82, 3·14 and 2·34, respectively, in the three experimental diets. The highest EPA:ARA level (3·82) in the lowest DHA:EPA diet could probably exert inhibition on the steroidogenesis in tongue sole via the regulation of PG production.

With respect to ARA, although the ARA content was designed to be constant among experimental diets, a slight increase in actual dietary ARA content (3·2, 3·4 and 3·8 % of TFA) existed with decreasing dietary DHA:EPA ratios. Moderate levels of ARA and its metabolites, PGE_2, have been widely demonstrated to stimulate gonadal steroidogenesis and maturation[4,19,20,23,32,33,36–40]. A recent study in our lab investigating the effects of dietary ARA on the gonadal steroidogenesis in tongue sole showed that, compared with the group without ARA supplementation, 15·44 % ARA (of TFA) in the diet

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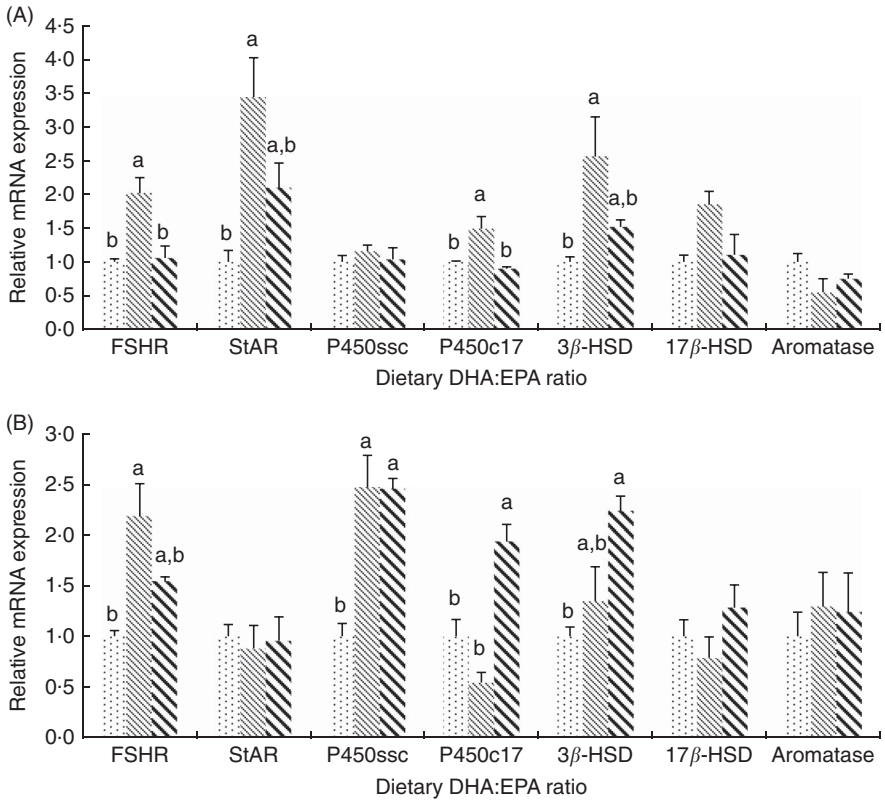

Fig. 2. Effects of dietary DHA:EPA ratios on the relative mRNA expressions of sex steroid-synthesising proteins in ovaries (A) and testes (B). Values are means of triplicate groups, with their standard errors represented by vertical bars. ⬚, 0·68; ▨, 1·09; ▩, 2·05. *FSHR*, follicle-stimulating hormone receptor; *StAR*, steroidogenic acute regulatory protein; *P450ssc*, cholesterol side-chain cleavage enzyme; *P450c17*, 17*α*-hydroxylase; *3β-HSD*, 3β-hydroxysteroid dehydrogenase; *17β-HSD*, 17β-hydroxysteroid dehydrogenase. [a,b] Mean values with unlike letters were significantly different ($P < 0·05$).

