CHAPTER - IV

MATURATION INDUCTION USING PROSTAGLANDIN AND STEROIDS

Serious bottlenecks to the development of shrimp culture are the scarcity of broodstock and the difficulty to mature them in captivity even when available. The current method for inducing maturation and spawning in penaeid shrimp is heavily dependent on the crude technique of eyestalk ablation (Aquacop, 1975; Primavera, 1978; Brown *et al.*, 1980; Emmerson, 1980a, 1983; Makinouchi and Primevera, 1987). However, eyestalk ablation is a traumatic method and results in poor quality larvae as it induces the female to go for precocious maturation even without proper nutrient accumulation (Harrison, 1997). The ablation also induces the female to spawn several times, but with larvae of decreasing vigour with succeeding spawnings (Emmerson, 1983; Choy, 1987). Weaker larvae, as a result of low quality egg and nauplii will be more susceptible to diseases, a problem which has been already crippling the industry.

Moreover, eyestalk ablation leads to many physiological anomalies due to the removal of organ producing/storing hormones controlling moulting, carbohydrate metabolism, osmoregulation, cardiac frequency, pigmentation etc. (Browdy, 1992; Charmantier-Daures *et al.*, 1994; Subramoniam *et al.*, 1998). So, a less traumatic technique, alternative to ablation, which naturally induces the maturation and spawning of mature females in captivity, would be a promising approach for quality larval production.

Number of researchers (Bomirski *et al.,* 1981; Quakenbush and Herrnkind, 1983; Meusy *et al.,* 1987; Charmantier *et al.,* 1997) studied the role of various glands as well as neurosecretions in controlling maturation, to develop practices that may induce maturation process. Continued research has proved that ovarian maturation in crustaceans is controlled by two antagonistic hormones, one stimulating and another inhibiting (Fingerman, 1997). Studies have proven that the gonad inhibiting hormone (GIH) is from the X organ-sinus gland complex of the eyestalk (Bomirski *et al.,* 1981; Quakenbush and Herrnkind 1983; Meusy *et al.,* 1987), and the gonad stimulating hormone (GSH) is from the thoracic ganglion and/ or tissue (Takayanagi *et al.,* 1986; Yano *et al.,* 1988).

The role of GIH on ovarian development in various crustaceans has been firmly established in that destroying the eyestalk using various means induces precocious gonadal development (Sithigorngul *et al.,* 1992, 1996; Soyez *et al.,* 1987). Recognition of the fact that the eyestalk is the source of GIH or VIH (vitellogenesis inhibiting hormone; Soyez *et al.,* 1987) led to eyestalk ablation, a common practice followed in commercial shrimp hatcheries to induce maturation.

Further research led to the identification of numerous steroids in the crustacean ovary and their involvement in stimulation of vitellogenesis. Number of sex steroid-like substances and enzymes involved in steroid metabolism have been detected in tissues of crab and shrimp (Kanazawa and Teshima, 1971; Teshima and Kanazawa, 1971a; Couch *et al.,* 1987; Fingerman, 1987; Sasser and Singhas, 1988; Shih, 1997; Shih and Wang, 1993; Carvalho *et al.,* 1994).

Few of the various steroid hormones identified in the ovaries of decapod crustaceans include estrogen (Hagerman *et al.,* 1957), estrone (Fairs *et al.,* 1990), 17 β-estradiol (Couch *et al.,* 1987; Fairs *et al.,* 1989) and progesterone and its related compounds (Kanazawa and Teshima, 1971; Ollevier *et al.,* 1986; Couch *et al.,* 1987). Many of the authors also correlated the concentration of these steroids to the development of ovary (Van Beek and De loof, 1988; Novak *et al.,* 1990; Fairs *et al.,* 1989). Junera *et al.* (1977) first proposed the presence of VSH (vitellogenesis stimulating hormone) in the ovary of amphipod, *Orchestia gammarella.* Since then number of workers have studied the effect of these steroids and number of other compounds on maturation or related activities.

