Available online at www.sciencedirect.com
ScienceDirect
Progress in Natural Science 18 (2008) 1453–1457
Progress in Natural Science
www.elsevier.com/locate/pnsc

Short communication

Immunocytological evidence for the presence of vertebrate FSH- and LH-like substances in the brain and thoracic ganglion of the swimming crab, *Portunus trituberculatus*

Huiyang Huang, Haihui Ye *, Shaojing Li, Guizhong Wang

Department of Oceanography, Xiamen University, Xiamen 361005, China

Received 21 January 2008; accepted 26 February 2008

Abstract

Investigating the distribution of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in the swimming crab, *Portunus trituberculatus*, helps in the understanding of its reproductive regulation mechanism. This study has detected the location of vertebrate FSH- and LH-like substances in the brain and the thoracic ganglion mass with immunocytochemical techniques. Their immunoreaction is very weak or negative during the immature period and the post-spawning period, while it is stronger in the mature period. Localization of FSH-like immunoreactive cells resembles that of LH-like cells. These immunoreactive cells were observed chiefly in the anterior median cell-cluster of the protocerebrum with stronger immunostaining. There were only a few in the inboard cell-cluster of the olfactory lobe of the deutocerebrum and the posterior cell-cluster of the tritocerebrum. Immunoreactive cells were located in all the three ganglia in the thoracic ganglion mass. These results indicated that substances resembling the vertebrate FSH and LH exist in *P. trituberculatus*, and their expression has some correlation with the developmental stage of ovaries, thus they might engage in the ovary development and ovulation.

Keywords: Follicle stimulating hormone (FSH); Luteinizing hormone (LH); *Portunus trituberculatus*; Brain; Thoracic ganglion mass; Immunocytochemistry

1. Introduction

The swimming crabs, *Portunus trituberculatus* (Crustacea: Decapoda: Brachyura), that are widely distributed in the coastal waters of China, Korea and Japan, reside in the benthal habitats with sand or pebbles. As one of the most common edible crabs, this species has been artificially propagated and cultured [1–4]. Owing to its commercial value, the reproductive biology of *P. trituberculatus* has been investigated by many researchers [5–11]. The reproduction of crustacean is known to be regulated by the endocrine and neuroendocrine mechanisms [12–14]. However, there have been few studies on the endocrinology of *P. trituberculatus*. Only Qin et al. investigated the structures of the Y-organ and the mandibular organ [15].

In vertebrates, the gonadotropins (GTHs), secreted by the adenohypophysis, are composed of the follicle stimulating hormone (FSH) and the luteinizing hormone (LH). These two hormones can combine with their corresponding receptors on the cytoplasmic membranes of gonadal cells, and then stimulate the gonadal development as well as the production of the steroid hormones (estrogen and androgen). Thus, they play an important role in spermatogenesis and oogenesis [16]. The function of FSH and LH is known relatively well in mammals, but it is much less known in other vertebrates, and it is nearly unknown in crustacean. It was reported by Zukowska that mammalian

* Corresponding author. Tel./fax: + 86 592 2185539.
E-mail address: haihuiye@xmu.edu.cn (H. Ye).

1002-0071/$ - see front matter © 2008 National Natural Science Foundation of China and Chinese Academy of Sciences. Published by Elsevier Limited and Science in China Press. All rights reserved.
doi:10.1016/j.pnsc.2008.02.016

FSH and LH have a stimulating effect on ovaries of sand shrimp *Crangon crangon*, FSH causing growth in a number of somatic cells of the ovary, and LH-meiosis in the sex cells [17]. Recently, vertebrate FSH and LH-like substances have been discovered in the brain of mud crab *Scylla serrata* [18]. With the immunocytochemical techniques, this study shows that vertebrate FSH and LH, relevant with the development of the ovary, have also been detected in the brain and the thoracic ganglion mass of *P. trituberculatus*. This study might be meaningful in understanding the neuroendocrine function in the reproduction of *P. trituberculatus*.

2. Materials and methods

2.1. Samples

The samples include three stages of *P. trituberculatus*: the immature period (milk-white ovaries), the mature period (orange ovaries) and the post-spawning period (most of the ova have been discharged). Five crabs were investigated in each stage. Female crabs were obtained from local vendors in Xiamen and the hatchery in Zhao'an County, Fujian Province.