Table 4. Gonad fatty acid compositions of tongue sole fed the experimental diets (% total fatty acids)*

	Female					Male				
Fatty acids	D:E-0·68	D:E-1·09	D:E-2·05	Pooled SEM	*P*	D:E-0·68	D:E-1·09	D:E-2·05	Pooled SEM	*P*
C14 : 0	1·6	1·5	1·4	0·04	0·235	1·5	1·5	1·6	0·05	0·677
C16 : 0	16·9	16·3	14·5	0·45	0·064	21·8[b]	25·9[a]	23·4[a,b]	0·67	0·009
C18 : 0	7·9	8·4	7·0	0·37	0·336	10·3	9·7	10·2	0·14	0·289
∑SFA	26·4	26·2	23·0	0·71	0·071	33·6[b]	37·2[a]	35·2[a,b]	0·59	0·009
C16 : 1*n*-7	3·1	2·6	2·6	0·12	0·025	1·7	1·8	1·5	0·14	0·752
C18 : 1*n*-9	22·0	22·6	21·7	0·45	0·747	10·9	11·0	11·3	0·28	0·875
C18 : 1*n*-7	3·9[a]	3·7[a,b]	3·2[b]	0·13	0·025	3·2[b]	4·6[a]	3·4[b]	0·24	0·002
∑MUFA	29·0	28·9	27·4	0·45	0·328	15·8	17·4	16·2	0·43	0·328
C18 : 2*n*-6	10·9	11·2	11·9	0·22	0·154	9·7	10·3	9·9	0·16	0·327
C20 : 4*n*-6	2·5	2·4	2·5	0·08	0·833	6·6	5·5	6·0	0·28	0·317
∑*n*-6 PUFA	13·5	13·6	14·9	0·27	0·333	16·3	15·8	16·0	0·22	0·721
C18 : 3*n*-3	0·9	0·9	1·1	0·03	0·250	0·6	0·6	0·5	0·05	0·589
C20 : 5*n*-3	5·4[a]	3·9[b]	3·6[b]	0·30	0·003	5·1[a]	2·8[b]	2·8[b]	0·40	0·001
C22 : 5*n*-3	4·1	4·0	4·2	0·06	0·143	4·1	3·5	3·8	0·13	0·116
C22 : 6*n*-3	11·4[b]	13·3[b]	17·1[a]	0·89	0·001	16·5[b]	18·8[a,b]	20·4[a]	0·63	0·009
∑*n*-3 PUFA	21·8[b]	22·1[b]	26·0[a]	0·76	0·011	26·3	25·1	27·1	0·39	0·081
∑*n*-3:∑n-6	1·6	1·6	1·8	0·05	0·239	1·6	1·6	1·7	0·03	0·403
DHA:EPA	2·1[c]	3·4[b]	4·9[a]	0·43	0·002	3·3[b]	6·9[a]	7·3[a]	0·69	0·003

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).
* Data represent the mean of triplicate groups.

enhanced the testosterone production in males, but 5·14 % was ineffective[27]. With regard to this, inhibitory effects of the diet with the lowest DHA:EPA ratio on gonadal steroidogenesis in this study may be less attributed to the slight ARA increment.

DHA is also an important regulator of fish gonadal steroidogenesis and maturation[18]. Similar to EPA, the inhibitory effects of high levels of DHA on gonadotropin-induced steroidogenesis have also been observed in *in vitro* studies

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Table 5. Liver fatty acid compositions of tongue sole fed the experimental diets (% total fatty acids)*

