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Dietary arachidonic acid differentially regulates the gonadal steroidogenesis in the marine teleost, tongue sole (*Cynoglossus semilaevis*), depending on fish gender and maturation stage

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ABSTRACT

A 3-month feeding trial with tongue sole *Cynoglossus semilaevis* broodstock was conducted before and during the spawning season to investigate the effects of dietary arachidonic acid (ARA) on the production of sex steroid hormones and gonadal gene expression of key proteins in steroidogenesis. Three isonitrogenous and isolipidic experimental diets were formulated to contain different ARA levels: the control diet without ARA supplementation (C, 0.58% ARA of total fatty acids (TFA)) and two diets with low (5.14% of TFA, ARA-L) or high ARA (15.44% of TFA, ARA-H) supplementation. The diets were randomly assigned to 9 tanks of 3-year-old tongue sole (10 females and 15 males in each tank). Fish were reared in a flowing seawater system and fed to apparent satiation twice daily. At the end of the feeding trial, tissue samples from mature females (MF, with spontaneous ovulation), immature females (IMF, early vitellogenesis), and mature males (MM, expressing milt) were collected to assay the production of sex steroid hormones, gonadal gene expression of sex steroid-synthesizing proteins, as well as the fatty acid profiles of gonad, liver and muscle lipids. Results showed that ARA supplementation significantly reduced the estradiol production in females, but stimulated the testosterone production in males. ARA supplementation significantly reduced the mRNA expression of aromatase in ovaries but significantly increased the gene expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) in testes. In mature ovaries, diet ARA-L significantly reduced the gene expression of follicle stimulating hormone receptor (FSHR), 3β-HSD, and 17β-HSD; however, in immature ovaries, it significantly increased the gene expression of FSHR, steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (P450ssc), 3β-HSD, and 17β-HSD. In all gonads, 17α-hydroxylase (P450c17) responded to dietary ARA differently from other sex steroid-synthesizing proteins. ARA was preferentially accumulated in tongue sole gonad lipids. ARA concentrations were highest in gonad, liver and muscle lipids of MM fish and lowest in MF fish. Compared to female tongue sole, males had higher DHA concentrations in gonad lipids, but lower concentrations in liver lipids. In conclusion, results suggest dietary ARA regulates sex steroid hormone synthesis in tongue sole broodstock, and accumulates in gonad lipids, depending on both fish gender and maturation stage. Dietary ARA supplementation appears more important for male fish than for female fish, and more important for immature females than for mature females.
Statement of relevance: This study is beneficial to the broodstock diet formulation.

1. Introduction

Lipid and fatty acid compositions of broodstock diets have been identified as major dietary factors that determine successful reproduction and survival of offspring in fish (Izquierdo et al., 2001). The important roles of n-3 long chain-polyunsaturated fatty acids (LC-PUFA) in fish reproductive processes have been demonstrated in a number of studies (Harel et al., 1994; Cerdá et al., 1995; Abi-Ayad et al., 1997; Navas et al., 1997; Almansa et al., 2001; Watanabe and Vassallo-Agius, 2003; Rodríguez-Barreto et al., 2012; Beirão et al., 2015; Butts et al., 2015; Luo et al., 2015); however, the importance of n-6 LC-PUFA, primarily arachidonic acid (ARA), has been relatively neglected. In the past decade, the functions of ARA in fish reproduction have been gaining more and more attention and it has been shown that moderate levels of ARA in broodstock diets exert significant positive effects on the spawning performance, egg quality, and offspring quality of several fish species including European sea bass *Dicentrarchus labrax* (Bruce et al., 1999), Japanese flounder *Paralichthys olivaceus* (Furuita et al., 2003), Atlantic halibut *Hippoglossus hippoglossus* (Mazorra et al., 2003), and rice field eel *Monopterus albus* (Zhou et al., 2011). The

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analysis of fatty acid profiles in gonads and gametes, as well as the dynamics of fatty acids during fish maturation and embryogenesis, additionally suggests the importance of ARA in fish reproduction (Grigorakis et al., 2002; Salze et al., 2005; Rodríguez-Barreto et al., 2012; Johnson, 2012; Støttrup et al., 2013; Hauville et al., 2015).

