CMFRI

Course Manual

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ENDOCRINOLOGICAL APPLICATIONS IN CRUSTACEAN BROODSTOCK DEVELOPMENT

28

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Introduction

Globally the scientific community is engaged in research on induced maturation of commercially important crustaceans aiming to the betterment of technologies over the already available eyestalk ablation techniques. In spite of all the technologies so far, there remains number of areas where further development would be highly desirable for optimizing commercial seed production.

Endocrine Control of Reproduction

The reproductive activity of crustaceans is under the control of neuroendocrine factors that in turn is influenced by other environmental factors. Hormones produced by the neuroendocrine organs present mainly in their ventral nervous system directly control reproductive processes in crustaceans. The crustacean endocrine system consists of classical epithelial-type endocrine glands and endocrine structures of neural origin, the neurosecretory cells and neurohaemal organs. This neuroendocrine component is of major significance with respect to both the number of hormones (neurohormones) and their broad array of roles. Reproduction in crustaceans is regulated by hormones from the eyestalks, brain, thoracic ganglia, androgenic glands, and ovaries and, perhaps, also by ecdysteroids. The X-organ sinus gland complex, which is the prime neuroendocrine center in the eyestalks of the crustaceans, produces hormonal factors that control many physiological processes including reproduction and it is the source of a gonad-inhibiting hormone (GIH). Hormones that produced from the brain and thoracic ganglion mainly influences the gonad development and spawning. Ovaries produce hormones that also have influence on the gonad development and spawning. Apart from these Y-organs, Mandibular Organs (MO) and androgenic glands also produce hormones that promote gonadal development directly or indirectly. It appears that a number of environmental signals can influence difference hormonal factors, which in turn regulate reproduction. The classical experiments of Panouse, (1943) on the female shrimp *Leander serratus* demonstrated for the first time that, removal of eyestalk during sexual inactivity led to the rapid increase in ovarian size and precocious egg deposition showed the presence of gonad-inhibiting hormone (GIH) in the eyestalk. Otsu showed the existence of the gonad-stimulating hormone (GSH) in the brain and thoracic ganglion. Based on these findings it was deduced that gonadal maturation in shrimp was regulated by two antagonistic neurohormones, (GIH and GSH). The major neuroendocrine organs that influence the reproduction are eyestalk X-organ sinus gland complex, Cerebral and thoracic ganglia, Mandibular organ, Androgenic gland, Y-organ and Ovaries.

Eyestalk X-organ sinus gland complex

The X-organ sinus gland complex in the eyestalk of crustaceans is a major neuroendocrine regulation center, which is responsible for synthesis of a number of neurohormones. It is the most thoroughly investigated part in crustacean endocrinology, as it is the center of many of the inhibitory hormonal factors (neuropeptides or neurohormones) that controls the physiological process of gonads. Similarly sinus gland is the principal neurohaemal organ involved in the storage and release of neurosecretory materials serving several endocrine functions. Now the eyestalk ablation technique is being practiced commercially for inducement of maturation and spawning of shrimps. Eyestalk ablation stimulates ovarian maturation in crustaceans by reducing the production of GIH, and thus permits maturation of the ovaries in females. Many workers suggest that reproductive maturation in the penaeids shrimp is regulated by a GIH from the X-organ – sinus gland complex in the eyestalks. Unilateral eyestalk ablation stimulates vitellogenic (Vg) synthesis and its secretion into the blood in immature *P. japonicus*. Eyestalk ablation also induces a rapid increase in yolk protein synthesis in *P. vannamei, P. indicus,* and *P. monodon.* These findings suggest the GIH, secreted by the X-

organ-sinus gland complex, inhibits Vg synthesis and secretion into the blood in female penaeids shrimp. Apart from these many workers have partially purified this hormone and characterized the bioactive factor as a peptide of three different molecular sizes: 2000, 5000 and 7000 Da. Chang *et al.* isolated and purified the peptide from sinus glands of shrimp *S. ingentis* that is responsible for inhibition of ovarian development and spawning. Quackenbush and Keeley have also isolated a factor from eyestalks of the shrimp *P. setiferus,* which has inhibitory effect on vitellin synthesis. Similar, studies have been carried out by Quackenbush in *P. vannamei*.

