Chinese Journal of Oceanology and Limnology
Vol. 24 No. 2, P. 142-146, 2006

In vitro study of neuroendocrine regulation over the testicular development in mud crabs S*cylla serrata**

YE Haihui (叶海辉)[†,††,**], HUANG Huiyang (黄辉洋)[†], LI Shaojing (李少菁)[†]
WANG Guizhong (王桂忠)[†], LI Qifu (李祺福)[††]
([†]*Department of Oceanography, Xiamen University, Xiamen 361005, China*)
([††]*Laboratory of Cell Biology, School of Life Science, Xiamen University, Xiamen 361005, China*)

Received July 11, 2004; revision accepted June 19, 2005

Abstract The reproductive endocrine activities of neuroendocrine organs and androgenic glands (AG) in male *Scylla serrata* were investigated with co-incubation technology. *In vitro* studies show that: (1) the AG in Stage Ⅲ can significantly accelerate the development of seminiferous tubules and spermic maturation; (2) the brain, thoracic ganglia and optic ganglia have no direct influence over the testicular development; (3) the brain and thoracic ganglia can significantly promote the growth of the AG cells and highly significantly boost the ratio of Type B cells, while the optic ganglia have no such effects. It is the first time for *in vitro* investigations to confirm that the brain and thoracic ganglia can regulate testicular development through AG in male crustaceans.

Key words: *Scylla serrata*; androgenic gland; neuroendocrine organs; reproductive endocrine; *in vitro* studies

1 INTRODUCTION

Ovarian development of female crustaceans is under the direct regulations of two neurohormones: gonad-inhibiting hormone (GIH) secreted by optic ganglia and gonad-stimulating hormone (GSH) secreted by brain and thoracic ganglia (Fingerman, 1997; Huberman, 2000). *In vitro* studies on *Uca pugilator*, *Procambarus clarkii* and *Scylla serrata*, have clarified that GIH and GSH have direct influence on the ovary, i.e. the ovary is the target organ of GIH and GSH (EastmanReks and Fingerman, 1984; Kulkarni et al., 1991; Jin et al., 2003). Male crustaceans are different from the females in that they have special endocrine glands—a pair of the androgenic glands (AG), which are correlative with male differentiation. It has been found that the morphology of AG would be changed significantly in male crustaceans with their nerve organs ablated or transplanted, or with the extracts of the nerve organs injected. Therefore it is concluded that GIH and GSH promote the testicular development through AG (Fingerman, 1997; Adiyodi and Adiyodi, 1970). However, it is also suggested that GIH and GSH may act directly on the testes (Payen, 1980). *S. serrata* is one kind of commercially important marine crabs in China. Whether the neurohormones regulate the testes directly or through AG indirectly is an ambiguous question. Based on our previous study on the development of AG and its secretion in *S. serrata* (Ye et al., 2003a, b), we examined a step further the reproductive endocrine in male *S. serrata* by co-incubation. Our present investigation indicates that the brain and thoracic ganglia in *S. serrata* promote the testicular development through AG. It is the first time for *in vitro* experiments to clarify that the brain and thoracic ganglia regulate testicular development through AG in male crustaceans.

2 MATERIALS AND METHODS

* This work was supported by the National Natural Science Foundation of China (No.30300269) and the Fujian Province Key Foundation Research Program (1998-2002).
** Corresponding author: haihuiye@163.com

2.1 Animals

Male mud crabs, *S. serrata*, with carapace length (c. l.) of 56–85 mm and body weight of 115–430 g were purchased from local vendors in Xiamen. They were then reared for 2–4 days in aquatic tanks containing sea water with good aeration and fed with fresh clams, *Ruditapes philippinensis*. The developmental stages of the testes and the AG were determined in morphology and histology (Ye et al., 2002; 2003a)

2.2 *In vitro* incubation

Experimental conditions: the conditions of dissection and incubation followed by Jin et al. (2003).

Incubation mode: the testicular piece about 1 m^3 volume in each vial was incubated with either the tissues from two AGs, one brain, two thoracic ganglia, two optic ganglia of one crab, or in the case of the control a piece of muscle approximately the same size as the other tissues.

The AG section (including the spermaducts) of about 5 mm in each vial was incubated with either the tissues from one brain, two thoracic ganglia, two optic ganglia of one crab, or in the case of the control a piece of muscle approximately the same size as the other tissues.

The experiments were performed three times.