Bomirski and Klek-Kawinska (1976) reported the stimulation of oogenesis in sand shrimp *Crangon crangon* when the animals were injected with human chorionic gonadotrophin (HCG). Progesterone stimulated vitellogenesis in both freshwater prawn, *Macrobrachium kistnensis* (Sarojini *et al.,* 1985), marine penaeid prawn, *Parapenaeopsis hardwickii* (Kulkarni *et al.,* 1979) and greasyback shrimp, *Metapenaeus ensis* (Yano, 1985). Stimulation of vitellogenesis by using estrone and estradiol was reported by Sarojini *et al.* (1986) and by using 17 α-hydroxyprogesterone by Nagabushnam *et al.* (1980) and Yano (1987). Tsukimura and Kamemoto (1988) reported an increase in oocyte diameter of hydroxyprogesterone-treated ovary of cultured *Litopenaeus vannamei.* Other than these neurotransmitter serotonin (5-hydroxytryptamine, 5-HT), eyestalk extracts, methyl farnesoate etc. have been tried to induce vitellogenesis (Ongvarrasopone and Pothiratana, 2001; Longyant *et al.,* 2003).

Meanwhile, D'Croz *et al.* (1988) dealing with the maturation stimulating effects of polychaete worms, isolated five prostaglandins and three related compounds from the polychaete, *Americonuphis reesei.* Two of the isolated substances (prostaglandin E and prostaglandin F) are linked to the gonad maturation in mollusc (Morse *et al.,* 1977). Prostaglandins have been proposed to play a role in reproduction as it does in mammals, certain fishes and insects (Sargent *et al.,* 1989; Sargent, 1995).

In this experiment, the prostaglandin E_2 (PGE_2) was used separately or in combination with steroids namely 17 α-hydroxy progesterone (HP) and human chorionic gonadotrophin (HCG), to stimulate maturation of *Fenneropenaeus indicus.* Objectives of the investigation include analysis of the effect of injecting the steroids and PE_2 alone or in combination, on ovarian development and on moulting cycle.

MATERIALS AND METHODS

Broodstock maintenance

The animals sourced from wild were kept for a day in quarantine tanks. The healthy animals were then transferred to broodstock holding tanks. The animals were acclimated to the hatchery conditions for 10 days prior to commencement of the experiment. The feeding was at *ad libitum* during the acclimation period and the animals were fed 5 times a day with frozen squid, fresh sardine, fresh cuttlefish and mussel. The healthy females within the weight range of 38-42 g and healthy males of 31-36 g were selected for the experiment. Other parameters related to maturation section operations are as explained in chapter 1.

Ovarian development

Ovary development stages (Plate.3B) were identified visually (table 17) according to Rao (1968). Visual observation of moulting stage was made according to Vijayan *et al.* (1997).

Table 17. Identification of different maturation stages using ovary colour appearance.

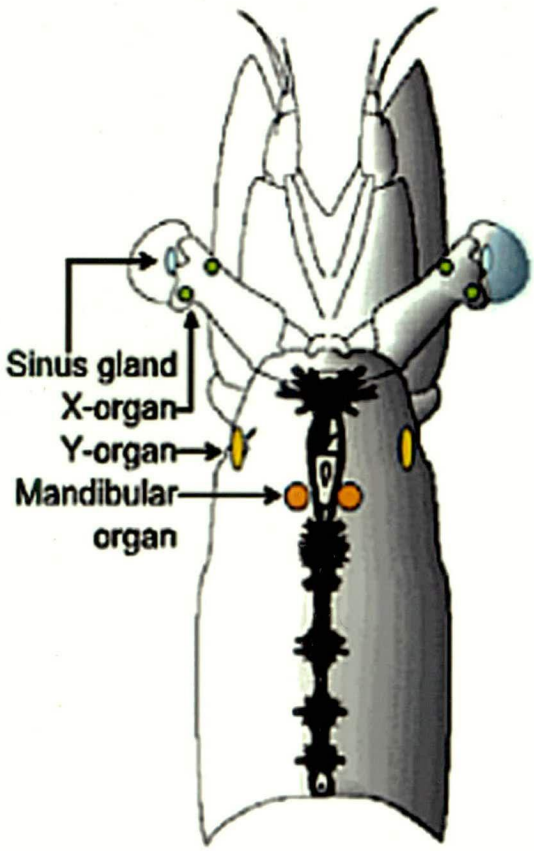
Maturation stage	Ovary colour and appearance
Stage I (immature)	Ovaries thin, translucent and unpigmented. Ovaries were not visible through the dorsal exoskeleton.
Stage II (early maturing)	Ovaries pale cream. The anterior and middle lobes of the ovary increase in size and the ovarian surface appeared to be granular. Ovary loses its transparency and now appeared opaque and was visible externally through dorsal exoskeleton upon careful examination.
Stage III (late maturing)	Ovary develops light green colour and shows increased granular appearance. The lobes seem to increase in dimension and fill up the body cavity.
Stage IV (mature / ripe)	Ovarian lobes distended. Ovary develops dark green colour and was clearly visible through the dorsal exoskeleton
Stage V (spent)	Ovary loose, flaccid in appearance and creamy in colour. Meanwhile, the ovary retained its opacity and was visible through dorsal cuticle.