Cerebral (Brain) and Thoracic ganglia

A second decapod reproductive hormone found in the brain and thoracic ganglia has been attributed the role of gonad stimulation. A considerable amount of research has been conducted in crustaceans that indicate the presence of gonad stimulating factors in cerebral and thoracic ganglia. Investigators like Otsu (1960); Oyama (1968), Hinch and Bennet (1979), Nagabhushanam *et al*. (1982); Eastman-Recks and Fingerman (1984); Takayanagi *et al*. (1986); Yano *et al*. (1988) and Yano (1992) have reported that the thoracic ganglion secretes the GSH in decapods. However, Gomez (1965); Gomez and Nayar (1965), Diwan and Nagabhushanam (1974); Nagabhushanam *et al*. (1982), and Yano and Wyban (1992) reported that besides the thoracic ganglia, the brain also secretes an ovarian growth accelerating hormone in crustaceans.

Otsu reported that the accumulation of yolk granules in oocytes was stimulated by repeated implantation of piece of thoracic ganglion in the immature female crab, *Potamon dehanni*. Injection of thoracic ganglion extract prepared from maturieng females is effective in increasing serum Vg in *P. japonicus* and suggest GSH stimulates Vg synthesis and or its secretion into the blood in penaeids shrimp. Yano showed that injection of thoracic ganglion extract prepared from vitellogenic females was effective in increasing serum Vg, even in immature females. This indicates that after initiation of vitellogenesis, higher amounts of GSH, which are increased by injection of thoracic ganglion extract, accelerate Vg synthesis and its release into the blood. This suggests that in penaeids shrimp, GSH, levels may increase further with the advancement of vitellogenesis, parallel to a decrease in the level of GIH. Yano and his associates have demonstrated that ovarian maturation of *P. vannamei* can be induced and accelerated by implantation of pieces of thoracic ganglion tissue prepared from female lobsters with developing ovaries. This results indicates that ovarian maturation is induced by a gonad-stimulating hormone (GSH) secreted by the neurosecretory cells of the thoracic ganglion of maturing females and that this GSH is not species specific in activity between this shrimp and lobster.

Further thoracic ganglion extract prepared from vitellogenic kuruma prawn females was fractionated by gel filtration high-performance liquid chromatography; high Vg-stimulating activity was detected in the fraction corresponding to a molecular weight of 10, 000. This fraction was inactivated by trypsin; therefore, the bioactive factor, GSH, may be characterized as a peptide hormone. Gomez (1965) and Gomez and Nayar (1965) reported that besides the thoracic ganglia, the brain also secretes an ovarian growth-accelerating hormone in the crab *Paratelphusa hydrodroous*. Demassieux and Balesdent (1977) have observed the cyclic variation in the function of B type neurosecretory cells of cerebral and nervous cord ganglia of an isopod *Ascellus aquaticus* in relation to reproduction. Again Yano and Wyban (1992) have observed that the administration of crude extracts of brain induced vitellogenesis in the oocytes of *P. vannamei*.

Eventhough the hormones from the brain of vitellogenic females also have a role in the induction of gonadial maturation, it was not so prominent as that of the hormone from the thoracic ganglion. However, it has been reported that formation of yolk granules, cortical crypts and germinal vesicle breakdown were found in maturing and mature oocytes of *P. vannamei*, which may be induced by a hormone secreted from brain, and it was similar to the development of oocytes induced by GSH secreted from thoracic ganglion (Yano and Wyban, 1992). Similarly it was reported in *P. japonicus* that brain and thoracic ganglion extracts prepared from vitellogenic females, were fractioned and different fractions were injected to vitellogenic females. Significant increase of vitellogenin concentration in the sera was detected, in the fractions, corresponding to the molecular wt of 1,000-2,000 for brain extract; and 10,000 for thoracic ganglion extract (Yano and Chinzei unpublished data, vide, Yano and Wyban, 1992). These above mentioned results

indicate that a hormone secreted from brain, which stimulates the ovarian maturation is different from GSH secreted from the thoracic ganglion.

Similarly Yano (1992) reported that, the ovarian maturation of *P. vannamei* is induced by the injection of brain extract and mentioned that ovarian maturation is stimulated directly by the thoracic ganglion extract, but not stimulated directly by the brain extract *in vivo*. Although in another study a supplement of thoracic ganglion extract to the culture medium was effective in keeping vitellogenic oocytes from degenerating, but brain extract was not effective in *P.japonicus in vitro.* These findings indicate that brain hormone is different from thoracic ganglion hormone and that the brain works through the thoracic ganglion in regulating vitellogenesis in penaeids shrimp. This suggests the presence of a brain hormone that stimulates the release of GSH in penaeids shrimp. Therefore it is nominated as a gonad-releasing hormone (GSH-RH) a possible hormone type in the brain.