The brains and thoracic ganglion mass were dissected free, and fixed in 10% neutral formalin solution for 4–6 h at 4 °C. The tissues were then embedded in paraffin after routine dehydration in alcohol and clearing in xylene. Serial sagittal and cross sections of 8 μm were mounted on clear glass slides.

2.2. Main reagents

Monoantibodies against vertebrate FSH and LH were products of Santa Cruz Company. Strept avidin–biotin-complex (SABC) kit was purchased from Wuhan Boster Biological Technology LTD. 3′,3′-Diaminobenzidine (DAB) was purchased from Sigma.

2.3. Immunocytochemistry staining

To detect FSH and LH, the sections were immunocytochemically stained by SABC method. The following procedure was performed. Sections were (1) incubated in 3% H_2O_2/PBS for 10 min to inactivate endogenous peroxidase at room temperature, then rinsed in PBS (pH 7.4) for 5 min; (2) incubated in normal goat serum (1:10 dilution) for 10 min to reduce non-specific binding at room temperature; (3) incubated in primary antisera for 1.5 h at 37 °C, then rinsed three times in PBS for 15 min; (4) incubated in biotinylated goat anti-rabbit IgG for 0.5 h at 37 °C, then rinsed three times in PBS for 15 min; (5) incubated in strept avidin-biotin-complex for 0.5 h at 37 °C, then rinsed three times in PBS for 15 min; (6) in 0.06% DAB-0.03% H_2O_2 for 5–10 min, the sections were rinsed thoroughly in tap water, then dyed in hematoxylin, dehydrated in alcohol, cleared in xylene and coverslipped; and (7) observed and photographed with an Olympus BH-2 microscope.

Two negative controls were included: (1) replacing the primary antibody with normal rabbit serum and (2) omitting the primary antibody in the reaction.

Three categories of immunoreaction were defined according to staining results: strong, weak, and none. They are indicated by brown, yellow, and none colors in the cytoplasm.

Table 1
Immunoreactive intensity of FSH and LH of *P. trituberculatus* in three different stages of ovary development

	FSH		LH	
	Brain	Thoracic ganglion mass	Brain	Thoracic ganglion mass
Immature period	+	+	–	–
Mature period	++	++	+	+
Post-spawning period	–	–	–	–

++ denotes strong immunoreaction; + denotes weak immunoreaction; – denotes no immunoreaction.

3. Result

3.1. Immunoreactive intensity of FSH and LH

Among the three developmental periods of the ovary, crabs in the mature period expressed stronger immunoreactive intensity of FSH- and LH-like substances. Crabs in the other two stages showed weak or even negative immunoreaction. In general, the intensity of immunoreaction for LH is weaker than that of FSH. The results are listed in Table 1.

3.2. Localization of FSH- and LH-like immunoreactive cells

Neurons in the brain and thoracic ganglion mass were classified into three types based on their sizes: small-, medium-, and large-sized neurons, which ranged about 15–20, 30–60, and 70–120 μm, respectively. The immunoreactivity was detected in small- and medium-neurons and not in the large neurons. The immunoreactivity was located in the cytoplasm of the neurons, not in the fibers surrounding the cell clusters. FSH- and LH-like immunoreactive cells were distributed in similar locations in the brain and in the thoracic ganglion mass of *P. trituberculatus*.

The brain constitutes of three parts: the protocerebrum, the deutocerebrum and the tritocerebrum. FSH- and LH-like immunoreactive cells were located mainly in the anterior median cell-cluster of the protocerebrum (Fig. 1a and b). Fewer immunoreactive cells were observed in the inboard cell-cluster of the olfactory lobe of the deutocerebrum (Fig. 1c), as well as in the posterior cell-cluster of the tritocerebrum (Fig. 1d).

The thoracic ganglion mass is formed by the fusion of the subesophageal ganglion, the thoracic ganglion and