	Female					Male				
Fatty acids	D:E-0·68	D:E-1·09	D:E-2·05	Pooled SEM	*P*	D:E-0·68	D:E-1·09	D:E-2·05	Pooled SEM	*P*
C14 : 0	1·4	1·3	1·3	0·07	0·681	1·1	1·2	1·4	0·05	0·095
C16 : 0	18·8	18·3	18·0	0·33	0·640	18·4	19·9	19·4	0·43	0·381
C18 : 0	13·6	13·4	13·1	0·41	0·925	14·3	12·8	12·8	0·46	0·368
∑SFA	33·8	33·0	32·4	0·32	0·209	33·7	33·9	33·6	0·37	0·956
C16 : 1*n*-7	1·8	1·8	1·7	0·15	0·964	1·5	1·5	1·3	0·10	0·716
C18 : 1*n*-9	14·9	14·6	15·4	0·32	0·642	11·8	11·1	11·8	0·27	0·585
C18 : 1*n*-7	3·7	3·8	3·4	0·08	0·246	2·5	2·6	2·0	0·18	0·491
∑MUFA	20·4	20·2	20·6	0·43	0·951	15·7	15·2	15·1	0·44	0·844
C18 : 2*n*-6	5·0^{b}	5·3^{a,b}	6·6^{a}	0·29	0·023	9·6	9·6	11·1	0·38	0·149
C20 : 4*n*-6	5·6	5·6	5·5	0·18	0·953	7·7	6·9	7·0	0·24	0·306
∑*n*-6 PUFA	10·8	11·0	12·1	0·31	0·107	17·3	16·4	18·1	0·49	0·428
C18 : 3*n*-3	0·3	0·4	0·4	0·03	0·189	0·5	0·4	0·6	0·05	0·447
C20 : 5*n*-3	6·0^{a}	5·4^{b}	4·7^{c}	0·19	0·001	8·6^{a}	6·7^{b}	5·6^{c}	0·44	0·000
C22 : 5*n*-3	2·6	2·3	2·5	0·08	0·308	3·0	2·6	2·8	0·12	0·538
C22 : 6*n*-3	20·1^{b}	22·4^{a}	22·6^{a}	0·48	0·013	16·1^{b}	18·6^{a}	18·4^{a}	0·45	0·005
∑*n*-3 PUFA	28·9^{b}	30·4^{a}	30·3^{a}	0·28	0·019	28·1	28·1	27·4	0·25	0·464
∑*n*-3:∑n-6	2·8	2·8	2·5	0·07	0·258	1·6	1·7	1·5	0·05	0·333
DHA:EPA	3·4^{c}	4·1^{b}	4·8^{a}	0·21	0·000	1·9^{c}	2·8^{b}	3·3^{a}	0·22	0·000

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).
* Data represent the mean of triplicate groups.

Table 6. Muscle fatty acid compositions of tongue sole fed the experimental diets (% total fatty acids)*

	Female					Male				
Fatty acids	D:E-0·68	D:E-1·09	D:E-2·05	Pooled SEM	*P*	D:E-0·68	D:E-1·09	D:E-2·05	Pooled SEM	*P*
C14 : 0	3·6	3·2	3·1	0·12	0·169	1·7	1·7	2·0	0·11	0·308
C16 : 0	22·8	21·1	22·0	0·36	0·125	21·6	24·4	22·3	0·63	0·176
C18 : 0	5·6^{b}	6·4^{a,b}	6·5^{a}	0·17	0·028	8·6	10·6	8·6	0·50	0·167
∑SFA	32·0	30·6	31·6	0·36	0·280	31·9^{b}	36·6^{a}	32·9^{a,b}	0·89	0·045
C16 : 1*n*-7	6·2	5·2	5·2	0·20	0·064	3·2	3·0	3·2	0·07	0·250
C18 : 1*n*-9	16·3	13·9	15·1	0·59	0·255	11·6^{b}	12·3^{b}	18·1^{a}	1·15	0·009
C18 : 1*n*-7	3·8^{a}	3·2^{b}	3·3^{b}	0·10	0·002	2·6^{b}	2·8^{a,b}	2·9^{a}	0·06	0·044
∑MUFA	26·3	22·3	23·6	0·76	0·066	17·3^{b}	18·0^{b}	24·2^{a}	1·20	0·006
C18 : 2*n*-6	6·0	6·1	7·9	0·40	0·067	9·6	8·7	9·6	0·24	0·206
C20 : 4*n*-6	2·1	2·8	2·6	0·15	0·082	4·9^{a}	4·4^{a,b}	3·4^{b}	0·26	0·018
∑*n*-6 PUFA	8·1	8·9	10·5	0·46	0·074	14·6^{a}	13·1^{b}	13·0^{b}	0·28	0·009
C18 : 3*n*-3	0·9	0·7	0·7	0·04	0·061	0·6	0·6	0·7	0·03	0·413
C20 : 5*n*-3	4·0^{a}	3·9^{a}	3·0^{b}	0·21	0·049	6·5^{a}	4·6^{b}	3·5^{c}	0·44	0·000
C22 : 5*n*-3	4·6	4·9	4·7	0·14	0·597	4·2	3·4	4·0	0·20	0·267
C22 : 6*n*-3	14·4^{b}	18·2^{a}	17·6^{a}	0·61	0·000	18·1	18·9	19·1	0·57	0·810
∑*n*-3 PUFA	23·7^{b}	27·8^{a}	26·0^{a,b}	0·65	0·009	29·5	27·5	27·3	0·69	0·398
∑*n*-3:∑n-6	2·9	3·2	2·5	0·15	0·138	2·0	2·1	2·1	0·06	0·865
DHA:EPA	3·7^{b}	4·6^{b}	6·0^{a}	0·36	0·002	2·8^{c}	4·1^{b}	5·4^{a}	0·41	0·002