PLATE - 3

A. Cephalothorax of shrimp - showing the position of vital neurosecretory organs

B. Stages of ovarian development

A



B

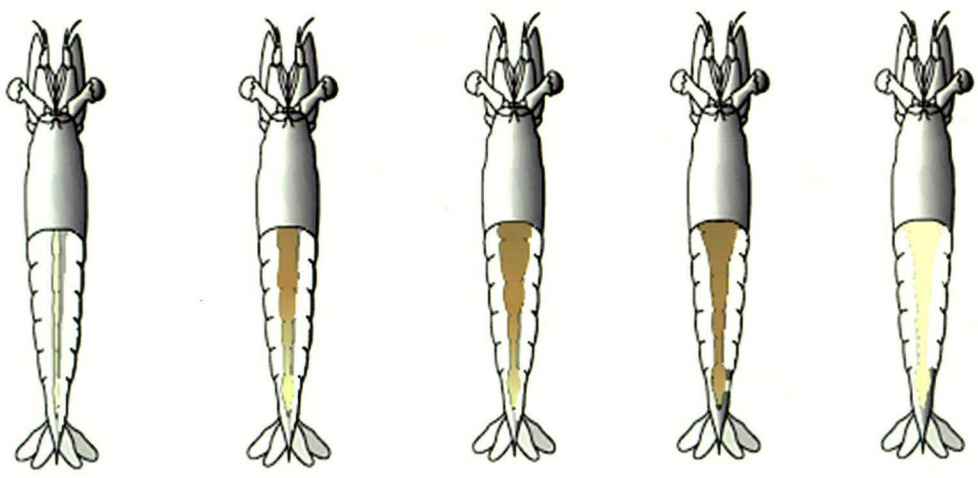


PLATE - 3

Experimental treatment

The selected animals from the broodstock holding tanks were transferred to the 2.5 m diameter experimental tanks. Care was taken to see that none of the females had ovary in the developing stage. Twelve different treatment combinations of PGE_2 and steroid hormones were used in the 6-weeks trial (Table.18). The PGE_2 and steroids individually or in combination were injected into the females using a 27 gauge needle connected to a 50 µl Hamilton syringe. The needle was inserted dorsally through the third abdominal tergum, parallel to the digestive tract, and into the muscle of the first abdominal somite. An interval of 8 days was maintained in the case of groups receiving second injection. For ablation treatment, one of the female eyestalk was cut off using red hot scissors before introducing them into the tank. No ablation was done for males. All treatments were in duplicate.

The prostaglandin E_2, HP and HCG were procured from Sigma-Aldrich, St. Louis, USA. (Product nos. P1451, H5752 and C8554 respectively). The dosage for PGE_2 was 0.1 µg/g body weight. For HP and HCG or combination which included these were given in doses of 0.01 µg hormone/g body wt. Both PGE_2 and HP were dissolved in ethanol at 0.2 µg/µl and 0.02 µg/µl concentration respectively, and HCG was dissolved in 3% saline solution at 0.02 µg/µl concentration. Earlier trials had proven that injecting ethanol did not affect maturation. Dosages were based on studies by Yano (1987) and Koskela *et al.* (1992).

During the experiment, animals were fed at the rate of 15% body weight/day, standardized during the acclimation period. Water exchange (150%/day) was started from the third day of the experiment. From the fifth day after injection, every alternate

evening the animals were checked for ovary development by external viewing, using an underwater torch. The IV^{th} stage animals (ready to spawn) if any, were transferred to 500 l spawning tanks and left overnight for spawning. The females were eye tagged individually and a water proof ink code was made on the cephalothorax, so as to differentiate moult or spawning by them. At the termination of the experiment, the females were weighed, the various visible stages of ovary development in females were recorded. The cephalothorax and abdomen of females were cut open to expose the ovary, and the same was removed and weighed. Ovary weight was used to calculate the gonado-somatic index (GSI) or ovarian index (OI).

RESULTS

Mortality and weight gain

The various treatments, the respective growth and mortality noticed at the end of 6 week experimental period are given in table 18. The statistical analysis did not show any significant mortality due to injection. Among the treatments, the increase in weight was significantly high for the combination of PGE_2 with HCG and HP. The highest weight gain was noticed in animals of treatment no.9 (first injection of PGE_2+HCG and second injection of HCG). Among all treatments, the ablated females recorded the lowest weight gain, but within the injected groups, the lowest weight gain was with the animals given single injection of HP (treatment no.3).

