Immunoreactivity of estrogen receptor alpha in brain and ovary of the short mackerel *Rastrelliger brachysoma* (Bleeker, 1851)

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Abstract. The reproductive physiology concerning the gonadotropin hormone (GTH) and its downstream target estrogen receptor alpha (ER α) is not well understood in the short mackerel *Rastrelliger brachysoma*, an economically important marine fish in Thailand. In this study, we tested the hypothesis that the expression of both GTH and ER α in the brain and ovary of *R. brachysoma* was as reported in other fish species. By applying immunohistochemical techniques, we identified the distribution of ER α -immunoreactive (ir) neurons in the brain and ovary of wild female *R. brachysoma* during the spawning season along with the distribution of GTHs-ir cells in the ovary. The nucleus lateralis tuberis in the diencephalon had a high number of ER α -ir neurons. In the mesencephalon, dense ER α -ir neuronal fibers were mainly found in the mesencephalic cells, stratum opticum, stratum fibrosum et griseum superficiale and stratum album centrale. Both the valvula and corpus cerebelli in the metencephalon contained ER α -ir neurons in granular and Purkinje cell layers as well as the molecular layer. The ER α -ir neurons were also observed in the medulla oblongata. In the ovary, weak ER α and moderate GTHs immunoreactivities were observed in follicular cells of oocytes in early and late vitellogenic stages. This information provides baseline data required to understand not only the activity of estrogen (E₂) on the brain but also the regulatory mechanism of the hypothalamo-pituitary-ovarian axis of *R. brachysoma*.

Keywords: central nervous system, estrogenic receptor, oogenesis, short mackerel

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a hypothalamic hormone known to play important roles in reproductive activity in various animals including fish. This hormone from scombrids, often called seabream GnRH (sbGnRH), has been an important target of aquatic research since the Family Scombridae contains many

aquaculture species such as *Scomber japonicus* Houttuyn, 1782 (Selvaraj *et al.*, 2009) and *Rastrelliger brachysoma* (Bleeker, 1851) (Senarat, 2015; Senarat *et al.*, 2019). In *R. brachysoma*, sbGnRH-immunoreactive (ir) neurons are present in three areas of the brain including nucleus periventricularis, nucleus preopticus and

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nucleus lateralis tuberis (Senarat, 2015; Senarat *et al.*, 2018). The sbGnRH-ir neuronal fibers project to the proximal pars distalis of the pituitary where gonadotropin hormone (GTH), specifically follicular stimulating hormone (FSH) and luteinizing hormone (LH), are produced (Senarat, 2015; Senarat *et al.*, 2018). These observations are consistent with those from other teleosts such as *Sparus aurata* Linnaeus, 1758 (Gothilf *et al.*, 1997), *Dicentrarchus labrax* (Linnaeus, 1758) (González-Martínez *et al.*, 2001; González-Martínez *et al.*, 2002) and other fishes (Guilgur *et al.*, 2006; Palevitch *et al.*, 2009). Given the well-known function of GTH to control the activity of the gonadal sex steroid hormone estrogen in teleosts (Huggard-Nelson *et al.*, 1996; Huggard-Nelson *et al.*, 2002), it must be reasonable to assume that the oocyte differentiation of *R. brachysoma* female is also under control of the hypothalamo-pituitary-gonad axis (HPG axis). Further clarification of the HPG axis will contribute to the establishment of efficient and sustainable aquaculture of this species.

Natural estrogens include estrone (E_1), 17β -estradiol (E_2) and estriol (E_3), and E_2 plays a particularly important role in the gonadal development and uptake of vitellogenin into oocytes (Gustafsson, 2003; Hess, 2003; Heldring *et al.*, 2007; Hara *et al.*, 2016; Liu *et al.*, 2017) via nuclear estrogen receptors (ERs) (Auchus and Fuqua 1994; Fukumoto *et al.*, 2013; Tsai and O'Malley, 1994). There are three reported isoforms of ER, namely $ER\alpha$, $ER\beta$ and $ER\gamma$ (Sabo-Attwood *et al.*, 2004; Nagler *et al.*, 2012), and only $ER\alpha$ is known to have a high affinity to estrogens in the ovary. The expression of $ER\alpha$ has been detected in the ovary of the rainbow trout *Oncorhynchus mykiss* (Richardson, 1836), indicating that estrogens regulate the oogenic development via $ER\alpha$ in this species (Nagler *et al.*, 2012). Interestingly, $ER\alpha$ has been also detected in the brain, especially the anterior preoptic area and the ventrolateral hypothalamus (Anglade *et al.*, 1994; Forlano *et al.*, 2005). It is therefore likely that estrogens exert a direct action on the brain via $ER\alpha$, creating a feedback loop to regulate the gonadal development (Anglade *et al.*, 1994; Bouma and Nagler, 2001; Radovick *et al.*, 2012).