Mandibular organ

Le Roux in 1968 first described the Mandibular organs (MO) in crustaceans and stated that these organs may have a role in moulting and / or reproduction because the cells display changes in ultra structure during vitellogenesis and molting cycle. The possible role of MO in reproductive activity as reviewed by Laufer and his associate, and found that there is evidence that the terpenoids, Methyl farnesoate (MF) and farnesoic acid (FA), both secreted by the Mandibular organ have not only a stimulatory effect on the synthesis/ secretion of ecdysteroids by the Y organ, but may also influence reproduction in male and female crustaceans, e.g., high levels of MF have been found in vitellogenic female and reproductively active male spider crabs. The level of MF synthesized in adult *L. emarginata* is higherst during vitellogenesis. MF is also reported to be synthesized by MO *in vitro*. It is also reported that implantation of active Mandibular organs in to juveniles induces ovarian growth and vitellogenesis, suggesting that MF may act as a gonadotropin in immature *Libinia emarginata.* Liu and Laufer found that the activity of MO is regulated by sinus gland neuropeptides. It was mentioned that MO-inhibiting hormonal compounds have the similar type molecular masses an amino acid composition as noticed with other sinus gland neuropeptides. Chang *et al.* by using a radio labeled photo affinity analog of MF found in *S. ingentis* the presence of MF binding proteins in the ovaries, testis and accessory glands in addition the haemolymph. Injection of MF increases the vitellogenic titer in eyestalk less spider crabs and has a positive effect on oogenesis in the crab, *Paratelphusa hydrodromous.*

Androgenic glands

Male crustaceans have a pair of androgenic glands and in most species one gland is attached to each sperm duct. The androgenic gland hormone in general not only regulates the spermatogenesis in the testes nut also is responsible for the development and maintenance of the secondary sexual characteristics in males. Mohammed and Diwan reported that bilateral andrectomy on sex reversal and inhibition of spermatogonial differentiation in the shrimp *P. indicus.* It was also reported that andrectomized male shrimps have lost their secondary sexual character and exhibited absence of sperm in the lumen of their testicular acini. In females GSH and GIH act directly on the ovaries but in males, these hormones appear to exert their effects on the testes only indirectly by directly affecting the androgenic glands. Many studies have bee devoted to the isolation and characterization of the AH, but its real chemical nature remained uncertain for a long time. Laufer and Landau reported that these glands produce several compounds including proteins, terpenes, hexahydroxy farnesylacetone and farnesylacetone and these have some roles in reproductive activities remain to be elucidated.

Y - organs

Chang has been reported the role of Y-organ in crustacean reproduction. Ecdysteroids or Ecdysone, a moulting hormone, is produced by the Y-organ in several crustaceans including the shrimp *P. vannamei.* A number of studies indicated that the involvement of ecdysteroids not only in the moulting process but also in the reproduction of crustaceans. The direct or indirect correlations between vitellogenesis ecdysteroids levels in haemolymph have been reported in some species however, the role of ecdysteroids in the regulation of vitellogenesis in female crustaceans is not known. Ecdysteroids may directly or indirectly participate in the regulation of spermatogenesis and induction of gonadal growth in males.

Ovaries

There is no evidence fro a testicular hormone in crustaceans, but there is evidence for an ovarian hormone that induces development of ovipositing setae and brood chambers. Again many researchers have reported that the ovarian tissue in most crustaceans, particularly in decapods produces vitellogenin-stimulating-ovarian-hormone (VSOH) and under the influence of this, the growth of oocytes takes place. Extirpation and implantation studies carried out in the amphipod, *O*rchestia *gammarella* gave indications for the presence of VSOH is produced in the follicle cells of the ovary and that stimulates the synthesis of vitellogenin. Besides VSOH, many ecdysteroids have been identified in the follicle cells and oocytes of the ovarian tissue. Yano described the endocrine control of vitellogenesis and reported that estradiol – 17 â is effective in increasing serum Vg in the Kuruma prawn, *M. japonicus.* The hormone estradiol - 17 â is generally distributed in the ovary of crustaceans and it was suggested that it is secreted from the ovarian follicle cells which in turn induce Vg synthesis in the ovary as a Vg-stimulating ovarian hormone in penaeids shrimp.

