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# Changes in brain and pituitary GnRH levels during a reproductive cycle in wild female Asian sea bass, *Lates calcarifer* (Bloch, 1790)

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## Abstract

Changes in the peptide levels of sea bream (*Sparus aurata*) GnRH (sbGnRH) in the olfactory bulb, telencephalon including the preoptic area, hypothalamus, optic tectum-thalamus and pituitary during a reproductive cycle were investigated in addition to plasma levels of testosterone (T), 17β- estradiol (E2) and 17α, 20b-dihydroxy-4-pregnen-3-one (DHP) in wild female Asian sea bass *Lates calcarifer* in Nha Phu lagoon, Vietnam from March to September, 2010. Ovaries were classified into five stages: II Recovering spent (March -April), III Maturing (May-June), IV Mature (June), V Ripe and ovulating (July- August) and VI Spent (September). The gonadosomatic index increased significantly from the stage II (1.62%±0.12) to stage V (8.48%±0.23) and dropped thereafter in stage VI (2.05%±0.25). In the olfactory bulb and telencephalon, sbGnRH levels in stage IV and stage V were slightly higher than those in the other stages, but no significant differences in sbGnRH levels were found among the ovarian stages. In the hypothalamus and optic tectum-thalamus, although significant differences in sbGnRH level were not found among the ovarian stages, the highest level of sbGnRH was determined in stage III (594.13±152.12 pg tissue$^{-1}$ and 501.37±124.35 pg tissue$^{-1}$, respectively). In the pituitary, sbGnRH level in stage III (6927.03±218.92 pg tissue$^{-1}$) was significantly higher than that in the other stages. In addition, plasma T, E2 and DHP levels increased significantly during ovarian maturation or ovulation with the highest level of T (2.91±0.31 ng ml$^{-1}$) and E2 (4.98±1.43 ng ml$^{-1}$) recorded in stage IV and DHP (389.6 ± 136.03 pg ml$^{-1}$) in stage V. The results showed that sbGnRH in the anterior brain and pituitary is involved in initiation and ovarian maturation in wild Asian sea bass.

**Keywords**: sbGnRH, Brain, Pituitary, Plasma steroid, Ovarian stages, Asian sea bass

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## Introduction

Reproduction in teleost fish is primarily regulated by gonadotropin- releasing hormone (GnRH) via the brain-pituitary-gonad (BPG) axis (Okuzawa and Kobayashi, 1999). GnRH activates the secretion of gonadotropin (GTH) in the pituitary. GTH in circulation stimulates the synthesis and production of steroid hormones which regulate gonadal development. Until now, eight GnRH forms have been identified in teleosts and a single species can possess two or three GnRHs within the brain depending on fish class (Sherwood and Adams, 2005). The discovery of multiple GnRHs has complicated the role of GnRH in the reproductive process in teleosts. An arising question is whether one or more than one GnRH form act in a synergistic manner to regulate reproduction of teleosts. It was commonly considered that the abundance of GnRH in the pituitary or the positive relationships between gonadal development and the amount of GnRH in the pituitary was an indicator of GnRH-releasing activity relating to reproduction of fish (Holland *et al*., 2001). Such researches were conducted in Perciform- the largest group of fishes and other fishes which coexist with three GnRH forms, i.e. salmon GnRH (sGnRH), chicken GnRH-II (cGnRH-II) and sea bream GnRH (sbGnRH)- the dominant form in the pituitary. However, the measurement of sbGnRH amount in the brain and pituitary during a reproductive cycle is limited in several female fish, because a large number of wild or reared animals will be scarified for experiments. Among the studied female fish, it was found that change in pituitary sbGnRH levels or brain sbGnRH mRNA levels was associated with ovarian development in a number of fish, such as the red sea bream *Pagrus major* (Senthilkumaran *et al*., 1999), barfin flounder *Verasper moseri* (Amano *et al*., 2008), turbot *Scophthalmus maximus* (Andersson *et al*., 2001), striped bass *Morone saxatilis* (Holland *et al*., 2001), Japanese flounder *Paralichthys olivaceus* (Pham *et al*., 2006b) and chub mackerel *Scomber japonicus* (Selvaraj et al., 2012a). Consequently, sbGnRH can be the primary regulator of gonadotropin activity and thus ovarian development, in these fishes.