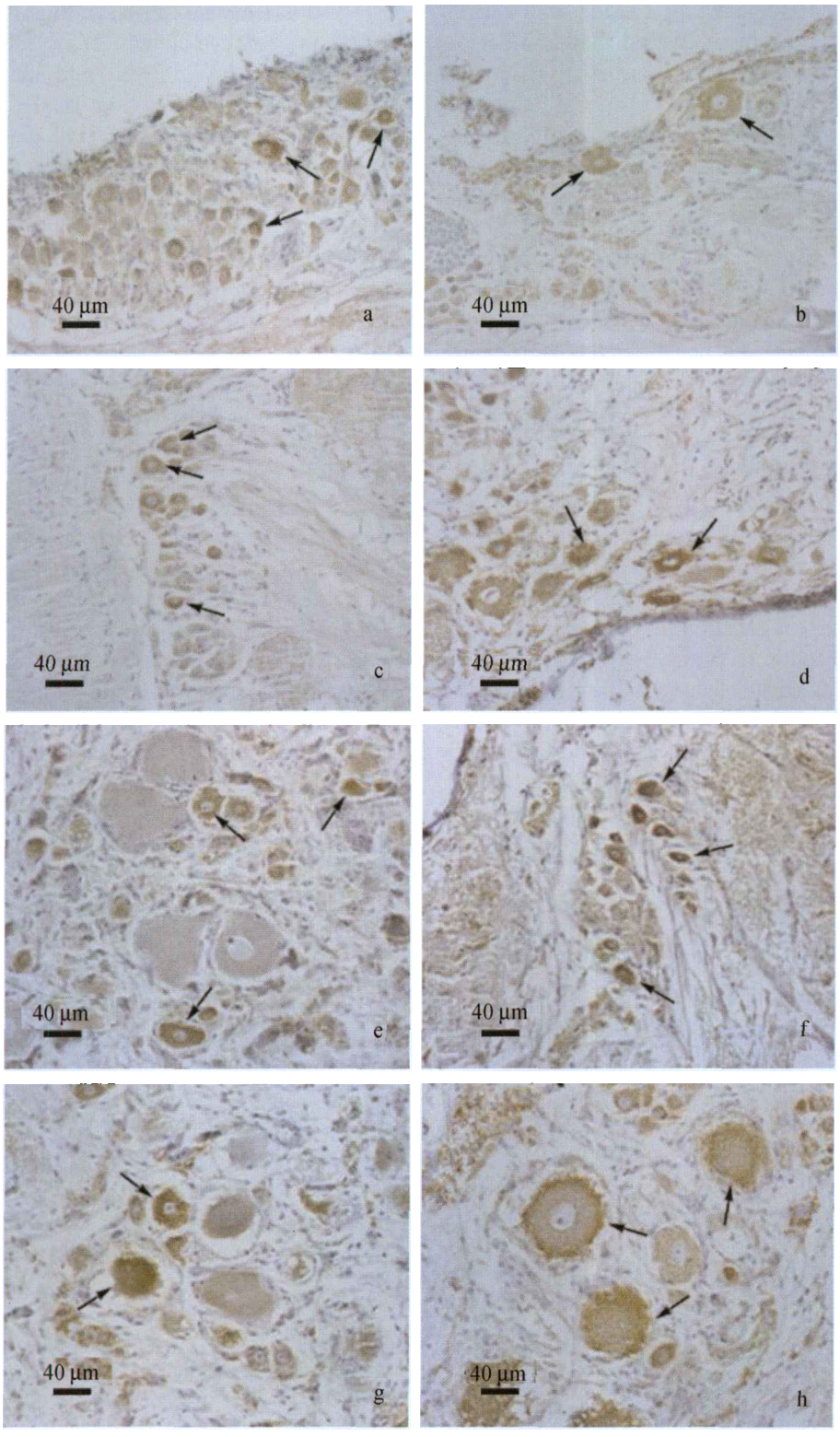

Fig. 1. FSH and LH immunoreactive cells in *Portanus trituberculatus*.

the abdominal ganglion. In the subesophageal ganglion, immunoreactive cells were often observed in the median part and in the sides of the ganglion (Fig. 1e). A large number of FSH- and LH-like immunoreactive cells were closely packed in the thoracic ganglion (Fig. 1f and g), and they were more dispersed in the abdominal ganglion (Fig. 1h).

4. Discussion

Hormonal regulation of breeding has been found in all higher groups including crustaceans [12–14]. It has been stated that mammalian FSH and LH could promote the growth of the oocytes and the meiosis in the germ cells in sand shrimp *Crangon crangon*. The response of ovaries to

exogenous FSH and LH indicates that GTH may act on the crustaceans [17]. It remains unknown that whether or not the crustaceans can themselves synthesize FSH and LH, and whether these two hormones engage in the neuroendocrine regulation on reproduction. Our results in this paper have answered the first question, i.e. crustaceans are capable of synthesizing vertebrates GTH-like substance. Associating with the immunostaining of the vertebrate FSH and LH in the brains of the mud crab *Scylla serrata* [18], the cockroach *Periplaneta Americana* [19] and the termite *Reticulitermes aculabialis* [20], we consider that GTHs probably participate in the neuroendocrine regulation on reproduction in arthropods.