Cytological Localization of the Regulating Neuropeptides in the Neuroendocrine System

By application of polyclonal antisera against GIH, it localized precisely the synthetic neuroendocrine cells in the X-organ of the medulla terminalis in the lobster *H. americanus* by Kallen and Meusy, (1989). Further more, Laverdure *et.al.,* visualized mRNA encoding GIH in neuroendocrine cells of the eyestalk of this lobster. De Kleijn *et al.,* studied the synthetic activity of the CHH and GIH producing cells in the x-organ sinus gland complex of the lobster at m RNA as well as at the protein level using the antisera for immunocytochemistry in combination with specifically prepared cRNA probes for *in situ* hybridization. This approach revealed that there is a frequent but not consistent cellular colocalization of the two neuropeptides (CHH and GIH) at the mRNA and protein level. This study also revealed GIH is present in males in equal numbers as in females. A comparable study on the eyestalk of lobster larvae reveals that the CHH/GIH producing cell system in larval and post-larval eyestalks is similar to that of adults. Specific antibodies localized GIH in the X-organ SG complex of the lobster *H. americanus* prominently at the meta-nauplius stage. Again neuropeptides were detected in the X-organ SG complex by using the technique of non-radioactively labeled cDNA probes in this lobster and its larvae, which has the property of GIH. The presence of GIH in embryos and larvae may be an indication of an inhibitory role before adolescence. Neuropeptides extracted from the eyestalks while administered into them have a negative effect on the growth of vitellogenic oocytes in the Prawn *P. varians* and cray fish *P. bouvrri.* Edomi *et al.,* while studying GIH of the Norway lobster (*Nephros norvegicus*) reported that the GIH is actively involved in gonad maturation process and plays a more complex role in control of reproduction and moulting. The possibility of the involvement of neurohumoral agents in relation to control of reproduction was debated for quite some time, but in recent years experimental evidences are available that 5-HT that is present in the nervous system including the X-organ sinus gland complex has a stimulatory effect on reproductive activity. It is also well known that biogenic amines release peptide neurohormones from neuroendocrine structures in several crustaceans. Serotonin has been found to induce the release of molt-inhibiting hormone from isolated eyestalks. This suggests that biogenic amines may stimulate the release of GIH from the X-organ – sinus gland complex in crustaceans.

Role of Neuropeptides / Neurotransmitters in Reproduction

Considerable research on crustacean neuropeptides /neurotransmitters has been conducted. 5-HT is a ubiquitous substance found in plants and animals. Gonad inhibiting hormone (GIH) produced from the X-organ sinus gland complex is the most important neuropeptides that regulates reproduction in crustaceans. A second decapod neurohormone found in the brain and thoracic ganglia, is the gonad – stimulating hormone (GSH). Studies on the chemistry of crustacean reproductive nerohomones (GIH and GSH) have been reported in recent years. Soyez *et al.* sequenced GIH from the American lobster, *Homarus americanus*. It consists of 77 amino acid residues, with a molecular weight of 9135 Da, and is structurally related to the crustacean hyperglycemic hormone (CHH) and the molt-inhibiting hormone (MIH), the three forming a family of neuropeptides unique to crustaceans. Yano reported that GSH from brains of maturing female 1000 – 2000 Da. However, Tensen *et al.* (1989) when purifying extracts of sinus glands from *H. americanus,* by using high – performance liquid chromatography (HPLC), found that GSH activity is present in the same to its hyperglycemic activity, a stimulatory action on the reproductive system. DeKleijn *et al.* (1995) in connection

with their finding that mRNAs for CHH are present in the ventral nerve cord of *H. americanus*, referred to the possibility suggested by Tensen *et al*. that CHH has GSH activity also. The known neurotransmitter in crustaceans are acetylchline (Ach), gamma-aminobutyric acid (GABA), glutamate, octopamine (OA), dopamine (DA), and 5 – hydroxytryptamine / serotonine (5 – HT). Among the neurotransmitter candidates tested for possible roles in crustacean reproduction 5 – HT, DA, GABA, and OA seem to be attracting more attention than the others.

5 – hydroxytryptamine (5 – HT, serotonin)

5 – HT is a ubiquitous substance, found in plants and animals. The presences of 5 –HT in the central nervous system of crustaceans were well documented. In *Procambarus clarkii*, the amount of 5 – HT is highest in the brain, following in decreasing order by the thoracic ganglia, suboesophageal ganglion, eyestalks, and abdominal nerve cord (Kulkarni and Fingerman 1992). In crustaceans, 5 – HT is known to function as both a neurotransmitter / neuromodulator and as a hemolymph – borne neuro – hormone. 5 – HT has been first identified as a neurotransmitter that stimulates release of some crustacean neurohormones, such as the CHH in crayfish, some colour change hormones and the molt – inhibiting hormone.