Asian sea bass, *Lates calcarifer* is a protandrous hermaphroditic fish which exhibits complex sexuality making it an interesting specimen for academic studies (Davis, 1982). This species with asynchronous ovarian development can spawn repeatedly in batches for several days (Almendras *et al*., 1988; Garcia, 1989). The fish is important for aquaculture and it has been introduced for breeding in some Asian countries, including Vietnam. For studies on reproductive endocrine of this species, changes in plasma and gonadal steroid hormones *in vitro* as well as androstenedione metabolism during the reproductive cycle and sex inversion process were investigated (Guiguen *et al*., 1993, 1995). On the other hand, three forms of GnRH, i.e. sGnRH, cGnRH-II and sbGnRH in the brain were detected and sbGnRH is the dominant form in the pituitary of the species. Moreover, anatomical observations revealed that sbGnRH-cells located in the preoptic area of the brain send the fibers into the pituitary. These results suggest a role of sbGnRH in regulating pituitary functions relating to reproduction in Asian sea bass (Pham *et al*., 2016). In the present study, to clarify the physiological role of sbGnRH in the reproduction in Asian sea bass, we measured sbGnRH levels in the anterior parts of the brain and pituitary in addition to plasma testosterone (T), 17β- estradiol (E2) and 17α, 20β- dihydroxy- 4-pregnen-3-one (DHP) levels in wild females during a reproductive cycle.

## Materials and methods

### *Fish and samplings*

Wild adult female Asian sea bass with a total length (TL) of 42-110 cm and a body weight (BW) of 4.8-14.0 kg were caught in Nha Phu lagoon ($12^o31´$-$12^o46´$N, $109^o15´$ - $109^o29´$E), Khanh Hoa province, Vietnam by gill net or fish hook with baits. The sea bass were collected every month from March to September in 2010 during the spawning season and the fish were held in a floating net cage in the lagoon for 1-2 days. The fish were then transported live within 1 hour to the Institute of Oceanography, Nha Trang, Vietnam with oxygen supplementation and were held in a $5m^3$-cement tank with running seawater for about 1-2 h. Animals were treated in accordance with the guidelines of the world society for the protection of animals in Vietnam.

The fish were anesthetized by immersion in ice water before the TL and BW were measured. Blood was collected from the caudal arteries of the fish into heparinized syringes. The blood samples were kept in ice and centrifuged at 4 $^o$C for 15 min at 3000 rpm to yield plasma. The plasma samples were stored at -30 $^o$C until the extraction of steroid hormones.

To collect brain and pituitary, the fish were then killed. The brains and pituitaries were removed quickly from the chondrocranium of decapitated fish. Six dissected parts of the brain (the olfactory bulb, telencephalon including the preoptic area, hypothalamus, optic tectum-thalamus, cerebellum and medulla oblongata), as shown in Fig. 1, were placed on dry ice and stored at -80$^o$C to extract GnRH. In this research, we measured sbGnRH peptide levels in the olfactory bulb, telencephalon including the preoptic area, hypothalamus, optic tectum-thalamus and pituitary.

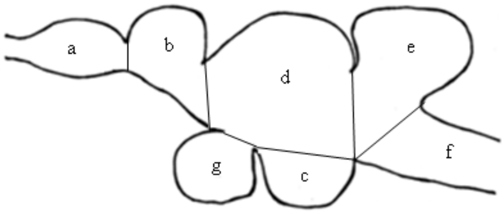

**Figure 1: Schematic diagram of a sagittal section of the Asian sea bass brain. (a) olfactory bulb, (b) telencephalon including the preoptic area, (c) hypothalamus, (d) optic tectum - thalamus including the midbrain, (e) cerebellum, (f) medulla oblongata and (g) pituitary.**

Ovaries were also removed and weighed to calculate gonadosomatic index (GSI=[ovarian weight including ovulated eggs)/(body weight]×100). Pieces of the ovaries were fixed in 10% neutral buffered formalin (pH 7.4) for histological analysis. Deeply frozen brain tissues and plasma were transferred to Japan on dry ice and analyses were carried out in the School of Marine Bioscience, Kitasato University.