To date, however, little information has been available on the mechanisms involved in the effects of dietary ARA on fish reproductive processes. The limited work suggests that dietary ARA modulates the oocyte maturation, ovulation, and the binding of sex steroids in plasma (Van Der Kraak and Biddiscombe, 1999; Sorbera et al., 2001; Patiño et al., 2003). Very few studies have been conducted to investigate the effects of dietary ARA on the synthesis of sex steroid hormones (Norambuena et al., 2013a), which is a very important process in fish reproduction. In mammals and birds, ARA has been shown to influence gonadal steroidogenesis (Johnson et al., 1991; Lopez-Ruiz et al., 1992). Some *in vitro* studies have demonstrated the modulation of testosterone synthesis by ARA, but the effects of ARA on estradiol synthesis is still lacking. Moreover, the *in vitro* studies have a limited ability to elucidate the real *in vivo* reproductive processes. Therefore, the present study was aimed to investigate the modulation of fish sex steroid hormone synthesis by dietary ARA through a broodstock feeding trial. At the same time, this study evaluates the effects of dietary ARA on the gene expression of key proteins involved in the key processes of sex steroid synthesis, i.e., the endocrine response to gonadotrophins, the delivery of cholesterol substrate, and biosynthetic reactions. These physiological processes play key roles in sex steroid synthesis, gonad maturation, and spawning performance; however, little information is available regarding their modulation by dietary nutrients.

This study was conducted on tongue sole (*Cynoglossus semilaevis*), an important aquaculture species in China. The demand for tongue sole has increased in the past decade; however, the unpredictable and variable reproductive performance of the species was a limiting factor to the successful mass production of juveniles. In recent years, our laboratory has investigated the variation in reproductive performance of this fish by altering dietary lipids (Xu et al., 2015) and fatty acid profile, including the dietary n-3/n-6 fatty acid ratio (Liang et al., 2014). As a following-up study, the present study is aimed at investigating the ability of dietary ARA, the primary n-6 LC-PUFA in fish gonads, to regulate sex steroid synthesis in tongue sole, as well as at elucidating the involved physiological processes. Along with evaluating changes in fatty acid profiles of gonad and other tissue lipids in response to experimental diets, this study provides needed information on the role of dietary fatty acids in tongue sole reproductive success.

2. Materials and methods

2.1. Experimental diets

Three isonitrogenous and isolipidic (55.5% crude protein and 13.0% crude lipid) experimental diets were formulated to contain different levels of ARA (Table 1). The control diet (C) was formulated using fish meal, casein, and wheat meal as protein sources, and soy lecithin, olive oil, and tristearin as lipid sources. An ARA enriched oil (ARA concentration, 41% of total fatty acids (TFA); in the form of triglyceride; Jiangsu Tiankai Biotechnology Co., Ltd., Nanjing, China) was supplemented to the control diet, replacing tristearin, to formulate two diets with low (ARA-L, with 1.43% (dry matter) ARA enriched oil) or high (ARA-H, with 4.43% ARA enriched oil) ARA content. A constant level of n-3 LC-PUFA enriched oil (containing 37% DHA and 21% EPA (of TFA); in the form of triglyceride; Hebei Haiyuan Health Biological Science and Technology Co., Ltd., Changzhou, China) was supplemented to all the diets to meet the n-3 LC-PUFA requirement. The diets were made, packed and stored following the common procedures in our laboratory (Xu et al., 2016). The fatty acid compositions of the experimental diets are presented in Table 2. The ARA content in C, ARA-L, and ARA-H was 0.58%, 5.14%, and 15.44% of TFA, respectively.

Table 1
Formulation and proximate composition of the experiment diets (g·kg^{-1} dry matter).