In recent years, in addition to identification and localization of 5 – HT by histochemical methods, precise measurements methods precise measurements of tissue concentration of biogenic amines are now possible. With the HPCL technique, 5 – HT was detected in the central nervous system and haemolymph of *P. leniusculus*. A series of experiments done elsewhere revealed that 5 – HT stimulates ovarian development when injected into the fiddler crab and red swamp crayfish. These crabs showed increased, dose dependent ovarian development. Supporting evidence for this was obtained in studies where the ovaries of crabs that received 5 – HT alone and with (5 – HT agonists) fenfluramine (5 – HT releaser), and fluoxetine (5 – HT potentiator) exhibited significant increase in ovarian index and oocyte size compared to the ovaries of untreated initial control crabs and saline-injected concurrent control specimens. Apart from these Sarojini *et al.* also reported supporting evidence for a neurotransmitter role of 5 – HT in stimulating GSH release in *P. clarkii.* In addition, 5 – HT stimulates testicular maturation in *P. clarkii* as shown by the use of a series of 5 – HT agonists and antagonists. % - HT and its agonists induce testicular maturation and help in the development of the androgenic glands. In contrasts, 5 – HT antagonists had no stimulatory effects on the testes or androgenic glands. This stimulatory action of 5 – HT on the testes and androgenic glands was hypothesized to be indirect, i.e. 5 – HT stimulates the release of GSH, which in turn activates the androgenic glands to synthesize and release androgenic gland hormone, and the androgenic gland hormone then triggers testicular maturation.

Dopamine (DA)

The presence of DA in the central nervous system of crustaceans is now well established. By use of an anti – DA antibody, the presence of DA – like neurons in the terminal abdominal ganglion, intestinal nerve, and axons in the hindgut musculature of *orconectes limosus* was demonstrated. HPLC analysis confirmed the presence of DA in these neurons. Using HPLC, DA was found in the brains and thoracic ganglia of the blue crab, *Callinectes sapidus* and *Uca panacea* (Fingerman). Like 5 – HT, DA appears to function both as a neurotransmitter / neuromodulator and as a haemolymph – borne neurohormone (Lingle, and Luschen *et al*., 1993). DA, when injected into female *P. carkii,* inhibited ovarian maturation. The inhibition was dose dependent. The DA injected individuals had a smaller mean ovarian index than the control. Different studies conclude the action of DA as 1) Inhibition of GSH release, thereby directly counter action the action of 5 – HT; 2). Stimulation of release of the GSH antagonist, GIH; or both the above.

Octapamine (OA)

Studies using thin layer chromatography showed the presence of OA in central nervous organs of H. americanus. Investigations with the use of radio labeled compounds and HPLC it was shown that OA is synthesized in the brain of *Orconectes*. Besides through HPLC it is detected in the eyestalks, brain and haemolymph of *P. leniusculus* and in the central nervous system of *U. panacea* and in the Y – organs of *C. maenas.* Many chromatographic and electrophoretic techniques were techniques were able to identify neurons in the second thaoracic ganglion of *H. americanus*. The role

of OA in reproduction appears to be at least in part stimulation of contraction of the ovarian walls, liberating the oocytes.

Red pigment – concentrationg hormone (RPCH)

RPCH was isolated from eyestalks of the prawn, *Pandalus borealis*. It was later found to be an octapeptide found in the brain and thoracic ganglia of the crayfishes and other crustaceans. In addition to its hormonal role in regulating pigmentation, appears to have another hormonal role, stimulation of MF synthesis in the mandibular organ and reported to be as a hormone involved in the regulation of crustacean reproduction to complement the two peptidergic neurohormones, GIH and GSH, that have well documented roles I controlling gonadal maturation in crustaceans.

Opioid peptides

The presence of opoid like substances were reported in many crustaceans in all the retinular cells, in nerve fibers, lamina gnaglionaris, sinus gland, optic peduncle etc. The potential involvement of an endogenous opioid system in the regulation of ovarian development in *U.pugilator* has been investigated *in vivo*. The ovarian maturation in male and female (ovarian and testes development) was found inhibited and the inhibition was reported as dose dependent. It is hypothesized that the opioid inhibition is 1) through the stimulation of GIH release, 2) inhibition of GSH release 3) both.

Conclusion

The endocrine control of crustacean reproduction is understood partially; however its application the commercial level is only through the eye-ablation technique. In order to find out other applications researchers are concentrated in the isolation of other hormones and their purifications. Further studies are on the way and we can hope a better technique for the crustacean brood stock development in the near future.