The purpose of research reported here is to investigate the brain histology in mature *R. brachysoma* along with the identification of $ER\alpha$

expression patterns in its brain. Additionally, the distribution of $ER\alpha$ - and GTHs-ir cells was determined in the ovary of *R. brachysoma*. This information provides baseline data required to understand not only the brain histology, but also an important role of E_2 on the brain and mature ovary of *R. brachysoma*.

MATERIALS AND METHOD

Fish collection. Sexually mature *Rastrelliger brachysoma* females ($n = 56$ for brain and ovarian histology; 5 out of 56 were used for immunohistochemistry), approximately 17-18 cm in total length (17.4 ± 0.65 cm in standard length, mean $\pm$ standard deviations) and 70-76 g in total weight (average 74.55 ± 2.81 g) were collected by bamboo strake trap from Samut Songkhram Province in the Upper Gulf of Thailand during the spawning season [January to February 2014 (Senarat, 2015; Senarat *et al.*, 2016)] ($13^\circ 16' 18.4''$ N, $100^\circ 02' 13.4''$ E). The main environmental factors related to reproductive activity are salinity and temperature (Senarat, 2015). During breeding season, the average ambient salinity was 31.75 ppt whereas the average temperature was 29.05°C (Senarat, 2015). All fish were euthanized by rapid cooling shock (Wilson *et al.*, 2009), and subsequently the brain and ovarian tissues were collected in field. In order to confirm that our specimens are fully mature, gonadosomatic indices (GSI) were calculated using the following formula ($GSI = [\text{gonadal weight}/\text{total weight} \times 100]$). All organs were kept in Davidson's solution for further histological analyses. The protocols used in this study were approved by the Animal Care and Use Committee of the Faculty of Science, Chulalongkorn University, Thailand (Protocol Review No. 1423003).

Histological and histochemical observations of the brain, pituitary gland and mature ovary.

Brain (telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon), pituitary gland and ovary were fixed in Davidson's fixative for about 24 hours at room temperature. After fixation, tissues were dehydrated through a graded series of ethyl alcohol and embedded in paraffin. Serial 4-5 μm

sections were cut by a rotary microtome. Finally, the serial longitudinal, horizontal and cross sections on all samples were stained with Harris's hematoxylin and eosin (H&E), and histochemically stained with Masson's trichrome (MT) and Periodic Acid-Schiff (PAS) (Bancroft and Gamble 2008). Brain sections were investigated for the histological structure and cellular composition, whereas ovary sections were examined for oocyte development under a light microscope following the guidelines by Dietrich and Krieger (2009) and Uribe *et al.* (2012). All tissue sections were photographed with a Leica DM1000 light microscope. Schematic diagrams of the pituitary gland were drawn by using Adobe Illustrator (version CS6).

Tissue preparation and immunohistochemistry. For immunohistochemical analyses, histological sections of *R. brachysoma* ($n = 5$) were mounted on poly-L-lysine coated slides. Brain (ER α) and ovarian [ER α and GTHs (follicular stimulating hormone (FSH) and luteinizing hormone (LH)] sections were deparaffinized, rehydrated and treated in 10 M citrate buffer (epitope-unmasking solution) for antigen retrieval. The sections were blocked for endogenous peroxidase with 3% H₂O₂ in 30% methanol for 3 min. Non-specific binding of proteins were blocked by incubating the section in a blocking solution (2% normal goat serum at room temperature for 30 min for ER α and 10% normal goat serum + 4% BSA in 1 M PBS for 2 hours for GTHs). The sections were then incubated with following rabbit primary antisera: ER α (1:100) (Thermo Scientific, Waltham, MA), anti-*Fundulus heteroclitus* (Fh) FSH β 50-60 or anti-Fh LH β (1:1000 and 1:2000 in 0.1 M PBS) for overnight at 4°C. After three washes in 0.1 M PBS, sections were incubated with EnvisionTM anti-rabbit IgG (ER α ; dilution 1:100; Thermo Scientific) or biotinylated goat anti-rabbit IgG solution (GTHs, dilution 1:500) for 30 min. Immunoreactive cells were visualized with 3,3'-diaminobenzidine (DAB) solution (Vector Laboratories, Burlingame, CA) and counterstained with Mayer's hematoxylin. The distribution of immunoreactive cells was observed and digital images were captured with a Leica digital 750 digital camera (Leica, Heidelberg, Germany). Distributions of GTHs-