### *GnRH measurement*

GnRH was extracted following a procedure which was briefly described by Pham *et al*, (2006a). The level of sbGnRH peptide in the extract was determined using a time-resolved fluoroimmunoassay (TR-FIA) according to Amano *et al*. (2004). Antiserum against sbGnRH (AS-691) was diluted 5000-fold. Parallelism of a typical standard curve of sbGnRH and the corresponding competition curves of brain and pituitary extracts of Asian sea bass were examined with serially twofold-diluted standard and brain and pituitary extracts in an assay buffer. sbGnRH peptide levels were expressed as both content (pg tissue$^{-1}$) and concentration (pg mgtissue$^{-1}$).

### *Plasma steroid hormone measurement*

To yield steroid extract, 200 µl of a plasma sample was extracted twice with 1 ml of diethylether. The completely dried extracts were reconstituted in 1 ml of assay buffer (0.05 M Tris, 0.9% NaCl, 0.5% bovine serum albumin (BSA), 0.05% $NaN_3$, 0.01% Tween 40, 20 µM diethylenetriamine- N,N,N´,N´ and N´-pentaacetic acid, pH 7.75) and stored at -30°C for assays. TR-FIA for T, E2 and DHP was performed according to Yamada *et al*. (1997).

### *Histological analysis*

The ovaries were dehydrated through serial ethanol concentrations, embedded in paraplast and sectioned at a thickness of 10 µm. The sections were then stained with hematoxylin-eosin following a routine procedure for microscopic examination. The ovarian developmental stages of the sea bass were classified based on the most advanced oocyte developmental stages according to Kuldeep and Pandey (1998). Oocyte diameter was measured using the Image J software provided free of charge by the National Institutes of Health (Bethesda, MD) via the internet. Fish were categorized into five groups according to the five ovarian stages (II-VI) as described in Table 1.

### *Statistics*

The data of TL, BW, GSI values, sbGnRH peptide and steroid hormone levels among ovarian stages were analysed using a one-way ANOVA, followed by the Tukey test. Significant differences were considered at $p<0.05$. The results were presented as the mean±standard error (SE).

## **Results**

### *Ovarian development and GSI*

No significant differences in the TL and BW among fish groups with various ovarian stages were observed (data not shown). Characteristics of ovarian stages in Asian sea bass are summarized in Table 1. The GSI of Asian sea bass are shown in Fig. 2. Stage II was found in fish collected in March and April. In May, the ovaries were in stage III. In June, a few fish remained in stage III, but most fish had reached the stage IV. The ovaries of fish in July and August were all in stage V and those in September were in stage VI.

**Table 1: Characteristics of ovarian developmental stages in wild female Asian sea bass collected in Nha Phu lagoon, Vietnam in 2010.**

| **Stages** | **II<br>Recovering spent<br>(Mar.-Apr.)** | **III<br>Maturing<br>(May-June)** | **IV<br>Mature<br>(June)** | **V<br>Ripe and ovulating<br>(Jul.-Aug.)** | **VI<br>Spent<br>(Sept.)** |
|---|---|---|---|---|---|
| Macroscopic characteristics | The ovaries are thick walled, translucent, with pinkish-red hue. | The ovaries increase in size, translucent and cream in colour. | The ovaries are markedly larger in size, with thinner wall. Yellow hue start appearing. | The ovaries are deep yellow in colour, with thin walls, distended and occupying most of the body cavity. | The ovaries are flaccid, reduced in size, with purple yellow colour. The wall becomes tough and wrinkled. |
| Histological characteristics | Chromatin nucleolus stage, perinucleolus stage oocytes dominate. | Perinucleolus stage oocytes are present; cortical alveoli stage predominate the population. | Yolky oocytes. Most of the oocytes are at early and late vitellogenic stages, Previtellogenic stages also coexist. | The oocytes undergo final maturation, migration of germinal vesicle and break down, coalescence of oil droplets. | Post ovulatory follicles; previtellogenic oocytes dominate along with a few residual yolky oocytes. |
| Largest oocyte diameter (µm) | 135-140 | 160 - 270 | 260 - 500 | 450 - 700 | 130-140 |

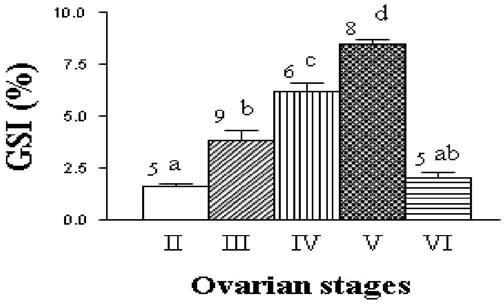

**Figure 2: Changes in the gonadosomatic index (GSI) (%) of wild Asian sea bass during ovarian development. Values are expressed as the mean±SE. Numbers above each bar indicate the number of fish analyzed. Different letters indicate a significant difference in mean values ($p$<0.05).**

The GSI values increased significantly from stage II (1.62%±0.12) at the beginning of the experiment, reaching a peak in stage V (8.48%±0.23) when the fish began to ovulate and dropped to a low value in stage VI (2.05%±0.25) after spawning ($p$<0.001).

### *Parallelism of sbGnRH*

Parallelism of the typical standard curve of sbGnRH and corresponding competition curves of brain and pituitary extracts of Asian sea bass are shown in Fig. 3. Displacement curves of these extracts paralleled the sbGnRH standard curve.

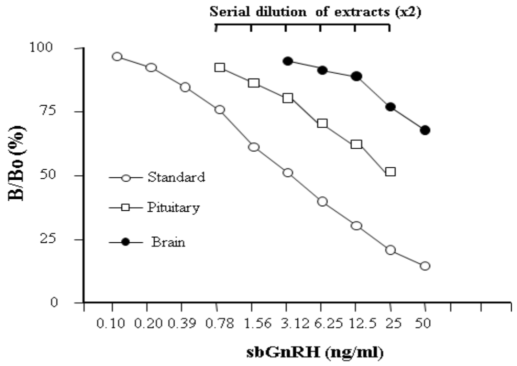

**Figure 3: Typical standard curves of sbGnRH and competition curves of serially twofold-diluted brain and pituitary extracts of the Asian sea bass. $B/B_o$, the binding rate (%).**

*Changes in sbGnRH levels in the brain and pituitary*

In the olfactory bulb, no significant differences in sbGnRH content (pg tissue$^{-1}$) and concentration (pg mg tissue$^{-1}$) were found among the different stages of ovarian development (Figs. 4a, 5a). A similar tendency in sbGnRH level was also found in the telencephalon. sbGnRH levels in the stages IV and V were slightly higher than those in the other stages (Figs. 4b, 5b).

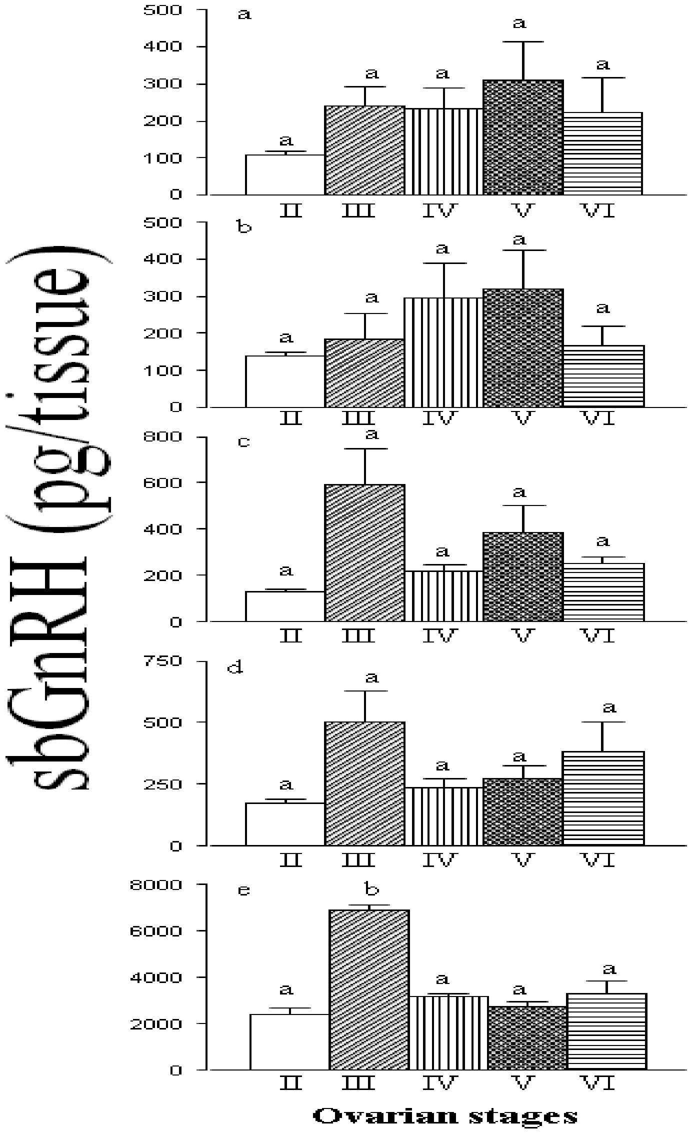

**Figure 4: Changes in sbGnRH contents (pg tissue$^{-1}$) in the olfactory bulb (a), telencephalon (b), hypothalamus (c), optictectum thalamus (d) and pituitary (e) of wild Asian sea bass during ovarian development. Values are expressed as the mean±SE. Different letters indicate a significant difference in mean values (*p*<0.05).**

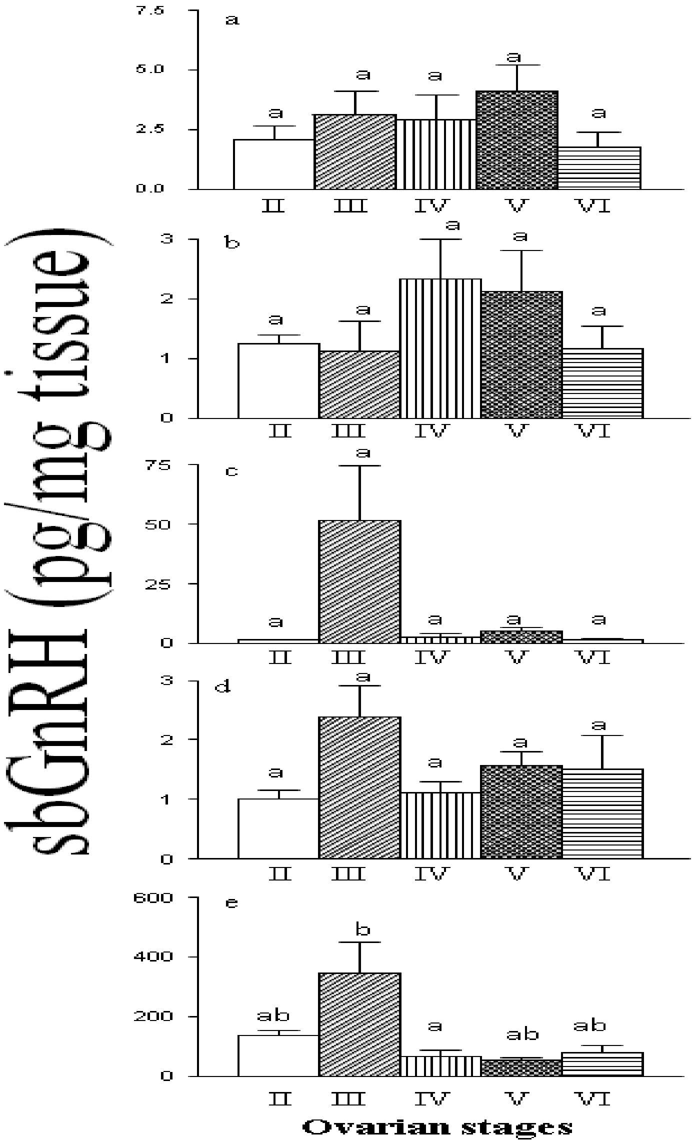

**Figure 5: Changes in sbGnRH concentrations (pg mg$^{-1}$) in the olfactory bulb (a), telencephalon (b), hypothalamus (c), optic tectum thalamus (d) and pituitary (e) of wild Asian sea bass during ovarian development. Values are expressed as the mean± SE. Different letters indicate a significant difference in mean values ($p$<0.05).**

Although the means of sbGnRH level are significantly different ($p$<0.05) by one-way ANOVA, both sbGnRH content and concentration in the hypothalamus were not significantly different among the ovarian stages. High levels of sbGnRH were determined in stage III (594.13±152.12 pg tissue$^{-1}$ and 51.74±23.0 pg mg$^{-1}$ tissue) (Figs. 4c, 5c). In the optic tectum-thalamus, sbGnRH content and concentration varied during the experimental period and high levels of this hormone were also recognized in stage III (501.37±124.35 pg tissue$^{-1}$ and 2.38±0.54 pg mgtissue$^{-1}$) (Figs. 4d, 5d). However, no significant differences were observed among the ovarian stages. In the pituitary, sbGnRH content (6927.03±218.92 pg tissue$^{-1}$) (Fig. 4e) and concentration (344.61±103.67 pg mg tissue$^{-1}$) (Fig. 5e) in stage III were the highest among the ovarian stages and there was a significant difference in sbGnRH content in this stage compared to the other stages ($p<0.01$).

*Changes in plasma steroid hormone levels*

Plasma steroid hormone levels of testosterone (Fig. 6a), estradiol (Fig. 6b) and DHP (Fi. 6c) showed a similar tendency in variations, increasing significantly from stage II to stage IV or V and decreasing thereafter ($p<0.05$). The levels of T and E2 were the highest in stage IV (2.91±0.31 ng ml$^{-1}$ and 4.98±1.43 ng ml$^{-1}$, respectively) and the level of DHP reached a peak in stage V (389.6±136.03 pg ml$^{-1}$).

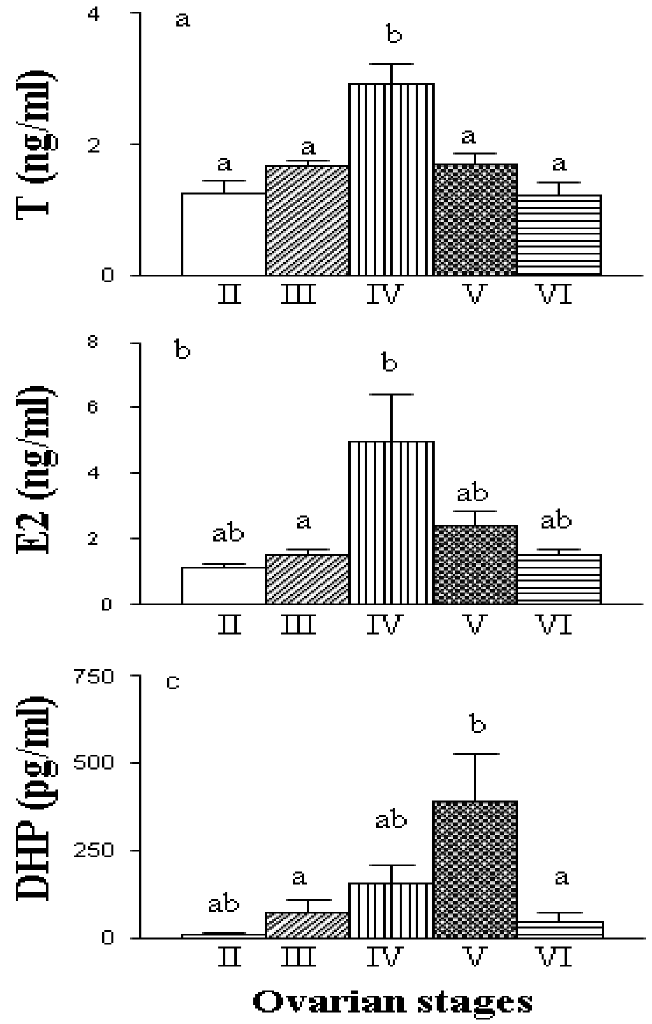

**Figure 6: Changes in steroid hormone level in plasma of wild Asian sea bass during ovarian development. (a) Testosterone (ng ml$^{-1}$), (b) Estradiol (ng ml$^{-1}$) and (c) DHP (pg ml$^{-1}$). Values are expressed as the mean±SE. Different letters indicate a significant difference in mean values ($p$<0.05).**

## Discussion

In the early months of the year before the spawning season, the ovary of Asian sea bass is still inactive with oocytes in the chromatin nucleolus and perinucleolus stages and low values of GSI. The brain and pituitary sbGnRH peptide levels are maintained at low levels during this period. In addition, the plasma levels of T and E2 remained low in the fish. The results of Hassin *et al*. (1999) and Holland *et al*. (2001) showed that in the pubertal female striped bass, the expression levels of the follicle stimulating hormone (FSH) and luteinizing hormone (LH) genes were very low during early oocyte growth and the pituitary sbGnRH peptide levels were low in the fish with small oocytes. Similar results were obtained for the barfin flounder where both sbGnRH levels in the brain and pituitary and plasma levels of T and E2 were very low in the young fish with oocytes in the perinucleolus stage (Amano *et al*., 2008). According to previous studies, the low peptide level of sbGnRH in the pituitary of immature wild female Japanese flounder, in multiple spawnings with oocytes at the chromatin nucleolus stage (Pham *et al*., 2006a) and the low immunostaining intensities of FSH and LH during the previtellogenic stage (Pham *et al*., 2008) were also observed. Taking all into consideration, it is possibly assumed that the endocrine system in reproduction is inactive in the early stages of ovarian development in Asian sea bass, similar to the above-mentioned cases.

The elevation of the sbGnRH level in the brain and pituitary in stage III with oocytes from the perinucleolus stage to cortical alveoli stage in early spawning season should be considered as an implementation of maturational initiation. The GSI, oocytes diameter and plasma level of T and E2 also began to increase in the Asian sea bass. In wild adult Japanese flounder, we found that the sbGnRH peptide levels in the brain and pituitary were highest in the previtellogenic stage at the onset of the spawning season (Pham *et al*., 2006b). In reared European male sea bass *Dicentrarchus labrax*, during the first spawning season, the pituitary sbGnRH level reached a peak in maturing fish (Rodriguez *et al*., 2000). On the other hand, in reared female barfin flounder (Amano *et al*., 2008) and captive chub mackerel (Selvaraj *et al*., 2012a), the sbGnRH peptide level in the pituitary increased in accordance with the increase in plasma levels of T and E2 and GSI in the vitellogenic fish. As known, T is the precursor of E2 which stimulates vitellogenin synthesis and GnRH is a primary stimulation of the synthesis of steroid hormones via inducing GTH secretion. sbGnRH can induce GTH secretion both *in vitro* and *in vivo* in several fish (Zohar *et al*., 1995; Gothilf *et al*., 1997; Forniés *et al*., 2003). Therefore, sbGnRH can be implicated in the activation of the endocrine-reproductive axis primarily through interneuronal pathways in Asian sea bass.

Levels of sbGnRH in the hypothalamus and pituitary in wild female Asian sea bass were considerable during periods, although they decreased, when the fish were undergoing vitellogennesis, maturation and ovulation from June to August. The results also showed that plasma T and E2 levels increased during vitellogenesis and declined before ovulation in the fish. Change in levels of these steroids in the present study is in agreement with that of Guiguen *et al*. (1993), who reported that plasma concentrations of T and E2 peaked during vitellogenesis in reared female Asian sea bass during the reproductive cycle. Moreover, it was shown that E2 induces *in vivo* vitellogenesis in Asian sea bass (Fazielawanie *et al*., 2013). A similar tendency in change of levels of these hormones was also observed in wild female Japanese flounder (Pham *et al*., 2006b). In addition, in the prepubertal female chub mackerel, an injection of GnRH-A exhibited a high level of FSH in the pituitary and increase in the perinucleolar oocyte diameter (Selvaraj *et al*., 2013). Both FSH and LH extracted from this species significantly stimulated E2 production in mid-vitellogenic stage follicles (Ohga *et al*., 2012). It was also found that in the European sea bass, an injection of native sbGnRH and [D-Ala6, Pro9Net]-sbGnRH triggered the plasma LH secretion in postvitellogenic and periovulatory females (Forniés *et al*., 2003). GTHs were involved in DHP production- a common maturation-inducing hormone (MIH) in most teleosts such as rainbow trout (Nagler *et al*., 1996), salmon (Martyniuk *et al*., 2006) and chub mackerel (Ohga *et al*., 2012). Our results also demonstrate the increase of plasma DHP level throughout oocyte maturation in the Asian sea bass. Furthermore, an injection or pellet implant of [D-Ala6, Pro9-NHEt] mammalian GnRH also induced final ovarian maturation and ovulation in this species (Almendras *et al*., 1988). Considering all these results, it can be concluded that synthesized sbGnRH could be rapidly utilized for GTH secretion, stimulating the production of the steroid hormones regulating oocyte maturation and ovulation in Asian sea bass. However, GnRH peptide level results from the temporary balance of synthesis, storage and release. In Asian sea bass and several multiple spawners, asynchronous ovary coexists several types of oocytes during spawning season; the synthesis and utilization of sbGnRH could be complicated because several processes relating to reproduction may occur at the same time. Consequently, a measurement of the brain sbGnRH mRNA level as found in the red sea bream (Okuzawa *et al*., 2003), barfin flounder (Amano *et al*., 2008) or brain kisspeptin mRNA level in chub mackerel (Selvaraj *et al*., 2012b) would provide an estimate of the activity of sbGnRH synthesis in Asian sea bass.

sbGnRH levels remain relatively high in the pituitary of the Asian sea bass toward the end of the spawning season when the fish was passing the regressing phase with the decreases in plasma steroid hormone levels. Similar to a number of perciformes and pleuronectiformes fish such as red sea bream (Senthilkumaran *et al*., 1999), turbot (Andersson *et al*., 2001), barfin flounder (Amano *et al*., 2008), Japanese flounder (Pham *et al*., 2006a,b), sbGnRH levels in the pituitary of Asian sea bass are always considerable compared to sGnRH and cGnRH-II regardless of the ovarian stages. An appreciable amount of sbGnRH in the pituitary in the regressing Asian sea bass could reflect an ability of sbGnRH neurons to store a certain amount of sbGnRH in this organ rather than the activity of sbGnRH synthesis in the brain when the BPG axis was becoming inactive in the regressing fish.

In summary, our study showed that sbGnRH levels in the brain and pituitary fluctuates in association with plasma steroid hormone levels during the reproductive cycle in the wild female Asian sea bass. The results indicated the involvement of sbGnRH in ovarian maturation in this species.

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