2.3 Observation and statistics

After 24-hour incubation, the samples of the AG and testes were fixed in Bouin solution and then embedded in paraffin. 6–8 μm sections were prepared and stained with Ehrlich hematoxylin and eosin. Results were from observation with Olympus BH-2 light microscope.

Ratio of mature sections: 100 transverse sections from testis samples were examined sporadically with optical microscope to count the number of transverse sections containing mature sperms, which was taken as one statistical datum. Ten such data were collected per experimental group, and they were eventually expressed in mean value ($\overline{X}$ ±SD) (Ye et al., 2002).

Ratio of Type B cells: 100 glandular cells from one section of AG were examined sporadically with optical microscope to count the ratio of Type B cells, which was taken as one statistical datum. Ten such data were selected per experimental group, and they were eventually expressed in their mean value ($\overline{X}$ ±SD) (Ye et al., 2003a).

The data were analyzed by means of Student's T-test with significance set at the 95% or 99% confidence interval. Standard errors of the means were also calculated.

3 RESULTS

3.1 *In vitro* incubations of AG and testes

The androgenic gland of *Scylla serrata* attach to the subterminal portion of the ejaculatory ducts, and their developmental process could be divided into 4 stages. In Stage I, the androgenic glands were short with a small amount of glandular cells. In Stage II, the glands were cord-shaped. In Stage III, the gland size reached the maximum, with hyperplasia in some portion. In Stage IV, the glands stopped growing and degenerated rapidly (Ye et al., 2003a).

The AGs in Stages II and IV had little influence on the testicular development (the difference was not significant (p>0.05). The AG in Stage III promoted highly the growth of the seminiferous tubules and the spermic maturation in the testes of the spermatocyte stage (p<0.01).

Table 1 *In vitro* incubations of AG with *S. serrata* testes

AG donator	Testes donator	Incubation mode	Results	
			Diameter of the seminiferous tubules [1] (μm)	Ratio of mature sections[2]
AG in Stage II, 56 mm c.l.	The spermatocyte stage, 56 mm c.l.	AG+T	128.34±11.50	0.12±0.05
		M+T	125.06±10.88	0.11±0.04
AG in Stage III, 72 mm c.l.	The spermatocyte stage, 72 mm c.l.	AG+T	173.94±16.83*	0.30±0.06*
		M+T	153.14±11.20	0.19±0.05
AG in Stage IV, 85 mm c.l.	The spermatid stage, 85 mm c.l.	AG+T	186.31±17.62	0.79±0.07
		M+T	185.34±19.55	0.76±0.07

AG: androgenic gland; T: testes; M: muscles; c.l.: carapace length. 1: n=100; 2: n=10; * highly significant difference with the control group (p<0.01)

3.2 *In vitro* incubations of neuroendocrine organs and testes

Neither the diameters of seminiferous tubules, nor ratio of mature sections were affected by the brain, thoracic ganglia or optic ganglia, and the difference was not significant (p>0.05).

3.3 In vitro incubations of neuroendocrine organs and AG

Compared with the control group, enlargement of the AG cells and the ratio of the B cells were promoted markedly (p<0.05), and remarkably (p<0.01) by the brain and thoracic ganglia. The optic ganglia have no effect on growth and secretion of the AG cells. The difference was not significant (p>0.05).

Table 2 *In vitro* incubations of neuroendocrinal organs with testes in *S. serrata*

Nervous organs donator	Testes donator	Incubation mode	Results	
			Diameter of the seminiferous tubules[1] (μm)	Ratio of mature sections[2]
Male crabs, 72 mm c.l.	The spermatocyte stage, 72 mm c.l.	B+T	151.75±13.93	0.23±0.08
		Th+T	154.71±12.59	0.22±0.05
		O+T	155.67±11.85	0.23±0.07
		M+T	152.18±12.95	0.22±0.05

B: brain; Th: thoracic ganglia; O: optic ganglia; T: testes; M: muscles. 1: n=100; 2: n=10.