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).
* Data represent the mean of triplicate groups.

with fish gonadal tissues[19,23]. Besides steroidogenesis, the gonadotropin-induced oocyte maturation in marine teleost (European sea bass *Dicentrarchus labrax*) was also observed to be inhibited by high levels of DHA[4]. The inhibitory effects of high DHA levels could partly contribute to the inhibitory effects of the highest DHA:EPA ratio on oestradiol production in female tongue sole in this study. The group with highest DHA:EPA (2·05) showed a decrease in oestradiol production in females compared with the group with the moderate DHA:EPA ratio (1·09). However, in testes of male tongue sole, the highest testosterone production was observed in the group with the highest DHA:EPA ratio. This indicated that, compared with females, male tongue sole may require more DHA and less EPA for the gonadal steroidogenesis. Although *in vitro* studies stated above showed the inhibitory effects of high levels of both EPA and DHA on gonadotropin-induced steroidogenesis[19,23], a very high level of DHA significantly stimulated the PGE production in testis cell of European sea bass at all the times of incubation[20]. To some extent, this might provide a clue for the relatively high DHA requirement in steroidogenesis and maturation of male fish. A significantly positive correlation between testis DHA content and serum testosterone concentrations has been reported in mature male European eel (*Anguilla anguilla*)[18], indicating that DHA might play positive roles in testosterone synthesis.

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The difference in effective DHA:EPA ratio between female and male tongue sole was in good accordance with the different accumulation of DHA and EPA between ovaries and testes. Testes had more DHA (average 18·5 *v.* 13·9 % of TFA) but less EPA (average 3·6 *v.* 4·3 %) accumulation than ovaries. Interestingly, in the liver of tongue sole, a reverse observation was made; that is, males had less DHA (average 17·7 *v.* 21·7 %) but more EPA (average 6·9 *v.* 5·4 %) than females. Preferable LC-PUFA were probably transferred to gonads from other tissues during maturation to meet the reproductive requirements[41]. Baeza *et al.* [17] reported that in the testis of European eel all primary LC-PUFA, that is, DHA, EPA and ARA, remained constant during the maturation process. *De novo* synthesis of EPA and DHA in the liver and probable subsequent transfer of them to the testis was also observed in that study. Differently, Japanese eel (*Anguilla japonica*) testes seemed to have a higher preference for EPA[42]. In addition, in the muscle of tongue sole the contents of both DHA and EPA were higher in males than in females. Higher muscle LC-PUFA contents in males than in females were also observed in mountain trout (*Salmo trutta macrostigma*)[43]. The sex-specific fatty acid preference in fish tissues must be closely correlated with the sex-specific bio-functions of fatty acids, and this could be regulated by the different sex hormone secretion between female and male fish[18,44–47].