immunoreactive cells were also confirmed by immunofluorescent staining, which used Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) as the secondary antibody at a dilution of 1:500 in blocking solution. These sections were washed and mounted in VECTA shield fluorescence mounting medium (Vector Laboratories) and then images were captured using a confocal laser scanning microscope (Olympus FV 1000; Olympus, Tokyo, Japan).

Positive and negative controls. In order to confirm the specific binding of antibody, positive and negative controls experiments were conducted. Standard tissue sections from breast cancer donated from the Songklanagarind hospital, Thailand, were used as the positive control for ER α because of the well-accepted presence of ER α in breast cancer cells. The negative control sections were processed in the same way except for the primary antibodies being replaced with pre immune sera.

Semiquantitative scoring and staining intensity. The staining intensities in the ovarian tissue were assessed by visual observation with following scores: – No immunoreactivity, + weak immunoreactivity, ++ moderate immunoreactivity and +++ strong immunoreactivity. At the same time, the intensity value of immunostaining was measured from three slides/10 areas/sample by an automated cellular image analysis system, Digimizer software, version 3.7.0. The grade of intensity was divided into 4 categories: Negative (1.00), weak intensity (0.720-0.999), moderate intensity (0.292-0.719) and strong intensity (0.00-0.291), as following the standard guideline of Qasi *et al.* (2011).

RESULTS AND DISCUSSION

Gravimetric analysis and ovarian development. Wild female *R. brachysoma* collected during the spawning season had an average GSI value of 1.65 ± 0.15 (mean $\pm$ SD). The ovarian developmental stage of our specimens was considered to be the late developmental stage, which comprised of prominent secondary growth oocytes. The

secondary growth oocytes were characterized by reddish-stain with acidophilic staining due to the accumulation of yolk deposition in the ooplasm. Large lipid globules were also observed in *Crenichthys baileyi* and *Empetrichthys latos* (Uribe *et al.*, 2012).

Histological organization of *R. brachysoma* brain.

Five major regions of the brain (telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon) have been previously described in *R. brachysoma* (Senarat *et al.*, 2016). However, the location of the diencephalon in the pituitary gland under transverse serial sections was not reported in detail in the earlier report (Senarat *et al.*, 2016). Prior to analyzing the protein distribution by immunohistochemistry, in this study, we further identified three nuclei in the diencephalon using detailed histological analyses on the brain of wild specimens ($n = 56$): The nucleus periventricularis, nucleus preopticus and nucleus lateralis tuberis. Based on the MT and PAS staining methods, neuronal populations in the nucleus preopticus-periventricularis were clearly distinguished from glial cells and were classified into three groups of different sizes: Small, medium and large (Figures 1A, 1B, 1D, 1F). The diameter of these neurons (calculated from randomly selected 10 cells per section) were $7.84 \pm 0.26 \mu\text{m}$, $30.6 \pm 2.11 \mu\text{m}$ and $50.9 \pm 1.36 \mu\text{m}$, respectively (mean $\pm$ SD). On the other hand, the glial cells represented the most abundant cells, which were distinguished from neurons by their small nuclei and surrounded by eosinophilic cytoplasm (Figures 1D and 1F).

The nucleus periventricularis (NPI) was located in the ventral part of the hypothalamus (Figures 1B and 1C). Only small neurons arranged in 12–15 layers were found in this region. The nucleus preopticus (Np) was located at the ventro-lateral region of the mesencephalon, where medium- and large-sized neurons were observed (Figures 1D and 1E). The nucleus lateralis tuberis (NLT) was found at the inferior lobe of the hypothalamus (i.e., in the ventral part of the nucleus periventricularis). The sagittal plane of this structure contained neurons of all sizes (small, medium and large; Figures 1F and 1G). A previous observation showed that all populations were positively reacted with anti-GnRH-like peptide antibody in *R. brachysoma* (Senarat *et al.*,

2019), and at present it is unclear how the functions of these three neuronal populations are different. Since these three nuclei are important regions for GnRH production in *R. brachysoma* (Senarat *et al.*, 2019) as well as other fishes (Peter *et al.*, 1991; Palmieri *et al.*, 2008), further investigation on these three types of neurons are required.