Table 3 *In vitro* incubations of neuroendocrinal organs with androgenic gland in *S. serrata*

Nervous organs donator	AG donator	Incubation mode	Results	
			Diameter of the AG cells[1](μm)	Ratio of the B cells[2]
Male crabs, 72mm c.l.	The spermatocyte stage, 72mm c.l.	B+AG	11.03±1.16*	0.53±0.13**
		Th+AG	11.46±1.42*	0.60±0.12**
		O+AG	10.17±0.48	0.42±0.10
		M+AG	9.99±0.43	0.35±0.08

B: brain; Th: thoracic ganglia; O: optic ganglia; AG: androgenic gland; M: muscles. 1: n=100; 2: n=10. * in significant difference (p<0.05); ** in highly significant difference with the control group (p<0.01)

4 DISCUSSIONS

4.1 Promotion of AG on testicular development

It has been confirmed that AG of crustaceans can stimulate and sustain testicular development and male differentiation (Nagamine et al., 1980; Malecha et al., 1992; Sagi et al., 1990; Li and Xiang, 1996; Qiu et al., 2000). Histological investigations of *S. serrata* has indicated that, before the testes enter the sperm stage, the glandular hyperplasia and the enlargement of glandular cells occur in the AG, and the ratio of Type B cells increases gradually. After the testes enter the sperm stage, AG does not grow any more, and the ratio of Type B cells increases dramatically, while the gland degenerates sharply. The requirement of testicular development for the AG hormone in *S. serrata* is in a pattern of low-high-low in temporal scheme (Ye et al., 2003a, b). In present study, AG in Stage II and Stage IV showed a trifle promotion over the testicular development, while in Stage III it has very significant promotion. This result is accordant with the morphological observation that AG in Stage II is in the developmental stage, AG in Stage III hit the peak secretion , and AG in Stage IV began to degenerate (Ye et al., 2003a). Investigations over the structure and function of AG in *S. serrata* is scientifically important for future studies on sexual reversal inducement and mono-sexualized aquaculture.

4.2 Regulation of neuroendocrine organs on AG activities

It has been confirmed by diversified experiments, such as the organic ablation and transplantation, hormone purification, and gene clone, that the X-organ-sinus gland complex is the synthesis center

of GIH, while the brain and thoracic ganglia are that of GSH (Fingerman, 1997; Huberman, 2000). The secretion of C_3 cells in X-organ of *S. serrata* reduces as ovary develops, and reaches the minimum when ovary enters the development stage and near-mature stage. Contrarily, the secretion of C_4 cells in thoracic ganglia boosts up as ovary develops, and reaches the maximum when ovary enters the two aforementioned stages. Hence it was postulated that C_3 and C_4 cells might correlate with the synthesis and releases of GIH and GSH (Shangguan and Li, 1995). With the technology in co-incubation, we have confirmed that the brain and thoracic ganglia are the sources of GSH, and they exert direct promotion over ovarian development; and the biological activities of GSH are strengthened gradually as the ovary develops (Jin et al., 2003).

Do GIH and GSH act on the testicular development directly or through the AG indirectly in male crustaceans? Due to lack of indication of the secretion of AG in previous studies, the investigation of the reproductive endocrine in male crustaceans was once impeded. Recently we found that the AG of *S. serrata* is holocrine and the glandular cells exist as Type B cells after the secretion; so we suggested that the ratio of the Type B cells could be a numerical indicator of the holocrine activities of AG (Ye et al., 2003a, b). In this study, the testes chosen as the samples were in spermatocyte stage, with evident changes occurred in both diameter of the seminiferous tubules and ratio of mature sections (Ye et al., 2002). In other words, the spermatocyte stage is a sensitive period during testicular development, and it is able to reflect the effect of hormonal regulation. As we can learn from Table 2, when neuroendocrine organs are co-incubated with testes, the neuroendocrine organs would have no influence on the testicular development. Therefore, it is suggested that GIH and GSH exert no direct regulation over the testes, which means that the testes are not the target organs of GIH and GSH. When the brain or thoracic ganglia are co-incubated with the AG, diameter of the AG cells rise significantly ($p<0.05$), and the ratio of Type B cells increases very significantly ($p<0.01$); these indicate that the GSH secreted by the brain and thoracic ganglia may have significant promotion to the development and secretion of AG. This result clarifies clearly that the target organ of GSH is the AG in male *S. serrata*; the GSH secreted by the brain and thoracic ganglia can stimulate the AG to secrete AG hormone, thus to accommodate the testicular development. In current investigation, *in vitro* incubation of the optic ganglia has no inhibitory affection on the AG. This may be owing to the low biological activities of GIH in optic ganglia of *S. serrata*, or the short incubation time. The ablation of the eyestalks would cause the hyperplasia of the AG and the aggrandizement of the testes in *S. serrata* with c.l. of 3.5–5.0 cm; however, no difference was observed in the testicular development when injected with the extracts of the brain and thoracic ganglia (Rangneker et al., 1971). This phenomenon should be attributed to high biological activities of GIH and low biological activities of GSH in early stage of testicular development. The structure of GSH in crustaceans is not yet well understood. Studies on the GSH of crustaceans would have great scientific importance to comprehend reproductive endocrine of crustaceans.