There have been intermittent reports of the presence of a gonad stimulating hormone (GSH) in the brain, and in the thoracic ganglion of different crustaceans, but up to now it has not been characterized.[21,22]. In our results, FSH- and LH-like immunoreactive cells, particularly in the protocerebrum of *P. trituberculatus*, are identical to that of *S. serrata*. The thoracic ganglion mass of brackyuras is fused by the subesophageal ganglion, the thoracic ganglion and the abdominal ganglion. It is the first report of FSH- and LH-like immunoreactive cells detected in the thoracic ganglion mass in crustaceans. Only the thoracic ganglion of crustaceans has long been regarded as reproduction regulator [12–14]. However, in this investigation, it is shown that vertebrate FSH- and LH-like immunoreactive cells were also present in the subesophageal ganglion and in the abdominal ganglion of P. trituberculatus as well. Whether or not these two ganglia also engage in the reproductive regulation? It should be one important aspect of crustacean endocrinology for future research.

In female vertebrates, FSH can hasten the growth, development and maturation of follicles, as well as promote the granulosa cells in the ovary to produce estrogen and induce the sensitivity of ova to other hormones, while LH can impel the release of mature ova from ovarian follicles [16]. Some gonadotropins (their chemical structures unclear) have also been found in the brains of insects. These hormones can modulate the maturation of gonads, sperms and ova of insects, and they can induce the ovary and testis to synthesize ecdysone. Ecdysone, in turn, can promote the synthesis of vitelloginin in fat-body tissues, and promote the differentiation of follicle cells or spermatogenic cells [23].

The immunoreaction of vertebrate FSH and LH in *P. trituberculatus* was stronger during the mature period and weaker in the immature period and the post- spawning period. This difference, correlating with specific ovary stages, indicated that these two hormones might be linked to the ovary development and spawning. Their weaker expression during the immature period may owe to an inactive phase of oogenesis and that the yolk had not been produced in great amount [11]. In the mature period, a great deal of yolk was synthesized and accumulated by the oocytes, which have rapidly increased in volume and become mature ova [11]. The high expression of vertebrate FSH and LH during this stage might indicate their participation in the development of oocytes, the generation of yolk, and the development and maturation of follicles. After spawning, the ova were largely discharged from the ovary, and then the ovary was degenerated [11]. After FSH and LH had fulfilled their function in the ovary maturation and ovulation, they showed a very weak expression as no immunoreaction was detected in our experiment. The immunoreaction expressions of vertebrate FSH and LH in *P. trituberculatus* resemble those in the vertebrates [16] and in the insects [19,20]. The results indicate a possible function of FSH and LH on the ovary in *P. trituberculatus*. It deserves further investigation as to whether GTH shares similar functions between the vertebrates and crustaceans.

There are three hypotheses about the secreting cells of FSH and LH in the vertebrates. FSH and LH are considered to be generated by the same kind of cells (two hormones from one single cell type), or two separate kinds of cells in the pituitary (each hormone from a different cell type), and GTH cells in the pituitary are regarded including FSH cells, LH cells and FSH-LH cells (two hormones from three types of cells) [24]. The locations of FSH- and LH-like immunoreactive cells are quite similar in our study; sometimes both showed immunoreaction in the two adjacent sections. These phenomena imply that there may be one kind of cells secreting both the hormones, or there are mono-hormone cells of FSH or LH in the nervous system of *P. trituberculatus*. The double immuno-labelling method should be used to recognize the FSH- and LH-like cells in *P. trituberculatus* in the future.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant Nos. 40406030 and 40776084) and by the Program for New Century Excellent Talents in Fujian Province University. The authors would like to thank Professor Paul K. Chien, Department of Biology, University of San Francisco, for his detailed comments and fruitful suggestions on this paper.

References

[1] Nogami K, Hamasaki K, Maeda M, et al. Biocontrol method in aquaculture for rearing the swimming crab larvae *Portunus trituberculatus*. Hydrobiologia 1997;358:291–5.

[2] Takeuchi T, Satoh N, Sekiya S, et al. Influence of the different feeding schedule of food organisms for larval swimming crab *Portunus trituberculatus*. Nippon Suisan Gakkaishi 1999;65(5):804–9.