The pituitary gland was located within the diencephalon and it appeared to be attached to the ventral region of the hypothalamus. The overall histological structure of the pituitary gland of *R. brachysoma* consisted of two components: the adenohypophysis and the neurohypophysis. The adenohypophysis consisted of a cluster of cells and also divided into three distinguishable areas; the rostral pars distalis, the proximal pars distalis and the pars intermedia (Figure 2 and schematic diagram 2A). Furthermore, penetration of the neurohypophysial process to the adenohypophyseal area was observed. The identification of cell types in the adenohypophysis using H&E, MT, and PAS staining methods did not easily identify the level of hormones in each cell. However, these methods could classify the pituitary cells into four types: acidophil I, acidophil II, basophil and chromophil, each of which had different distributions in the adenohypophyseal area (Figures 2B and 2J). Acidophil I and II cells were the major component in rostral pars distalis. The acidophil I cells were stained in red-orange and were mainly located in the border of the neurohypophysis; they had oval shape nuclei. The acidophil II cells were relatively small when compared with acidophil I. These cells were localized in the central – ventral parts of RPD. They had spherical nuclei and were stained in red in the MT stain. The acidophil I, II and basophil cells were commonly observed in proximal pars distalis. It is well known that basophils are considerably homologous to the somatotrope and gonadotrope, as previously described in other teleosts (Shimizu *et al.*, 2003; Yan and Thomas, 1991). The important role of gonadotrope is to produce GTHs (Shimizu *et al.*, 2003). Hence, the activity of estrogens could play a crucial role in the regulation via ER receptor for estrogen feedback. This pathway then controls LH secretion in the pituitary gland (Gieske *et al.*, 2008).

Figure 1. Schematic diagrams of the brain (Br) (A) and light photomicrographs of three nuclei in the diencephalon: nucleus periventricularis (NPI, B-C), nucleus preopticus (Np, D-E) and nucleus lateralis tuberculi (NLT, F-G). Sn = small-sized neuron, Msn = medium-sized neuron, Lsn = large-sized neuron. Gc = glial cells, Ol = optic lobe, Cb = cerebellum. Scale bar: 50 μm (B), 100 μm (D, F).

Figure 2. Schematic diagram of the pituitary gland (A) and light photomicrographs of pituitary histology stained using MT (B-F) and H & E (H-J) with composed of two distinct areas including the adenohypophysis (Ap) and the neurohypophysis (Np). Ac1 = Acidophilic cell type 1, Ac2 = Acidophilic cell type 2, Bc = basophilic cell, Bv = blood vessel, Cc = chromophilic cell, Gc = granular cord, Hy = hypothalamus, PI = pars intermedia, PPD = proximal pars distalis, RPD = rostral pars distalis. Scale bar: 50 μ m (B, D, F, H, I, J).

Immunohistochemical localization of ER α in the brain. No ER α -ir cells were found in the telencephalon (Figure 3A), whereas all other regions (diencephalon, mesencephalon, metencephalon and myelencephalon) tested contained ER α -ir cells. In the mesencephalon, intense ER α signals were observed in the cytoplasm of the neurons, while nucleus was only weakly stained (Figures 3B-3C). Dense ER α -ir fibers were found in the stratum opticum, stratum fibrosum et griseum superficiale and stratum album centrale of the optic tectum (Figure 3D). The strong expression of ER α in the optic tectum suggests the potential role of estrogen in the visual system, although further study is needed to understand the function. In the diencephalon, strong immunoreactivity was observed in small-, medium- and large-sized neurons of the nucleus lateralis tuberis (Figure 3E).

Immunoreactivity of ER α in the metencephalon was located in the Purkinje cell layer in both the corpus cerebelli (Figures 3F-3H) and valvula cerebelli (Figure 3I). Intense ER α immunoreactivity was also observed in the granular layer of metencephalon (Figure 3G). In addition, the stained structures were dendrites of Purkinje cells in both the valvula cerebelli (Figure 3I) and corpus cerebelli under high magnification (Figures 4A-4C). Lastly, the ER α immunoreactivity was detected in the neurons of the medullar oblongata (Figures 4D-4H) and in the gonadotropic cells of the pituitary gland (Figures 4F-4G).

Immunohistochemical localization of ER α and GTHs in *R. brachysoma* ovaries. The overall histological analysis on the ovary showed that our specimens contained oocytes in several developmental stages, including previtellogenic stage, early vitellogenic stage and late vitellogenic stage (Figure 5A). Weak immunoreactivity of ER α was observed in follicular cells of oocytes in early and late vitellogenic stages (Figure 5B, Table 1). Interestingly, an intense GTHs immunoreactivity was observed in follicular cells of oocytes (Figures 5C-5H). The intensity of LH and FSH

immunoreactivities measured by the Digimizer software was generally consistent with visual observation (Figures 5F-5H, Table 1).

Positive and negative controls. For the positive controls, ER α immunoreactivity was observed in breast cancer sections (Figure 6A). No immunoreactivity of ER α was detected in the brain in negative control experiments (Figures 6B-6D). There were no FSH- and LH-ir cells observed in the negative control sections of the ovarian tissue (Figures 6E-6G).

In this study, rabbit polyclonal antisera against ER α was used for the identification of ER α in the brain and ovary of *R. brachysoma* with appropriate positive and negative controls described above (see Figure 6). The positive reactions were found in different regions of *R. brachysoma* brain including the diencephalon and the metencephalon (Figures 2-3). These distribution patterns of ER α are quite similar to those in other teleosts, for example *Macropodus opercularis* (Linnaeus, 1758) (Davis *et al.*, 1977), *Carassius auratus* Linnaeus, 1758 (Kim *et al.*, 1978) and *O. mykiss* (Anglade *et al.*, 1994), as well as in higher vertebrates (Morrell *et al.*, 1975), indicating that estrogenic actions take place in the brain of *R. brachysoma* to support the reproductive performance. Specifically, an intense reaction of ER α was observed in the nucleus preopticus of the diencephalon in this study as also reported in several fish species including *O. mykiss* (Anglade *et al.*, 1994) and *Oryzias latipes* (Zempo *et al.*, 2013). It would be of interest to explore whether ER α exerts specific functions in the nucleus preopticus. We also observed intensive ER α staining in Purkinje cells in the Purkinje layer, in neuronal cells in the granular layer, and some layers of the optic tectum and medullar oblongata. These results identified the potential brain regions involved in the E₂ pathway for *R. brachysoma*, although further studies are required to explore its function. The localization of ER α in the central and peripheral nervous systems has also been reported in *Porichthys notatus* (Forlano *et al.*, 2005).

Figure 3. Schematic diagram (upper) and light micrographs of the estrogen receptor α immunoreactivity (lower) of *Rastrelliger brachysoma* brain. (A) telencephalon, (B-D) mesencephalon, (E) diencephalon, (F-I) metencephalon. 1 = stratum marginale, 2 = stratum opticum, 3 = stratum fibrosum et griseum superficiale, 4 = stratum album central, 5 = stratum griseum central, 6 = stratum periventriculae, Cc = corpus cerebelli, Ct = cytoplasm, Gl = granular layer, Mc = mesencephalon, Ml = molecular layer, Nf = neuronal fibers, NLT = nucleus lateralis tuberis, Nu = neurons, Nuc = nucleus, Pc = Purkinje cells, Po = pre-optic area, Tc = telencephalon, Vc = valvula cerebelli. Scale bar, A-E, G-I = 50 μ m; F = 30 μ m.

Figure 4. Light micrographs of the distribution of estrogen receptor α immunoreactivity in the metencephalon (A-C), myelencephalon (D-E) and pituitary gland (F-G) of *Rastrelliger brachysoma*. Cc = corpus cerebelli, Ic = immunoreactive cells, Gl = granular layer, Me = myelencephalon, Ml = molecular layer, Nf = neuronal fibers, Nu = neurons, Pc = Purkinje cells, Pg = pituitary gland. Scale bar, H = 200 μm ; C, E = 100 μm ; A, D, I = 50 μm ; F, G = 20 μm .

Figure 5. Light photomicrograph showing the ovarian histology stained with MT (A) and ER α , FSH and LH immunoreactivities in the ovarian tissue. B: ER α ; C-E: LH; F-H: FSH. Ev = early vitellogenic stage, Fc = follicular cell, Lv = late vitellogenic stage, P = peirucleolar stage, Z = zona pellucida. Scale bar; 20 μ m (A-H).

Table 1. Summary of the presence of ER α -ir, LH - ir and FSH - ir in oogenic stages and their follicular cells of *Rastrelliger brachysoma*.

Immunoreactivity	Steps of oogenic stages and follicular cells			
	Oocyte (Ev)	Fc (Ev)	Oocyte (Lv)	Fc (Lv)
ER α -ir	-	+ Weak (0.84 $\pm$ 0.04)	-	+ Weak (0.79 $\pm$ 0.04)
LH - ir	+ Weak (0.75 $\pm$ 0.17)	++ Moderate (0.67 $\pm$ 0.08)	+ Weak (0.78 $\pm$ 0.03)	++ Moderate (0.67 $\pm$ 0.08)
FSH - ir	+ Weak (0.74 $\pm$ 0.05)	++ Moderate (0.61 $\pm$ 0.03)	+ Weak (0.79 $\pm$ 0.08)	++ Moderate (0.70 $\pm$ 0.11)

Note- + weak immunoreactivity, ++ moderate immunoreactivity, +++ strong immunoreactivity, - no immunoreactivity (Fc = follicular cells, Ev = early vitellogenic stage, Lv = late vitellogenic stage)

Figure 6. Light photomicrograph showing the positive control of ER α immunoreactivity on breast cancer sections (A) and negative controls (B-H). Ca = cortical alveoli stage, Cb = cerebellum, Fc = follicular cell, Hy = hypothalamus, Lv = late vitellogenic stage, Ot = optic tectum, P = perinucleolar stage, Z = zona pellucida.

In vertebrates, E₂ has multifunctional roles such as gonadal development and uptake of vitellogenin into the oocytes (Gustafsson, 2003; Hess, 2003; Heldring *et al.*, 2007). Our current and previous (Senarat, 2015) results suggest that E₂

also functions in the brain, possibly in relation to GnRH release, because ER α immunoreactivity was detected in the sbGnRH-ir cells. Although there are few reports regarding the physiological roles of this colocalization (Forlano *et al.*, 2005;

Oakley *et al.*, 2009), the existence of the E₂ feedback to GnRH-producing cells has been proposed. It is currently difficult to figure out whether the feedback is positive or negative (Sower, 2005; Oakley *et al.*, 2009; Kenealy and Terasawa, 2012), but the involvement of GABA and/or Kisspeptide has been also proposed as the mechanism for the interaction of E₂ and GnRH (Sower and Baron, 2005). In further works, it would be of interest to investigate the co-localization of GABA, GnRH, kisspeptin and ER in the brain to determine the consequences of their interactions.

Another important observation of the current work relates to the moderate immunoreactivity of LH and FSH detected in the follicular cells in vitellogenic stages compared to those in early oocyte differentiation stages (i.e., oögonia to lipid and cortical alveoli stage). These results are consistent with a previous hypothesis that FSH, but not LH, stimulates vitellogenin uptake to follicular cells in rainbow trout (Nagahana, 1994). FSH is also considered to be involved in the testosterone synthesis and its conversion to E₂ by aromatase in follicular cells, which further stimulates the final oocyte maturation. We assume that ER α , FSH and LH coordinately control the oocyte maturation in *R. brachysoma* ovary. However, this hypothesis needs to be tested in future studies that employ detailed observation of the activity and expression levels of enzymes involved. It is also important to use *R. brachysoma* specimens of various maturation stages since the present study used only those in the spawning season.

In this study, we demonstrated the immunolocalization of ER α in the brain of *R. brachysoma*, which indicates that the brain is one of the target organs of estrogens. We also detected ER α and GTHs (FSH and LH) in the follicular cells in the ovary of *R. brachysoma*. To our knowledge, this is the first clear-cut evidence of GTHs-ER α expression in follicular cells, which suggests that follicular cells have a more complicated function than previously thought. Detailed functional analyses on follicular cells will help us understand the reproductive physiology of *R. brachysoma*.

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