References

Adiyodi, K. G. and R. G. Adiyodi., 1970. Endocrine control of reproduction in decapod Crustacea. *Biol. Rev.* **45**: 121-164.

EastmanReks, S. and M. Fingerman, 1984. Effects of neuroendocrine tissue and cyclic AMP on ovarian growth *in vivo* and *in vitro* in the fiddler crab, *Uca pugilator*. *Comp. Biochem. Physiol.* **79A**: 679-684.

Fingerman, M., 1997. Crustacean endocrinology: a retrospective, prospective and introspective analysis. *Physiological Zoology* **70**(3)**:** 257-269.

Huberman, A., 2000. Shrimp endocrinology: a review. *Aquaculture* **191**: 191-208.

Jin, Z. X., H. H. Ye, S. J. Li, H. Y. Huang and G. Z. Wang, 2003. Role of nervous organs in stimulating ovarian maturation in the mud crab, *Scylla serrata*: *in vitro* studies. *Marine Science* **27**(1): 72-74.

Kulkarni, G. K., L. Glade and M. Fingerman, 1991. Oogensis and effects of neuroendocrine tissue on *in vitro* synthesis of protein by the ovary of the red swamp crayfish, *Procambarus clarkii* (Girard). *J. Crust. Biol.* **11**: 513-522.

Li, F. H. and J. H. Xiang, 1996. Primary study of the structure and the function of the androgenic gland of *Penaeus chinensis*. *Science Bulletin of China* **41**(15): 157-164.

Malecha, S. R., P. A. Nevin, P. Ha, L. E. Barck, Y. Lamadrid-Rose, S. Masuno and D. Hedgecock, 1992. Sex- reversed freshwater prawns, *Macrobrachium rosenbergii*. *Aquaculture* **105**: 201-218.

Nagamine, C., A. W. Knight, A. Maggenti and G. Paxman, 1980. Effect of androgenic gland ablation on the male primary and secondary sexual characteristics in the ma-

laysian prawn, *Macrobrachium rosenbergii* (de Man) (Decapoda, Palaemonidae) with first evidence of induced feminization in a non-hermaphroditic decapod. *Gen. Comp. Endocrinol*. **41**: 423-441.

Payen, G.. G., 1980. Experiment studies of reproduction in Malacostraca crustaceans. In: Adams T. S. & Clark W. H. ed, Recent Advance in Invertebrate Reproduction. Elsevier North-Holland, New York. 187-196.

Qiu, G. F., P. Wu and Y. D. Lou, 2000. Structure and function of the androgenic gland in *Eriocheir sinensis*. *Journal of Fisheries of China* **24**(2): 108-112.

Rangneker, P. V., M. N. Madhyastha and A. N. Latey, 1971. Hormonal control of reproduction in male crab, *Scylla serrata* (Forskal). *J. Anim. Morphol. Physiol*. **18**(1): 17-29.

Sagi, A., D. Cohen and Y. Milner, 1990. Effect of androgenic gland ablation on morphotypic differentiation and sexual characteristic of the male freshwater prawns, *Macrobrachium rosenbergii*. *Gen. Comp. Endocrinol.* **77**: 15-22.

Shangguan, B. M. and S. J. Li, 1995. Cytological studies on neurosecretory cell of the X-organ in *Scylla serrata*, *Acta. Oceanol. Sinica* **14**(2): 283-290.

Ye, H. H., S. J Li, H. Y. Huang and G. Z.Wang, 2002. Histological studies on testicular development of the mud crab, *Scylla serrata*. *Zoological Research of China* **23**(2): 141-144.

Ye, H. H., S. J. Li, H. Y Huang, G. Z. Wang and Q. W. Lin, 2003a. Histological studies on the development of androgenic gland in mud crab, *Scylla serrata*. *Journal of Fishery Science of China* **10**(5): 376-381.

Ye, H. H., S. J Li, H. Y. Huang, G. Z. Wang and Q. W. Lin, 2003b. Holocrine activity of androgenic gland in the mud crab, *Scylla serrata. Journal of Fishery Science of China* **10**(5): 431-433.