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Dietary fish oil replacement by soybean oil: Effect on plasma vitellogenin, sex steroids and ovarian steroidogenesis in Chinese strip-necked turtles (*Mauremys sinensis*)

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Abstract

In order to investigate the effects of dietary fish oil replacement, the turtles (*Mauremys sinensis*) were fed four experimental diets for 10 months: FO (100% fish oil), FSO (70% fish oil and 30% soybean oil), SFO (30% fish oil and 70% soybean oil) and SO (100% soybean oil), sampled at pre-vitellogenesis, vitellogenesis and post-vitellogenesis. The results showed that plasma gonadotropin-releasing hormone (GnRH) levels were the highest at pre-vitellogenesis, which promoted the secretion of gonadotropin and sex steroids. Therefore, plasma luteinizing hormone (LH) and estrogen (E_2) levels were significantly increased at post-vitellogenesis ($p < 0.05$), while follicle-stimulating hormone (FSH) levels increased at vitellogenesis ($p < 0.05$). The FO and FSO groups had significantly higher GnRH and E_2 levels than the other two groups ($p < 0.05$). In addition, plasma vitellogenin (Vtg) levels significantly increased at vitellogenesis and post-vitellogenesis ($p < 0.05$), which were significantly higher in the groups of FO and FSO than SO ($p < 0.05$). Moreover, the expression levels of hepatic estrogen receptor α (Erα) mRNA were significantly increased at vitellogenesis and post-vitellogenesis while ovarian Cyp19α1α mRNA were significantly increased at post-vitellogenesis ($p < 0.05$), and both were the lowest in SO. Taken together, the replacement of fish oil with 66.7% soybean oil is feasible.

KEYWORDS
Chinese strip-necked turtle, fish oil, soybean oil, steroidogenesis, vitellogenin

1 | INTRODUCTION

Continual development of the aquafeed industry increased the demand for fish oil and has caused great pressure on fishery resources (Levavisivan, Biran, & Fireman, 2006). Soybean oil has garnered perhaps the most attention because of its widespread availability, competitive prices and generally favourable nutrient composition (Hardy, Dabrowski, & Hardy, 2010). However, high dietary soybean oil, mainly due to the high content of linoleic acid (18:2n-6) and the lack of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Nutrition, 1993), reduced reproduction in fish (Ling, Kuah, Tengku Muhammad, Kolkovski, & Shu-chien, 2006; Peng et al., 2015; Zhang, Li, Zhu, & Deng, 2013). The lipid composition in broodstock diets can affect vitellogenesis, as production of vitellogenin by the liver requires a supply of long-chain fatty acids (March, 1993). Fatty acids, especially PUFAs, as the precursors for prostaglandin synthesis, can modulate the expression patterns of many key enzymes involved in both prostaglandin and steroid metabolism (Murdoch, Hansen, & Mcpherson, 1993). Notably, there is presently a lack of data on the effect of

alternative lipid sources on the vitellogenesis of freshwater turtle species.

More studies on fish reproduction have revealed that n-3 PUFAs and their metabolites, which are rich in fish oil produced from cyclooxygenase and lipoxygenase, have modulatory effects on steroid metabolism (Nunez, Haourigui, Martin, & Benassayag, 1995), which in turn control ovulation, steroidogenesis (Sorbera, Asturiano, Carrillo, & Zanuy, 2001), and fecundity rates (Bogevik et al., 2014; Zhang et al., 2013). Gonadotropin-releasing hormone (GnRH) was directly affected by dietary fatty acid (Olsen, Dragnes, Myklebust, & Ringø, 2003). The primary role of GnRH after its release from the hypothalamus is to stimulate the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary, thus regulating the hypothalamic-pituitary-gonadal axis. In turn, stimulation of this axis mainly leads to gametogenesis and steroid hormone production in the gonads (Okubo & Nagahama, 2008). In females, FSH stimulates aromatase expression in follicle granulosa cells, which increases estrogen (E_2) production and follicular growth. E_2 stimulates hepatic vitellogenin (Vtg) synthesis, which is essential in oocyte development (Lubzens, Young, Bobe, & Cerdà, 2010). Sex steroid hormones play important roles in vertebrate reproduction. In several teleost species, E_2 and T, which are produced in gonadal tissues under the control of GtH, are essential in gametogenesis (Nagahama & Yamashita, 2008). Only a few studies have investigated the effects of dietary PUFAs on hormone synthesis during gonadogenesis in turtles (Custodia-Lora, Novillo, & Callard, 2004; Pakdel et al., 1997).

The Chinese strip-necked turtle (*Mauremys sinensis*) is a novel aquaculture species in China because of its high medicinal and edible values. In spite of important technical developments and intense research in turtles, some bottlenecks still limit the expansion of its industry (Qiu, Sheng, Hong, & Shi, 2015). The information on nutritional requirements of this species is very sparse, although it is essential to develop a commercial diet for this important aquaculture species. Additionally, there is no information available on the use of alternative lipid sources in the practical diets of this important turtle species. In the present study, the effect of replacement of FO with SO on ovarian steroidogenesis was investigated by analysing the plasma levels of hormones (including GnRH, FSH, LH and E_2) in the HPG axis and plasma Vtg levels in Chinese strip-necked turtles. In addition, to understand the molecular mechanism of ovarian development, we measured the gene expression levels of ovarian Cyp19α1α and hepatic Erα (estrogen receptor alpha) following replacement of FO with SO in the diet.

2 | MATERIALS AND METHODS

2.1 | Experimental diets

Four iso-nitrogenous, iso-energetic and iso-lipidic (approximately 45% crude protein and 8% crude fat) experimental diets were formulated (Table 1): (a) 100% fish oil (FO), (b) 66.7% fish oil and 33.3% soybean oil (FSO), (c) 33.3% fish oil and 67.7% soybean oil (SFO), and (d) 100% soybean oil (SO). Fish meal and soybean meal were used as protein sources; fish oil and soybean oil were used as lipid sources. The ratio of n-3/n-6 LC-PUFA concentrations of groups FO, FSO, SFO and SO were 3.18, 1.95, 1.25 and 1.04 respectively. All ingredients were thoroughly mixed and mechanically extruded in 2-mm pellets. The pellets were oven-dried at 25°C to obtain a moisture level of approximately 100 g × kg^{-1} and stored in airtight plastic bags at −20°C.

2.2 | Experimental design

Female Chinese strip-necked turtles (4 year of age, body weight 1557 ± 307 g) were bought from Hongwang Agricultural Farming Co. (Wenchang, Hainan Province, China) and randomly divided into four groups according to the proportion of fish oil (F) and soybean oil (S) of the diets, with 18 turtles per 2 m^3 pool, and triplicate pools were allocated to each dietary treatment. Turtles were fed two times per week (because of low metabolism in turtles), and the experiment lasted for 10 months (from early May to February). Water quality parameters, temperature, pH, alkalinity, NH_4^+ ranged from 20–27°C, 7.2–7.4, 103–115 mg/L and 0.018-0.025 mg/L, respectively, and there were no significant influences of dietary treatments on these parameters.

TABLE 1 Ingredients and proximate compositions of the experimental diets

	Content (%)			
Ingredient	**FO**	**FSO**	**SFO**	**SO**
Steamed fish meal	54	54	54	54
Soybean meal	17	17	17	17
Wheat middling	23	23	23	23
Gluten	2	2	2	2
CaH_2PO_4	1.5	1.5	1.5	1.5
Choline	0.25	0.25	0.25	0.25
Multi-vitamin[a]	0.15	0.15	0.15	0.15
Vc	0.1	0.1	0.1	0.1
Multi-mineral[b]	1	1	1	1
Fish oil	1	0.67	0.33	0
Soybean oil	0	0.33	0.67	1
Total	100	100	100	100
Nutrient levels				
Crude protein	44.28	44.36	44.25	44.31
Crude fat	8.16	8.34	8.29	8.21
n-3 PUFA	2.05	2.14	1.77	1.37
n-6 PUFA	0.65	1.10	1.41	1.31
n-3/n-6	3.18	1.95	1.25	1.04

[a]Multi-vitamin (kg): VA 10,000,000 IU, VB_1 20 mg, VB_2 25 mg, VB_6 12 mg, VB_{12} 100 mg, VD_3 3,750,000 IU, VE 50 IU, VK_3 12 mg, biotin 10 mg, folic acid 2.5 mg, *D*-pantothenic acid 40 mg, nicotinic acid 80 mg.
[b]Multi-mineral (kg): K 90 g, Mg 27 g, Cu 750 mg, Fe 13.5 mg, Mn 1.2 g, Zn 38.5 g, I 120 mg, Se 50 mg, and Co 100 mg.

2.3 | Sample collection

Turtles from each treatment were sampled in July and November 2014 and February 2015 to evaluate pre-vitellogenesis, vitellogenesis and post-vitellogenesis respectively (according to our preliminary study on the histological structure of ovary). Six turtles per replicate were anaesthetized at −20°C crymoanesthesia for 0.5–1 h after starvation for 24 h. Blood was collected by carotid puncture using pre-heparinized syringes fitted with 5 ml needles and centrifuged at 1 2000 *g* for 5 min at room temperature. Plasma was stored at −20°C for the measurement of GnRH, FSH, LH, E_2 and Vtg levels. Ovary and liver samples of two turtles per replicate were quickly removed and frozen immediately in liquid nitrogen and stored at −80°C for RNA extraction.

2.4 | Biochemical analyses

2.4.1 | GnRH, FSH, LH, E_2 and Vtg levels

Plasma levels of GnRH, FSH, LH, E_2 and Vtg were measured with an ELISA (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, 40 μl of a plasma sample, 10 μl of biotinylated anti-antibodies (anti-GnRH, FSH, LH, E_2 and Vtg) and 50 μl of streptavidin-HRP were added to each well, mixed gently and incubated at 37°C for 1 h. After incubation, the supernatant was discarded and each well was washed five times with washing buffer. Then, 50 μl of solutions A and B were added in this sequence to each well, mixed gently and incubated at 37°C in the dark for 10–15 min. To stop the reaction, 50 μl of stop buffer was applied to each well. Plates were read at 450 nm by a microplate reader (Spectramax M5 Molecular Devices, USA) within 15 min. The standard curve was established according to the manufacturer's instructions (Peng et al., 2015).

2.4.2 | Cyp19α1α and Erα mRNA levels

Total RNA was isolated from hepatic and ovarian tissues with Trizol reagent (Invitrogen, USA) following the manufacturer's protocol and was treated with RNase free DNase I (Fermentas, Lithuania, EU) to remove genomic DNA. The integrity of total RNA was checked by observing the band intensity of 28S RNA and 18S RNA on 2.0% agarose gels. The purity and concentration of total RNA were measured with a Nanodrop spectrophotometer (Thermo Scientific). First-strand cDNAs were synthesized from 1 μg of total RNA using a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's protocol. Table 2 shows the primers used for quantitative real-time RT-PCR (qRT-PCR). The feasibility of qRT-PCR systems were verified before the target genes were quantitated. The melting curve, amplification curve and standard curve of each gene were gained by ABI QuantStudio™ Real-Time PCR software. Reactions were carried out in a 20 μl volume containing 1 × SYBR Premix EX Taq™ II (Takara, Dalian, China), sense and anti-sense primers (0.4 μM) and target cDNA (4 ng). Amplification of Cyp19α1α and Erα was performed in a Step-One Plus Real-Time PCR System (ABI, USA) with the following conditions, 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, and 60°C for 30 s. The relative quantification of gene expression was analysed with the two-ddCt methods.

2.5 | Statistical analysis

Data are presented as the mean ± SE. Differences between the different dietary groups at the same stage of vitellogenesis and differences between the different vitellogenic stages within the same diet group were tested using a one-way analysis of variance (ANOVA) followed by Turkey's multiple comparison (SPSS Inc., Chicago, IL, USA) respectively. A value of $p < 0.05$ was considered a significant difference.

3 | RESULTS

3.1 | Plasma gonadotrophin releasing hormone (GnRH) levels

From Figure 1, we can see that plasma GnRH levels continuously decreased during vitellogenesis in all four groups. Plasma GnRH levels at post-vitellogenesis were significantly lower than those pre-vitellogenesis or at vitellogenesis ($p < 0.05$), with no significant differences in plasma GnRH levels between the pre-vitellogenesis and vitellogenesis stages. During the three periods, plasma GnRH levels in the FO and FSO groups were significantly higher than the other groups, and there were no significant differences between the FO and FSO groups or the SFO and SO groups.

3.2 | Plasma FSH and LH levels

Within the same dietary group, plasma FSH levels in the FO and SO groups at vitellogenesis were the highest in the three vitellogenic stages, however, those in FSO and SFO groups at vitellogenesis were not significantly different from those at pre-vitellogenesis ($p < 0.05$). At pre-vitellogenesis, there were no significant differences in plasma FSH levels among these four dietary groups, while at post-vitellogenesis, the FO group were significantly higher than the SO group ($p < 0.05$) (Figure 2).

Plasma LH levels continuously increased during vitellogenic stages and were significantly increased at post-vitellogenesis ($p < 0.05$). At pre-vitellogenesis, there were no significant differences in plasma LH levels among these four dietary groups. However, at vitellogenesis, plasma LH levels in the FO and FSO groups were significantly higher than those in the other groups, while at post-vitellogenesis, the FO group was significantly higher than the SO group ($p < 0.05$).

3.3 | Plasma E_2 level

During the vitellogenic stages within the same dietary group, plasma E_2 levels continuously increased (Figure 3). Furthermore, the levels were the highest in the stage of post-vitellogenesis ($p < 0.05$). Among these four dietary groups, the levels in the FO and FSO

TABLE 2 Primer sequences for PCR

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product (bp)
β-actin	GCACCCTGTGCTGCTTACA	CACAGTGTGGGTGACACCAT	190
Erα	TCAATGGAACACCCAGGAAAAC	CATCATACGGAACCGAGCAGAA	132
Cyp19α1α	CAGGATGATGTAATTGATGGC	TGAAAGTAGCGAGAAGGCAC	149

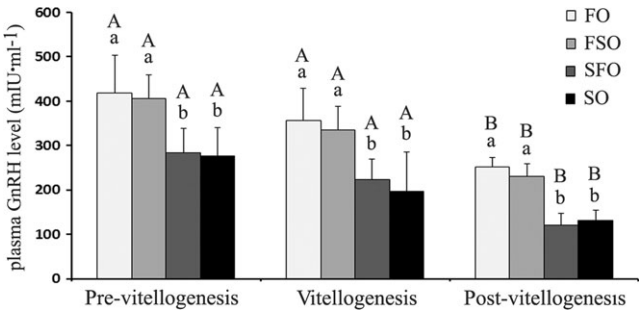

FIGURE 1 Plasma GnRH level during vitellogenesis in Chinese stripe-necked turtles fed different experimental diets. Different uppercase letters represent significant differences among vitellogenic stages within the same diet group, and different lowercase letters represent significant differences among diet groups at the same vitellogenic stage ($p < 0.05$)

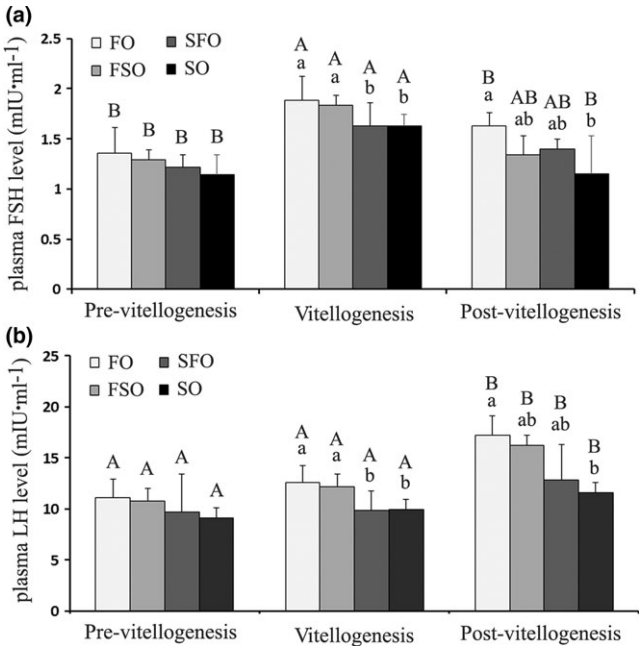

FIGURE 2 Plasma FSH and LH levels during vitellogenesis in Chinese stripe-necked turtles fed different experimental diets. (a) Plasma FSH. (b) LH levels. Different uppercase letters represent significant differences among vitellogenic stages within the same diet group, and different lowercase letters represent significant differences among diet groups at the same vitellogenic stage ($p < 0.05$)

groups were significantly higher than those in the other groups during the vitellogenic stages, and there were no significant differences between the FO and FSO groups or between the SFO and SO groups.

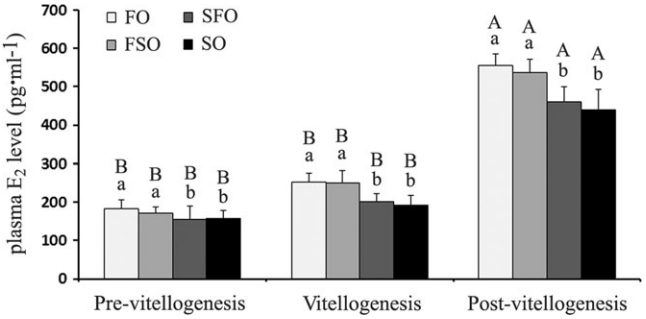

FIGURE 3 Plasma E2 levels during vitellogenesis in Chinese stripe-necked turtles fed different experimental diets. Different uppercase letters represent significant differences among vitellogenic stages within the same diet group, and different lowercase letters represent significant differences among diet groups at the same vitellogenic stage ($p < 0.05$)

3.4 | Plasma Vtg levels

During the vitellogenic stages within the same dietary group, plasma Vtg levels continuously increased (Figure 4), especially in the FO and FSO groups. However, in the SFO and SO groups, there were no significant differences at vitellogenesis and post-vitellogenesis.

At pre-vitellogenesis, plasma Vtg levels were the lowest in the SO group. At vitellogenesis and post-vitellogenesis, plasma Vtg levels in the FO and FSO groups were significantly higher than the SO group.

3.5 | *Cyp19a1a* and *Era* mRNA levels

The correlation coefficient of standard curves were greater than 0.99, and all genes amplification efficiency were 98%–99%. Combined with the melting curve and amplification curve, the established method of qRT-PCR were sensitivity and accuracy (data not shown). The expression levels of ovarian Cyp19α1α and hepatic Erα mRNA levels are shown in Figure 5. In these four dietary groups, the expression levels of Cyp19α1α mRNA were significantly increased by ovarian development ($p < 0.05$). There were no significant differences in the expression levels of Cyp19α1α mRNA between pre-vitellogenesis and vitellogenesis. At vitellogenesis and post-vitellogenesis, those in the FO and FSO groups were significantly higher than the other groups ($p < 0.05$). At pre-vitellogenesis, the FO and FSO groups were significantly higher than the SO group ($p < 0.05$).

The change of the expression levels of hepatic Erα mRNA were the same as that in ovarian Cyp19α1α. At pre-vitellogenesis, there were no significant differences in the expression levels of Erα mRNA. However, at vitellogenesis and post-vitellogenesis, the levels

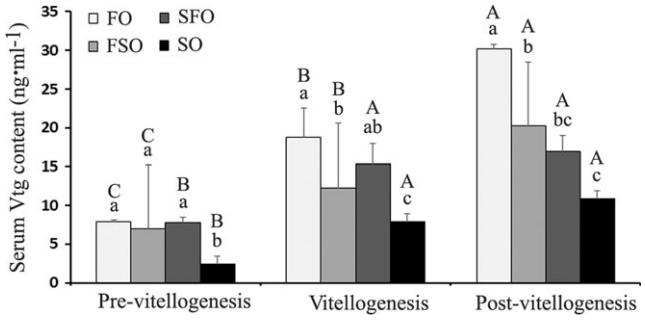

FIGURE 4 Plasma Vtg levels during vitellogenesis in Chinese stripe-necked turtles fed different experimental diets. Different uppercase letters represent significant differences among vitellogenic stages within the same diet group; different lowercase letters represent significant differences among diet groups at the same vitellogenic stage ($p < 0.05$)

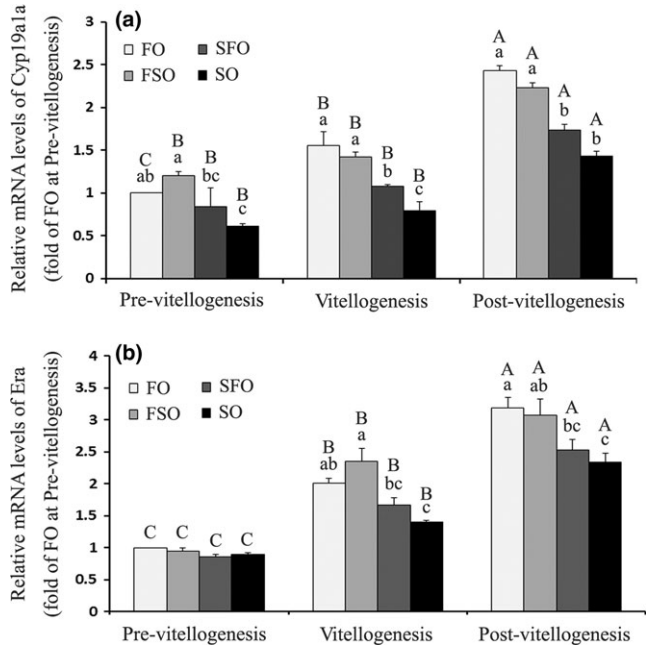

FIGURE 5 Effect of dietary n-3 LC-PUFA on ovarian Cyp19a1a (a) and hepatic Era (b) expression levels in Chinese stripe-necked turtles during vitellogenesis. Different uppercase letters represent significant differences among vitellogenic stages within the same diet group; different lowercase letters represent significant differences among diet groups at the same vitellogenic stage ($p < 0.05$)

in the FO and FSO groups were significantly higher than the SO group ($p < 0.05$).

4 | DISCUSSION

4.1 | Hypothalamic-pituitary-gonadal (HPG) axis

The HPG axis refers to the hypothalamus, pituitary gland, and gonadal glands that adjust and affect each other. The axis constitutes an integral and intercoordinated endocrinium, which controls development, reproduction and ageing in vertebrates (Villeneuve et al., 2007). The anterior portion of the pituitary gland produces LH and FSH, and the gonads produce estrogen and testosterone (Burns & Matzuk, 2002). In females, FSH and LH act primarily to activate the ovaries to produce estrogen and regulate the ovarian cycle, however, gonadal steroid hormones (such as E_2 and T) modulate LH and FSH levels via feedback to the anterior pituitary, as well as to the hypothalamus (Millar et al., 2004). In mice lacking GnRH or GnRH receptors, FSH levels, as well as LH levels, are 60%–90% lower in males and females (Wu, Wilson, Busby, Isaac, & Sherwood, 2010). GnRH injection in rats with low endogenous GnRH increased FSH transcription 4-fold (Dalkin et al., 2001). Tsai and Licht (Tsai & Licht, 1993) pointed out that injection of GnRH could cause an increase in LH levels in red-eared slider turtles (*Trachemys scripta elegans*), while a low concentration of GnRH could cause a change in FSH levels. In our study, the levels of plasma GnRH were the highest at pre-vitellogenesis, which could activate the secretion of LH and FSH. Therefore, plasma FSH and LH significantly increased in the four dietary groups at vitellogenesis and post-vitellogenesis, and similar results were obtained with plasma E_2 which were positively correlated with ovarian growth and development (Oppen-Berntsen et al., 1994; Taranger et al., 1999). However, with the increase in plasma E_2 levels, the levels of plasma GnRH decreased because estrogen forms a negative feedback loop by inhibiting the production of GnRH in the hypothalamus. Previous reports have shown that E_2 regulates gonadotropin secretion and exerts either positive or negative feedback effects on FSH levels at different developmental stages (Larsen & Swanson, 1997; Leaver et al., 2008). In vitellogenic rainbow trout and sea bass, E_2 leads to lower plasma FSH levels (Mateos, Mañanos, Carrillo, & Zanuy, 2002; Saligaut et al., 1998). In this study, the reduction in plasma FSH levels at post-vitellogenesis was attributed to the negative feedback effect of E_2. Among the three vitellogenic periods, plasma GnRH levels in the FO and FSO groups were significantly higher than the other groups, suggesting that a long-term dietary deficiency in n-3 LC-PUFAs reduces steroid production (Navas et al., 1998). Nevertheless, the SO group had lower plasma FSH, LH, and E_2 levels than the FO and FSO groups, which coincided with the research on fishes such as sea bass (Peng et al., 2015).

4.2 | Vtg synthesis

In oviparous organisms, the HPG axis is commonly referred to as the hypothalamus-pituitary-gonadal-liver axis (HPGL-axis) in females. Many egg-yolk and chorionic proteins are synthesized heterologously in the liver and are necessary for oocyte growth and development. The major precursor of yolk proteins is Vtg, a phospho-lipoprotein synthesized in the liver under the influence of gonadotropin-induced ovarian estrogen, primarily17β-estrdiol (E_2). It is transported via blood to the ovary (Hiramatsu et al., 2002). In this study, the increase in plasma Vtg levels during vitellogenesis was accompanied by high plasma E_2 levels, consistent with studies on sea bass and

silver pomfret (Mañanós, Zanuy, & Carrillo, 1997; Navas et al., 1998; Peng et al., 2015). This study also revealed that Vtg levels in the FO and FSO groups were significantly higher than those in the SO group at vitellogenic and post-vitellogenic stages, implying that the rate of hepatic Vtg synthesis decreased when dietary fish oil was replaced with 100% soybean oil, which may lead to the deficiency of dietary n-3 PUFA. Previous studies have shown that dietary n-3 LC-PUFA was associated with the synthesis of Vtg (Navas et al., 1998; Peng et al., 2015; Zhang et al., 2013).

4.3 | Gene expression of ovarian *Cyp19α1α* and hepatic *Erα*

In response to GtH (FSH and LH), cholesterol is converted into T and E_2, which is catalysed by the enzyme encoded by *Cyp19α1α* (Anderson, Swanson, Pankhurst, King, & Elizur, 2012; Lubzens et al., 2010). Therefore, Cyp19α1α expression is critical in maintaining appropriate T/E_2 ratios (Cheshenko, Pakdel, Segner, Kah, & Eggen, 2008). In this study, Cyp19α1α expression levels significantly increased at post-vitellogenesis, consistent with the plasma E_2 levels. The actions of estrogens are mediated by estrogen receptors (Erα and Erβ), which belong to the nuclear receptor (NR) superfamily (Cheng et al., 2015). In the wildlife females of a newt and in males of tilapia, hepatic Erα may be primarily involved in the induction of Vtg synthesis (Davis et al., 2008; Ko et al., 2008). The ability of E_2 to induce hepatic expression of its own receptors is documented in a turtle (Custodia-Lora et al., 2004; Pakdel et al., 1997). The expression of the Vtg gene is up-regulated by E_2 and is parallel to the increase in ER expression in the liver (Davis et al., 2007; Flouriot, Pakdel, & Valotaire, 1996; Menuet et al., 2004; Pakdel, Féon, Le, Le, & Valotaire, 1991). In our study, the expression of hepatic Erα increased significantly, which is consistent with the synthesis of Vtg. However, the mechanism underlying the association between dietary fatty acids and hepatic Erα expression levels is not well understood.

5 | CONCLUSION

Hormone synthesis in the HPG axis during gonadogenesis in Chinese strip-necked turtles was as follows: the plasma GnRH level was the highest in the period of pre-vitellogenesis, and then plasma FSH , LH and E_2 levels were significantly increased at vitellogenesis or post-vitellogenesis. However, with the increase in plasma E_2 levels, the levels of plasma GnRH and FSH were decreased because estrogen forms a negative feedback loop by inhibiting the production of GnRH in the hypothalamus. The replacement of FO with SO retards ovarian development in Chinese strip-necked turtles by lowering plasma FSH and LH levels and by decreasing E_2 production and hepatic Vtg synthesis accompanied by gene expression of ovarian Cyp19α1α and hepatic Erα. However, no significant differences were found in the FSO and SFO groups, which indicates that the replacement of fish oil with 66.7% soybean oil in Chinese strip-necked turtles is feasible.

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CONFLICT OF INTEREST

There is no conflict of interest of any authors in relation to the submission.

AUTHOR CONTRIBUTIONS

MH, CS and LD designed the experiments and interpreted the data and wrote the article. CS, NL and WL performed the experiments with assistance and advice from HS. DW and HS revised the manuscript. All authors have read the manuscript and approved submitting it to your journal.

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