Besides the sex-specific effects of DHA:EPA, another interesting observation of this study was that the DHA:EPA ratio inducing the highest sex steroid production in both females and males was >1·0. This indicated that DHA may be superior to EPA in promoting fish steroid hormone synthesis. Very little information has been available about the relative effectiveness of DHA and EPA in regulating fish steroidogenesis, although several *in vivo* studies with fish species such as gilthead seabream (*Sparus aurata*), silver pomfret (*Pampus argenteus*) and European eel have investigated the effects of total LC-PUFA or *n*-3 LC-PUFA on sex steroid production[24–26]. A feeding trial with Siberian sturgeon (*Acipenser baeri*) showed that serum oestradiol was higher in females fed the diet with a DHA:EPA ratio of 1·9:1 compared with the ratio of 1:1·9[22], providing another evidence for the superiority of DHA to EPA in inducing sex steroid production. Similar to steroidogenesis results, some studies have also indicated the superior effect of DHA to EPA on other reproductive parameters. In a study with European sea bass, the PUFA-enriched diets with higher DHA content but lower EPA content induced the better-quality eggs and larvae (although reduced the spawning parameters) compared with the control diet[48], whereas another study with male European sea bass showed that the wet diet containing a higher EPA content resulted in lower spermiation performance and lower sperm quality as compared with the PUFA-enriched diet with a lower EPA content[3]. However, contradictory results were also observed in other fish species. A study with domesticated common sole (*Solea solea*) breeders showed that egg EPA concentration was positively correlated with larval viability, but egg DHA concentration was negatively correlated with hatching rate and larval viability[49]. To date, the relative effects of DHA and EPA on steroidogenesis and other reproductive parameters is highly unknown, and more future studies are needed.

Regarding the regulation of sex steroid-synthesising proteins by dietary DHA:EPA ratios, in tongue sole ovaries the gene expression of *FSHR, StAR, P450c17* and *3β-HSD* was significantly influenced by dietary treatments; however, the gene expression of *17β-HSD* and aromatase was not significantly different among experimental groups. The effects of diets on *17β-HSD* and aromatase may be masked by the endogenous inhibition of these enzymes in the time point of sampling. Tongue sole has group-synchronous ovarian development. Shifts in gonadal steroidogenesis in the group-synchronous gonadal development have been observed in several fish species such as European sea bass, rainbow trout (*Oncorhynchus mykiss*) and striped bass (*Morone saxatilis*)[50–53]. At the very beginning of ovulation, during which the samples were obtained in the present study, maturation-inducing progestagens may reach peaks, whereas the oestradiol production may already be decreasing[53]. Inhibition of *17β-HSD* and aromatase might be involved in the endogenous down-regulation of oestradiol production at this time point, considering that these two enzymes are two of the most important and rate-limiting enzymes in oestradiol synthesis.

In the testes, although dietary DHA:EPA ratios influenced gene expressions of several proteins such as *FSHR*, *P450ssc, P450c17* and *3β-HSD*, only the regulation of *3β-HSD* correlated well with the testosterone concentration results. This was in agreement with our previous studies investigating the steroidogenesis-regulating effects of ARA in this fish[27]. These results indicate that *3β-HSD*, which converts pregnenolones to progesterones, may play a key role in the regulation of testosterone synthesis by fatty acids[54,55]. Peñaranda *et al.*[56] have reported the gene expression of *cyp11a1* (*P450ssc*) and *cyp17-I* (*P450c17*), which are important enzymes initiating the testosterone synthetic pathway, correlated well with the testosterone synthesis during the maturation of European eel testis. However, in the present study, only a general positive correlation was observed between testosterone production and *P450ssc* or *P450c17* gene expression. As in ovaries, the aromatase gene expression in testes was also not influenced by dietary DHA:EPA ratio, but this may be related to the fact that the aromatase mRNA was less abundant in the testis[57].