[3] Arai D, Hamasaki K, Maruyama K, et al. Relationship between mass mortality of megalops and morphogenesis of zoeas in the seed production of swimming crab *Portunus trituberculatus* (Brachyura: Portunidae). Nippon Suisan Gakkaishi 2004;70(4):567–72.

[4] Hamasaki K, Fukunaga K, Kitada S. Batch fecundity of the swimming crab *Portunus trituberculatus* (Brachyura: Portunidae). Aquaculture 2006;253(1–4):359–65.

[5] Hamasaki K, Imai H, Akiyama N, et al. Ovarian development and induced oviposition of the overwintering swimming crab *Portunus*

trituberculatus (Brachyura: Portunidae) reared in the laboratory. Fisheries Sci 2004;70(6):988–95.

[6] Xue JZ, Wu HX. The mechanisms and correlated morphological character of the egg attachment of the swimming crab *Portunus trituberculatus*. Acta Zool Sinica 2004;50(5):873–9, [in Chinese].

[7] Zhu DF, Wang CL, Yu HW, et al. Morphological and structural changes of sperm during the acrosome reaction in the swimming crab *Portunus trituberculatus*. Acta Zool Sinica 2004;50(5):800–8007, [in Chinese].

[8] Zhu DF, Wang CL, Yu HW. Oogenesis, oocyte activation and early cleavage of *Portunus trituberculatus* (Miers). Oceanol et limnologia sinica 2005;36(5):421–9.

[9] Zhu DF, Wang CL, Xue LY, et al. Observation on the sperm–egg interaction of *Portunus trituberculatus* by scanning electron microscopy. Acta Zool Sinica 2006;52(1):196–201, [in Chinese].

[10] Yang F, Xu HT, Dai ZM, et al. Molecular characterization and expression analysis of vitellogenin in the marine crab *Portunus trituberculatus*. Comp Biochem Physiol B Biochem Mol Biol 2005;142(4):456–64.

[11] Wu XJ, Yao GG, Yang XZ, et al. A study on ovarian development of *Portunus trituberculatus* in the East China Sea during the first reproductive cycle. Oceanol et limnologia sinica 2007;29(4):120–7, [in Chinese].

[12] Fingerman M. Endocrine mechanism of crustacean. J Crust Biol 1987;7(1):1–24.

[13] Fingerman M. Crustacean endocrinology: a retrospective, prospective and introspective analysis. Physiol Zool 1997;70(3):257–69.

[14] Huberman A. Shrimp endocrinology: a review. Aquaculture 2000;191(1–3):191–208.

[15] Qin K, Kang XJ, Guo MS, et al. Microstructures and ultrastructures of Y-organ and mandibular organ in *Portunus trituberculatus*. J Oceanogr Taiwan Strait 2007;26(1):76–84, [in Chinese].

[16] Chen D. Fertilization biology. Beijing: Science Press; 2000, p. 20–22.

[17] Zukowska A. Effect of hypophyseal gonadotropins (FSH and LH) on the ovaries on the sand shrimp *Crangon crangon* (Crustacea: Decapoda). Mar Biol 1981;63(3):241–8.

[18] Ye HH, Huang HY, Li SJ, et al. Immunorecognition of vertebrate FSH and LH in brain of *Scylla serrata*. Prog Nat Sci 2006;16(6):768–70, [in Chinese].

[19] Verhaert P, De Loof A. Substances resembling peptides of the vertebrate gonadotropin system occur in the central nervous system of *Periplaneta americana*: immunocytological and some biological evidence. Insect Biochem 1986;16(1):191–7.

[20] Su XH, Xi GS, Li K. Immunocytochemical localization of gonadotropins in termite brains. Acta Zool Sinica 2004;50(2):240–4, [in Chinese].

[21] Yano I. Ultraintensive culturte and maturation in captivity of penaeid shrimp. CRC handbook of mariculture crustacean aquaculture, 2nd ed., vol. 1. Boca Raton: Florida CRC Press; 1993. p. 89–313.

[22] Liao JY, Zhang Y, Sun JX, et al. Partial isolation and bioassay of gonad stimulating hormone from the brain of *Macrobrachium rosenbergii*. J Fisheries China 2001;25(1):5–10, [in Chinese].

[23] De Loof A, Baggerman G, Breuer M, et al. Gonadotropins in insects: an overview. Arch Insect Biochem Physiol 2001;47(3):128–9.

[24] Cui S, Xia GL. Cellular type of pituitary gonadotropes and their changes during sheep estrous cycle. J China Agric Univ 1998;3(5):114–8, [in Chinese].