Ingredient	C	ARA-L	ARA-H
Fish meal	600.0	600.0	600.0
Casein	120.0	120.0	120.0
Wheat meal	156.2	156.2	156.2
Vitamin premix[1]	10.0	10.0	10.0
Mineral premix[2]	10.0	10.0	10.0
Monocalcium phosphate	10.0	10.0	10.0
Choline chloride	5.0	5.0	5.0
L-ascorbyl-2-polyphosphate	5.0	5.0	5.0
Mold inhibitor[3]	1.0	1.0	1.0
Ethoxyquin	0.5	0.5	0.5
Soy lecithin	15.0	15.0	15.0
Olive oil	10.0	10.0	10.0
n-3 LC-PUFA enriched oil[4]	13.0	13.0	13.0
ARA enriched oil[5]	0.0	14.3	44.3
Tristearin[6]	44.3	30.0	0.0
Proximate composition			
Crude protein	558.1	556.4	559.0
Crude lipid	128.7	129.8	130.1
Ash	127.9	128.5	129.3

[1] 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; alpha-tocopherol, 120 mg; wheat middling, 13.67 g.
[2] Mineral premix (mg or g/kg diet): $MgSO_4 \cdot 7H_2O$, 1200 mg; $CuSO_4 \cdot 5H_2O$, 10 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $FeSO_4 \cdot H_2O$, 80 mg; $MnSO_4 \cdot H_2O$, 45 mg; $CoCl_2 \cdot 6H_2O$ (1%), 50 mg; $NaSeSO_3 \cdot 5H_2O$ (1%), 20 mg; $Ca(IO_3)_2 \cdot 6H_2O$ (1%), 60 mg; zoelite, 13.485 g.
[3] Mold inhibitor: contained 50% calcium propionic acid and 50% fumaric acid.
[4] n − 3 LC-PUFA enriched oil: containing 37% DHA and 21% EPA (of total fatty acids); in the form of triglyceride; Hebei Haiyuan Health Biological Science and Technology Co., Ltd., Changzhou, China.
[5] ARA enriched oil: containing 41% ARA (of total fatty acids); in the form of triglyceride; Jiangsu Tiankai Biotechnology Co., Ltd., Nanjing, China.
[6] Tristearin: HUDONG Daily Chemicals Co., Ltd., Jiaxing, China.

2.2. Experimental fish and feeding procedure

Three-year-old tongue sole *Cynoglossus semilaevis* broodstock, which have been reared with formulated feeds from the early juvenile stage, were used in the present study. Prior to the start of the feeding trial, experimental fish were reared in concrete tanks (25 m^3) and fed the control diet for 7 days to acclimate to the experimental conditions. At the onset of the feeding trial, experimental fish were distributed into 9 polyethylene tanks (diameter: 230 cm, height: 100 cm) and each diet was randomly assigned to triplicate tanks. Each tank had 25 fish (10 female and 15 male) and the tanks were supplied with flowing filtered seawater at a rate of 50 L min^{-1}. Fish were hand-fed to apparent

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

Fatty acid	C	ARA-L	ARA-H
C14:0	3.5	3.5	3.1
C16:0	24.8	22.0	17.4
C18:0	25.1	19.2	6.8
∑SFA	53.4	44.6	27.3
C16:1n-7	2.4	2.7	2.7
C18:1n-9	10.9	12.3	14.7
C20:1n-9	0.5	0.5	0.6
∑MUFA	13.8	15.5	18.0
C18:2n-6	5.2	6.0	7.9
C20:4n-6	0.6	5.1	15.4
∑n-6 PUFA	5.8	11.1	23.4
C18:3n-3	0.8	0.7	0.8
C20:5n-3	3.8	4.3	4.1
C22:6n-3	8.8	8.7	8.9
∑n-3 PUFA	13.3	13.7	13.8
∑n-3/∑n-6	2.3	1.2	0.6

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid.