Evidences for involvement of growth hormone and insulin-like growth factor in ovarian development of starry flounder (*Platichthys stellatus*)

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Abstract Although gonadotrophins are major regulators of ovarian function in teleosts and other vertebrates, accumulating evidence indicates that the growth hormone (GH)-insulin-like growth factor (IGF) axis also plays an important role in fish reproduction. As a first step to understand the physiological role of the GH-IGF system in the ovarian development of starry flounder (*Platichthys stellatus*), the expression profiles of GH and IGF messenger RNAs (mRNAs) and plasma GH, IGF-I, estradiol-17β (E2), and testosterone (T) levels during the ovarian development were investigated. The developmental stages of ovaries were divided into five stages (II, III, IV, V, and VI) by histological analysis. The hepatosomatic index (HSI) and gonadosomatic index (GSI) values increased and peaked at stage IV and stage V, respectively, and then declined at stage VI. Pituitary GH mRNA levels decreased sharply at stage III and raised to top level at stage VI. The hepatic IGF-I mRNA levels ascended to maximum value at stage V and then declined significantly at stage VI. However, the hepatic IGF-II mRNA levels remained stable and increased significantly at stage VI. In contrast, the ovarian IGF-I mRNA levels increased gradually and peaked at stage VI. The ovarian IGF-II mRNA levels were initially stable and increased significantly at stage V until the top level at stage VI. Consistent with the pituitary GH mRNA levels, plasma GH levels reduced sharply at stage III and remained depressed until stage V and then raised remarkably at stage VI. Plasma IGF-I level peaked at stage V and then declined to initial level. Plasma E2 level peaked at stage IV and then dramatically descended to the basal level. Plasma T level peaked at stage V and then declined significantly back to the basal level. Based on statistical analysis, significant positive correlations between hepatic IGF-I mRNA and GSI, ovarian IGF-II mRNA and hepatic IGF-II mRNA, ovarian IGF-I mRNA and ovarian IGF-II mRNA, and plasma IGF-I and plasma T were observed, respectively. These results suggest that the GH-IGF system may be involved in the ovarian development of starry flounder; GH and IGFs appear to play distinct roles in the regulation of the ovarian development in paracrine/autocrine manners. These findings extend our knowledge of the roles of the GH-IGF axis on reproduction regulation in fish.

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Introduction

It is well known that the growth of vertebrates including fish is primarily controlled by the growth hormone (GH)-insulin-like growth factor (IGF) system. The GH-IGF system of fish is particularly complex due to genome duplication in their evolution, resulting in multiple forms of GH, GH receptor (GHR), and IGF (Reindl and Sheridan 2012; Wang et al. 2015). The classical view of the GH-IGF system holds that GH released from pituitary gland stimulates synthesis and secretion of IGF-I principally from the liver through binding to GHRs and IGF-I, in turn, stimulates cell growth and differentiation in a variety of target tissues via distinct IGF receptors (Reindl and Sheridan 2012; Wood et al. 2005). The regulation of growth has focused on the production and release of GH from pituitary gland, and the intracellular signaling mechanisms mediating multifactorial neuroendocrine control of GH secretion have been well demonstrated (Canosa et al. 2007; Chang et al. 2012; Gahete et al. 2009; Wang et al. 2014). Moreover, the IGF system clearly plays an essential role in the growth regulation of vertebrates (Beckman 2011; Moriyama et al. 2000; Wood et al. 2005).

In addition to being involved in growth regulation, there is accumulating evidence that the GH-IGF system also plays an important role in fish reproduction (Bevers and Izadyar 2002; Hull and Harvey 2014; Reinecke 2010; Silva et al. 2009; Wood et al. 2005). Specific localization of GHR messenger RNA (mRNA) in tilapia ovary was identified, and the GHR mRNA level was significantly greater in the immature oocytes, as compared with matured oocytes (Kajimura et al. 2004). Similarly, the localization of IGF-I receptor was reported in ovarian follicle of gilthead sea bream (Perrot et al. 2000) and sterlet (Wuertz et al. 2007). Moreover, expression of IGF-I receptor in the ovaries of sterlet elevated at the onset of vitellogenesis (Wuertz et al. 2007). Both IGF-I mRNA and IGF-I immunoreactivity were demonstrated to be present in the ovary of several fish species, including tilapia (Berishvili et al. 2006; Schmid et al. 1999), sea bream (Kagawa et al. 1995; Perrot et al. 2000), and sterlet (Wuertz et al. 2007). In addition, IGF-II mRNA and IGF-II immunoreactivity occurred only in granulosa cells of late follicle stages in tilapia (Schmid et al. 1999). Recently, a novel IGF subtype, IGF-III, was discovered in fish and the expression of IGF-III mRNA

was gonad specific (Wang et al. 2008). Further investigations showed that IGF-III was involved in gonadal development (Baroiller et al. 2014; Berishvili et al. 2010) and oocyte maturation (Li et al. 2015; Li et al. 2011). Moreover, GH could stimulate ovarian steroidogenesis by increasing the aromatase activity in spotted seatrout (Singh and Thomas 1993) and GH had a direct modulatory effect on gonadotropin-stimulated steroid production in goldfish (Van der Kraak et al. 1990). On the other hand, IGF-I also played a role in regulation of ovarian steroidogenesis in coho salmon (Maestro et al. 1997) and white perch (Weber et al. 2007), and both IGF-I and IGF-II induced oocyte maturation in red sea bream (Kagawa et al. 1994) and southern flounder (Picha et al. 2012). In addition, IGF-I and IGF-II increased germinal vesicle breakdown (GVBD), a marker for resumption of meiosis, in oocytes of striped bass (Weber and Sullivan 2000) and southern flounder (Picha et al. 2012). Collectively, the previously mentioned studies indicate that GH-IGF axis appears to play a crucial role in the formation, differentiation, and function of fish ovary (Lubzens et al. 2010).

Starry flounder (*Platichthys stellatus*), which belongs to the order Pleuronectiformes, is an economically important marine fish species and a popular table fish in East Asia due to its tasty flesh. In addition, starry flounder features an asynchronous ovarian development, and oocytes at different developmental stages are simultaneously present in a single ovary, thus providing a unique model for reproductive biology study. In our previous studies, the full-length complementary DNAs (cDNAs) encoding GH, IGF-I, and IGF-II from starry flounder were isolated and their spatial expression pattern in adult organs and temporal expression profile throughout embryonic development were examined (Xu et al. 2015; Zang et al. 2014). However, the mode of actions of GH-IGF axis in the ovarian development in this species remains unknown. Moreover, the natural spawning of this species still cannot be attained under artificial conditions; the underlying mechanisms for gonadal growth and reproduction should be clarified urgently, which is beneficial for sustainable development of farming industry. Accordingly, the aims of the present study were (1) to characterize the ovarian development of starry flounder and (2) to examine the changes of GH and IGF mRNAs along with their peptides during the ovarian development, as the first step to understand the role of the GH-IGF axis in the reproduction regulation of starry flounder.

Materials and methods

Experimental fish

All of the animal experiments were approved by the Animal Care and Use Committee of Chinese Academy of Fishery Sciences. Sexually matured female starry flounder (total length [TL] = 34.1 ± 0.5 cm and body weight [BW] = 868.4 ± 49.7 g) were sampled from a local flatfish hatchery in Qingdao, China. The fish were reared in an indoor concrete tank (25 m^3 in volume) supplied with flow-through seawater (water exchange rate 400 % per day; salinity 27–31 ppt; pH 7.8–8.2). The seawater was sand filtered and decanted prior to tank supply. The fish were fed to satiation twice daily with a commercial dry diet (Shengsuo Aquafeed Co., Ltd., Yantai, China) at a rate of 2–3 % of BW. All of the fish were exposed to natural photothermal regimes, and water temperature ranged from 10 to 26 °C as seasons changed.

Histological analysis of ovarian development

Four females were sampled at monthly intervals between January 2013 and August 2013. All fish were anesthetized with MS222 (180 mg/L) and measured, blood was drawn from the caudal vein, and the plasma obtained by centrifugation at 10,000 rpm (4 °C) for 5 min and stored at −30 °C until analysis. Following being sacrificed by decapitation, the ovaries and livers were removed and weighed to calculate the gonadosomatic index (GSI = [gonad weight / (BW − Viscera weight)] × 100) and the hepatosomatic index (HSI = [liver weight / (BW − Viscera weight)] × 100). In order to verify the developmental stages of ovaries, histological analysis of the ovaries was performed as previously described (Shi et al. 2015). Briefly, the ovaries were transferred to 70 % ethanol after being fixed for 24 h in Bouin's solution. Following paraffin embedding, the ovaries were serially sectioned at 5 μm and stained with hematoxylin and eosin (H&E). The ovarian developmental stages were identified under light microscopy (Leica DM500, Germany). The maturity of the females with the asynchronous-type ovary was classified according to the most advanced type of oocytes found in the ovary as described in previous studies (Garcia-Lopez et al. 2007; Shi et al. 2015), and five phases of ovarian development were identified, as follows: previtellogenesis (stage II), vitellogenesis (stage III), late vitellogenesis (stage IV), maturation (stage V), and post ovulation (stage VI).

RNA extraction, reverse transcription, and real-time quantitative PCR

Pituitaries, livers, and ovaries were collected from fish at different ovarian developmental stages following anesthetization and decapitation. The tissues were immediately snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Total RNAs were extracted using RNAiso reagent (Takara, Dalian, China) according to the manufacturer's instructions. The purity and yield of RNA were assessed by a NanoDrop 2000C spectrophotometer (Thermo Scientific, USA) with 260:280 ratios of 1.8–2.0. The integrity of RNA was determined on 1 % agarose gel electrophoresis with ethidium bromide (EB) staining. Subsequently, cDNA synthesis and real-time quantitative PCR were performed as previously described (Xu et al. 2015). In brief, the first-strand cDNA was synthesized with PrimeScript First-Strand cDNA Synthesis Kit (Takara, Dalian, China) according to the manufacturer's protocol. Gene expression levels were determined with real-time quantitative PCR assay. The primers used in this study are listed in Table 1. No amplification was observed with nontemplate as a negative control to confirm that the reagents were not contaminated. The specificity of the primers was evaluated by the melting curve analysis, and the PCR products were determined on 2 % agarose gel electrophoresis with EB staining. Five-fold serial dilutions of standards containing the qPCR amplicon were conducted to estimate PCR efficiency as previously described (Schmittgen and Livak 2008). Beta-actin was used as the internal reference, and beta-actin levels remained stable throughout the study. The relative gene expression levels were normalized to beta-actin levels and calculated by the comparative Ct method (Schmittgen and Livak 2008).

Assay of plasma hormones

The plasma levels of E2 and T were measured using a validated radioimmunoassay, as described elsewhere (Xu et al. 2012). The assay detection limits were 1.8 pg/mL for E2 and 22 pg/mL for T, respectively. The intra- and inter-assay coefficients of variation were 1.9 and 7.4 % for E2 and 2.1 and 7.7 % for T, respectively. The plasma levels of GH and IGF-I were measured by commercially available ELISA kits (Cusabio,

**Table 1** Information of primers used in real-time quantitative PCR

Name	Primer sequence(5′–3′)	Amplicon size (bp)	PCR efficiency	GenBank accession no.
GH F	AGCTAACTGCACCCTGTAGCC	140	2.03	KC709504
GH R	TTGGAATGCACAACCAACAT			
IGF-I F	GAGACCTTCACCGGGACATA	87	2	KC709503
IGF-I R	TAATTTCGTCCACCCGTGTT			
IGF-II F	CCACCATCACCACCACAAC	179	1.96	KM821276
IGF-II R	GCGACTGGACGAAGACATC			
β-Actin F	AGAGGTTCCGTTGTCCAGAG	90	1.94	JN226149
β-Actin R	TGATGCTGTTGTAGGTGGTCTC			

Wuhan, China) according to the manufacturer's protocols. The assay detection limits were 312.5 pg/mL for GH and 31.25 pg/mL for IGF-I, respectively. The intra- and inter-assay coefficients of variation were typically less than 15 % for GH and IGF-I, respectively.

Statistical analysis

All data were expressed as mean ± SEM and analyzed by one-way ANOVA followed by Duncan's multiple comparison test using SPSS 16.0 software

Fig. 1 Representative photomicrographs of the ovarian development of starry flounder. **a** Previtellogenesis, ovary at stage II; **b** vitellogenesis, ovary at stage III; **c** late vitellogenesis, ovary at stage IV; **d** maturation, ovary at stage V; and **e** post ovulation, ovary at stage VI. *YP* yolk plates, *FM* follicle membrane
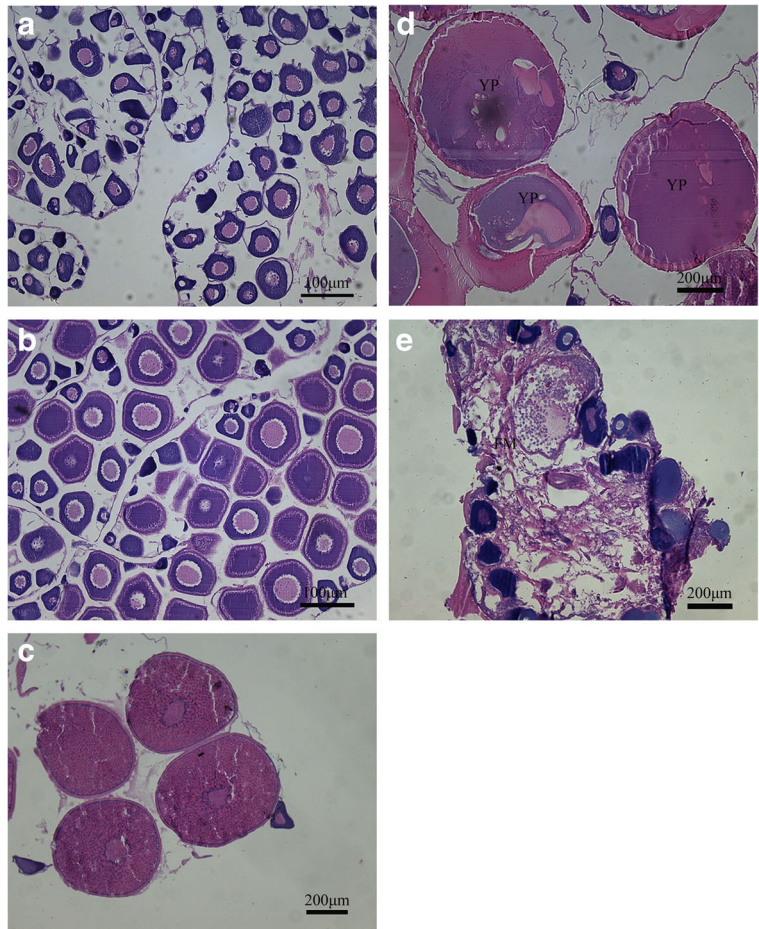
(Chicago, IL, USA). Levels of correlation were determined by calculating the Pearson correlation coefficient (r). Statistical significance was considered as p value of <0.05.

Results

Characterization of the ovarian development of starry flounder

The ovary developmental stages were systematically determined by histological analysis. The ovarian development of starry flounder is asynchronous, and oocytes of different developmental stages can be simultaneously found in the same ovary. As shown in Fig. 1, five phases of ovarian development were identified: previtellogenesis (stage II, Fig. 1a), vitellogenesis (stage III, Fig. 1b), late vitellogenesis (stage IV, Fig. 1c), maturation (stage V, Fig. 1d), and post ovulation (stage VI, Fig. 1e).

Changes in GSI and HSI during ovarian development

As shown in Fig. 2a, GSI was low at stages II and III and increased progressively to peak level at stage V. Thereafter, GSI declined post ovulation (stage VI). HSI increased gradually with the ovarian development and peaked at stage IV and then declined gradually to the initial level at stage VI (Fig. 2b).

Gene expression of GH and IGF mRNAs during ovarian development

As shown in Fig. 3a, the pituitary GH mRNA levels decreased sharply at stage III and remained depressed until stage V and raised to top level after spawning (stage VI). The hepatic IGF-I mRNA levels ascended progressively to the maximal value at stage V and then declined significantly post ovulation (stage VI) (Fig. 3b). However, hepatic IGF-II mRNA levels remained stable during the ovarian development and increased significantly after spawning (stage VI) (Fig. 3c). In contrast, the ovarian IGF-I mRNA levels increased gradually with ovarian development and peaked at stage VI (Fig. 3d). The ovarian IGF-II mRNA levels were initially stable and increased

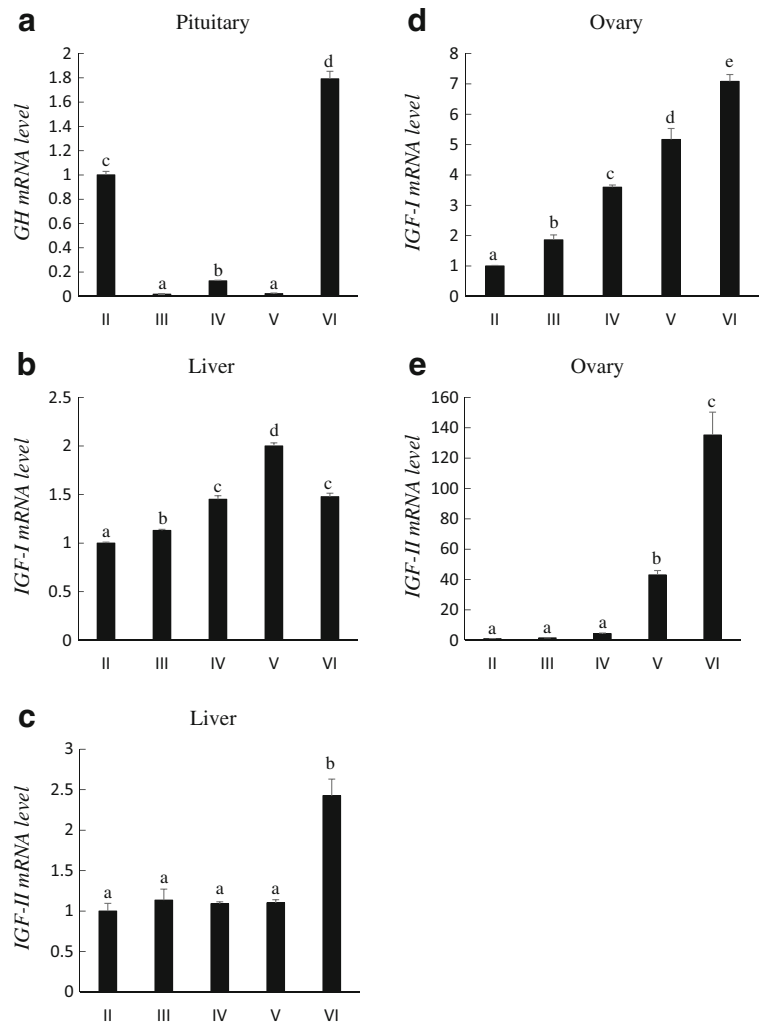significantly at stage V until the top level at stage VI (Fig. 3e).

Changes in plasma levels of hormones during ovarian development

We evaluated the plasma levels of GH, IGF-I, E2, and T at various ovarian stages. Consistent with the pituitary GH mRNA levels, plasma GH concentration reduced sharply at stage III and remained depressed until stage V and then raised remarkably post spawning (stage VI) (Fig. 4a). Plasma IGF-I concentration increased progressively, peaked at stage V, and then declined to initial level (Fig. 4b). Plasma E2 concentration remained low at stages II and III, raised rapidly to the top level at stage IV, and then descended gradually to the basal level (Fig. 4c). Plasma T concentration remained stable before stage IV, raised sharply to the
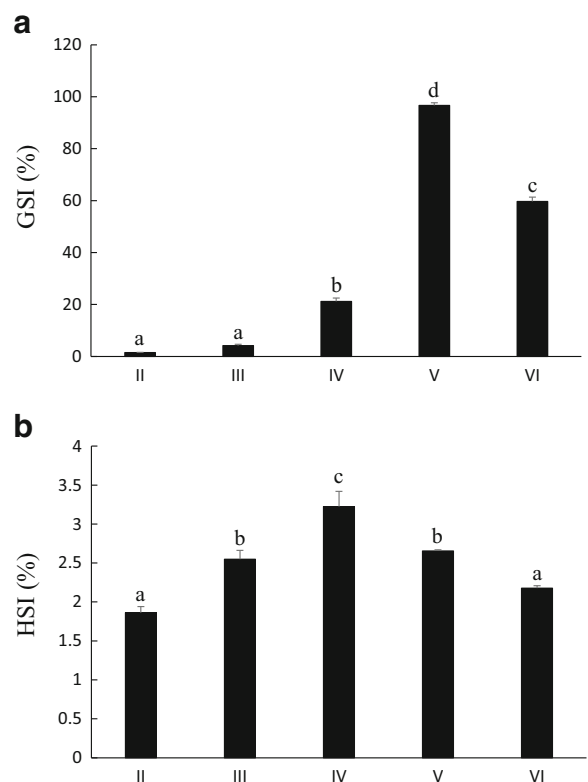
Fig. 2 Changes in GSI and HSI at different ovarian developmental stages of starry flounder. Each *bar* represents the mean ± SEM ($n = 4$). Groups with *different letters* are significantly different from each other ($p < 0.05$)

Fig. 3 Relative gene expression levels of pituitary GH mRNA (**a**), hepatic IGF-I (**b**), and IGF-II (**c**) as well as ovarian IGF-I (**d**) and IGF-II (**e**) mRNAs at different ovarian developmental stages of starry flounder. Each *bar* represents the mean ± SEM (*n* = 4). Groups with *different letters* are significantly different from each other (*p* < 0.05)

Correlation analysis among reproductive parameters

As shown in Table 2, there was a significant positive correlation between hepatic IGF-I mRNA and GSI, between ovarian IGF-II mRNA and hepatic IGF-II mRNA, between ovarian IGF-I mRNA and ovarian IGF-II mRNA, and between plasma IGF-I and plasma T, respectively. Other correlations were not significant though there was a strong correlation among some factors.

maximum level at stage V, and then significantly reduced back to the basal level at stage VI (Fig. 4d).

Discussion

The present study characterized the ovarian development of starry flounder and analyzed the expression profiles of GH and IGF mRNAs as well as plasma hormone levels during the ovarian development of starry flounder. Our data suggest that the GH-IGF system plays an important role in the ovarian development of starry flounder.

Starry flounder displayed an asynchronous ovarian development, and oocytes of different developmental stages could be simultaneously found, suggesting that starry flounder was a multiple batch spawner. On one hand, GSI is an important indicator of gonad development in fish. In our study, GSI in starry flounder began

Fig. 4 Changes in plasma levels of GH (**a**), IGF-I (**b**), E2 (**c**), and T (**d**) at different ovarian developmental stages of starry flounder. Each *bar* represents the mean ± SEM (*n* = 4). Groups with *different letters* are significantly different from each other ($p < 0.05$)
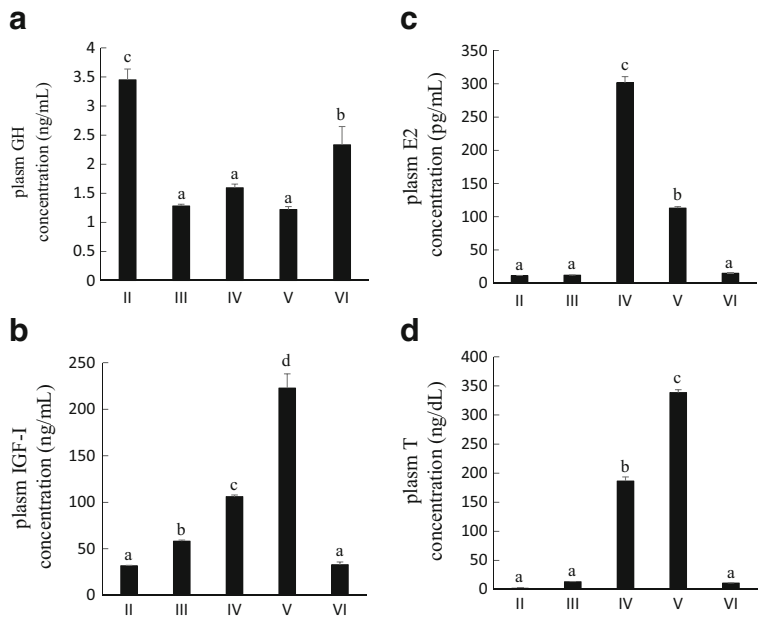
to increase significantly at stage IV and reached the highest value at stage V and then declined sharply after spawning, displaying a relation with ovarian development. The variation of GSI was consistent with those of Korean spotted sea bass (Lee and Yang 2002), amberjack (Micale et al. 1999), and Atlantic cod (Dahle et al.

2003). On the other hand, HSI is a good indicator of liver energy content (Dahle et al. 2003). Starry flounder HSI increased continuously and peaked at stage IV, which is likely to reflect vitellogenesis in the liver and preparation for ovarian development. The decrease of HSI at stage V suggested that vitellogenin stored in the

Table 2 Correlation analysis among reproductive parameters

Correlation coefficient (r)	GSI	HSI	Pituitary GH mRNA	Hepatic IGF-I mRNA	Hepatic IGF-II mRNA	Ovarian IGF-I mRNA	Ovarian IGF-II mRNA	Plasma GH	Plasma IGF-I	Plasma E2	Plasma T
GSI	1	0.142	0.036	*0.947**	0.335	*0.802*	0.589	−0.396	*0.727*	0.081	*0.706*
HSI		1	*−0.679*	0.438	−0.289	0.142	−0.276	*−0.774*	0.528	*0.876*	*0.612*
Pituitary GH mRNA			1	−0.229	0.807	0.414	0.744	0.692	−0.626	−0.477	−0.584
Hepatic IGF-I mRNA				1	0.128	0.713	0.378	−0.583	0.87	0.364	0.875
Hepatic IGF-II mRNA					1	*0.779*	*0.954**	0.133	−0.372	−0.322	−0.349
Ovarian IGF-I mRNA						1	*0.891**	−0.279	0.275	0.093	0.315
Ovarian IGF-II mRNA							1	0.067	−0.115	−0.295	−0.103
Plasma GH								1	*−0.637*	−0.398	−0.59
Plasma IGF-I									1	0.443	*0.978***
Plasma E2										1	0.599
Plasma T											1

Strong correlations ($|r| > 0.6$) are in italics

***Correlation is significant at the 0.05 level (two tailed); correlation is significant at the 0.01 level (two tailed)

liver had been transferred to gonad, and the decline of HSI after spawning (stage VI) was associated with the reduction of metabolic activity in the liver (Maddock and Burton 1998; Sol et al. 1998).

A strong positive correlation of pituitary GH mRNA levels with plasma GH levels was found in our study. Both pituitary GH mRNA levels and plasma GH levels remained depressed during the ovarian development and regained higher levels after spawning, implying that GH had no direct effects on ovarian development. In agreement with our hypothesis, treatment of previtellogenic ovarian fragments from short-finned eel with recombinant human IGF-I resulted in increased oocyte diameters, whereas no such effect was seen after treatment with GH (Lokman et al. 2007). In contrast, GH seems to control ovarian development via regulating expression of IGF-I in the liver or ovary (Duan et al. 1993; Duguay et al. 1994; Lokman et al. 2007). The GH treatment in the prereproductive ovary of sea bream induced a significant increase of IGF-I expression (Gioacchini et al. 2005). In rainbow trout, IGF-I mRNA levels increased in the liver and other extra-hepatic tissues in response to recombinant bovine somatotropin treatment (Biga et al. 2004). In addition, the binding capacity of GH to GHR present in the ovary of rainbow trout increased during the ovarian development (Gomez et al. 1999). Based on the previously mentioned studies, we hypothesized that a low concentration of plasma GH was associated with the inhibition of somatic growth, while an ovary-specific increase of the binding capacity of GHR contributed to the ovarian development of starry flounder.

Both IGF-I and IGF-II mRNAs were detected in the liver and ovary at various stages of ovarian development of starry flounder, which was in line with previous reports in tilapia (Berishvili et al. 2006; Schmid et al. 1999) and gilthead sea bream (Perrot et al. 2000). A strong positive correlation of hepatic IGF-I mRNA levels with plasma IGF-I levels was observed in this study. Both hepatic IGF-I mRNA levels and plasma IGF-I levels increased gradually, reaching the maximal level at stage V and then dropped after spawning, suggesting that IGF-I mainly participated in vitellogenesis and oocyte maturation in starry flounder. However, hepatic IGF-II mRNA levels remained constant during the ovarian development, implying that IGF-II derived from the liver may not be involved in the

ovarian development. Consistent with previous studies in rainbow trout (Bobe et al. 2003; Bobe et al. 2004), ovarian levels of IGF-I and IGF-II mRNAs increased with ovarian development, suggesting that locally produced IGFs play important roles in the paracrine/autocrine regulation of ovarian development. Moreover, the significant correlation between ovarian IGF-I mRNA levels and IGF-II mRNA levels was observed, which may indicate a cooperative function relationship between IGF-I and IGF-II in ovarian development of starry flounder.

Sex steroid hormones are important regulators of sexual maturation (Lubzens et al. 2010). E2 controls the synthesis of vitellogenin and vitelline envelope proteins in the liver (Davis et al. 2007; Larsson et al. 2002). In this study, peak levels of E2 appeared around the start of spawning period and a subsequent decrease during spawning was observed, which was in accordance with a number of periodic spawners (Harmin et al. 1995; Johnson et al. 1991; Scott et al. 1998). T also plays a role in the synthesis of vitellogenin (Sundararaj et al. 1982). A significant positive correlation between serum T levels and serum IGF-I levels was observed in our study, and the increase of sex steroid hormone levels might be associated with the enhancement of IGF-I levels. IGF-I promoted E2 production by stimulating aromatase activity and cytochrome P450 aromatase gene expression in the ovarian fragments of red sea bream (Kagawa et al. 2003). Similarly, IGF-I increased T and E2 production in ovarian fragments of white perch in vitro (Weber et al. 2007). Furthermore, the plasma IGF-I levels positively correlated with plasma levels of E2 and T in chum salmon (Onuma et al. 2009). Collectively, these data suggest that IGF-I plays an essential role in steroidogenesis during vitellogenic period. The role of IGF-II in steroidogenesis awaits further studies in teleosts.

In summary, we characterized the ovarian development of starry flounder and analyzed the expression profiles of GH and IGF mRNAs as well as the plasma hormone levels during the ovarian development of starry flounder. GSI increased during the course of ovarian development and reached a maximum during spawning in starry flounder, while HSI arrived at the peak level before spawning. These changes were associated with changes in the gene expression of GH and IGF mRNAs along with their peptides and sex steroids. These results indicate that paracrine/autocrine actions of the GH/IGF-I axis

play an important role in the development of the ovary in starry flounder.

Compliance with ethical standards All of the animal experiments were approved by the Animal Care and Use Committee of Chinese Academy of Fishery Sciences.

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