Regarding the difference between sexes in effective DHA:EPA ratio inducing the highest gene expression of steroidogenic proteins, the results were consistent with the steroid concentration results for some proteins such as *FSHR, StAR, P450c17* and *3β-HSD* in ovaries and *P450c17* and *3β-HSD* in testes; that is, the DHA:EPA ratio of 1·09 induced the highest gene expressions in ovaries, whereas the ratio of 2·05 induced the highest values in testes. This, to some extent, confirmed that males may require more DHA and less EPA than females for steroid synthesis. The difference in gonadal steroidogenesis between males and females may contribute to the sex-specific modulation of sex steroid-synthesising proteins by dietary fatty acids; however, very little information has been available about the difference in steroidogenesis between ovaries and testes of this fish. The commonly occuring sex reversal in juveniles of this fish makes this issue more complicated[58,59]. In this study, the gene expression of *StAR*, the rate-limiting transport protein translocating cholesterol from the outer mitochondrial

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membrane to the inner mitochondrial *P450scc* site, was regulated by experimental diets in females but not in males. This difference could be related to the fact that ovaries and testes may use cholesterol substrate supplied in different ways[60]. In mammals, for the steroid synthesis, the ovaries preferentially use cholesterol supplied from plasma lipoprotein via the scavenger receptor class B type I-mediated endocytic pathway, whereas the testicular Leydig cells rely heavily on the use of endogenously synthesised cholesterol[61–63]. In this regard, the difference in cholesterol substrate abundance between ovaries and testes may interfere with regulation of *StAR* by fatty acids. Contrary to *StAR*, the gene expression of *P450ssc* was regulated by diets in males but not in females. This may be related to the fact that the gene expression of *P450ssc* (*cyp11a1*) correlates better with testosterone production[56].

With respect to the tissue fatty acid compositions, besides DHA and EPA, ARA also differently accumulated in the females and males, as observed in our previous studies with this fish[27]. Males had much higher ARA contents than females in all tissues analysed. Male average *v.* female average was 6·0 *v.* 2·5 % (of TFA), 7·2 *v.* 5·6 % and 4·2 *v.* 2·5 % in gonad, liver and muscle, respectively. Several studies have indicated that ARA was metabolised differently in male and female fish and suggest that ARA may be more important for the reproductive success of males[40,64]. Higher *n*-6:*n*-3 PUFA ratios have also been shown to be more beneficial to male fish than to female fish[3,13,65].

Besides the LC-PUFA, oleic acid (C18 : 1*n*-9, OA) was another important fatty acid with different accumulation between female and male tongue sole. For all tissues, females had obvious high OA contents than males. Female average *v.* male average for OA was 22·1 *v.* 11·1 % (of TFA), 15·0 *v.* 11·6 % and 15·1 *v.* 14·0 % in gonad, liver and muscle, respectively. Gonad had higher OA content variation between sexes (content in ovary is two times that in testis) than liver and muscle, and on the other hand ovaries had the highest OA content among all the tissues. OA is a major energy source during egg and larval development[66]. Significant positive correlations between the OA content of fish eggs and egg viability or hatching percentages have been observed in gilthead seabream broodstock[67]. Therefore, the high OA accumulation in tongue sole ovaries must be a preparation for the energy demand in egg and larval development. Different from OA, palmitic acid (C16 : 0, PA), another important energy fatty acid, had no significant difference between sexes in the liver and muscle contents. However, testes had much higher PA content than ovaries (average 23·7 *v.* 15·9 % of TFA). *De novo* synthesis of PA has been observed in the liver of male European eel during spermatogenesis[17]. PA might have special physiological or energetic roles in the testosterone synthesis or spermatogenesis in male fish, but the precise mechanisms need to be elucidated by future studies.

In conclusion, the present results suggest that the dietary DHA:EPA ratio, possibly combined with the dietary EPA:ARA ratio, differentially regulated sex steroid hormone synthesis in mature female and male tongue soles. Females seemed to require more EPA but less DHA than males for gonadal steroidogenesis. The LC-PUFA accumulations in fish gonads and the response of gene expressions of sex steroid-synthesising proteins to dietary DHA:EPA confirmed the regulation of sex steroid hormone production by dietary DHA:EPA.

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H. X. and M. L. designed the study. H. X. analysed the data and wrote the manuscript. L. C. carried out the feeding trial and the real-time PCR. Y. W. and Y. Z. were involved in the real-time PCR and the fatty acid analysis. All the authors read and approved the final manuscript.

The authors declare that there are no conflicts of interest.

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