Moulting

The ablation treatment recorded the lowest moulting cycle period, and was significantly lower than rest of the treatments, except no. 6 and 10 (table 19). Generally,

Table 18. Mortality and change in weight of *F. indicus* females at the end of six weeks experiments for twelve treatments

No.	Injection 1	Injection 2	Total females (no)	Mortality (%)	Initial wt (g)	Change in wt.(g)
1.	Control		20	25.0	38.61 ± 0.19	2.94 ± 0.09 ^{a}
2.	PGE_2		20	20.0	37.22 ± 0.20	2.57 ± 0.15 ^{ab}
3.	HP		20	15.0	38.46 ± 0.21	1.69 ± 0.15 ^{bc}
4.	HCG		20	25.0	37.42 ± 0.22	1.81 ± 0.18 ^{bc}
5.	PGE_2+HP+HCG		20	30.0	38.97 ± 0.19	3.15 ± 0.15 ^{a}
6.	PGE_2+HP		20	30.0	39.49 ± 0.22	4.25 ± 0.12 ^{d}
7.	PGE_2+HCG		20	25.0	38.86 ± 0.23	4.98 ± 0.13 ^{d}
8.	PGE_2+HP	HP	20	20.0	38.63 ± 0.20	4.32 ± 0.13 ^{d}
9.	PGE_2+HCG	HCG	20	30.0	39.31 ± 0.18	5.18 ± 0.12 ^{d}
10.	PGE_2+HP	PGE_2	20	25.0	37.67 ± 0.23	4.44 ± 0.55 ^{d}
11.	PGE_2+HCG	PGE_2	20	30.0	38.56 ± 0.22	4.96 ± 0.16 ^{d}
12.	Ablated		20	35.0	39.52 ± 0.21	1.04 ± 0.01 ^{c}

Means (±S.E.) marked with different superscripts are significantly different ($P<0.05$)

Table 19. Mean moult cycle duration, final ovary development stage and mean ovarian index of *F. indicus* broodstock from the twelve treatment groups.

Treatment Group	Moult cycle (d)	Ovary development stage					Ovarian index
		I	II	III	IV	V	
1. Control	15.95 ± 0.17^{a}	9	-	4	2	-	2.560 ± 0.589
2. PGE_2	14.72 ± 0.29^{a}	4	6	3	2	1	2.879 ± 0.509
3. HP	15.81 ± 0.20^{a}	9	4	3	1	-	1.894 ± 0.453
4. HCG	15.20 ± 0.17^{ac}	4	7	2	1	1	2.311 ± 0.485
5. PGE_2+HP+HCG	15.64 ± 0.26^{ac}	5	5	4	-	-	2.069 ± 0.391
6. PGE_2+HP	14.14 ± 0.14^{bc}	3	6	3	2	-	2.694 ± 0.523
7. PGE_2+HCG	14.67 ± 0.20^{ac}	7	-	4	2	2	2.734 ± 0.701
8. PGE_2+HP HP	14.88 ± 0.23^{ac}	7	5	4	-	-	1.818 ± 0.364
9. PGE_2+HCG HCG	15.29 ± 0.23^{ac}	3	7	1	3	-	3.064 ± 0.598
10.PGE_2+HP PGE_2	14.00 ± 0.23^{bc}	5	8	-	2	-	2.170 ± 0.516
11.PGE_2+HCG PGE_2	14.57 ± 0.24^{ac}	4	5	4	1	1	2.563 ± 0.487
12.Ablation	12.85 ± 0.21^{b}	3	3	-	6	1	3.867 ± 0.819

Means marked with different superscript are significantly different ($P<0.05$)

there was a reduction in the moulting cycle period with treatments that included PGE_2. Data analysis between the PGE_2-treated and non PGE_2-treated (excluding ablation treatment) showed significant difference ($P<0.001$) in moulting cycle. The PGE_2 treated animals recorded an average moulting cycle of 14.73 ± 0.095 days, while the non-PGE_2 groups gave an average of 15.67 ± 0.11 days. However, a comparison between PGE_2-treated and ablated animals showed that the ablated females had significantly lesser ($P<0.001$) moulting cycle period.

Ovarian development

During the experimental period out of the 163 females (after mortality reduction) 48 females showed ovarian development (stage III and IV) and 5 of them had spawned. On the basis of ovarian index (table 19), the best response to injection was exhibited by individuals of treatment 9 (PGE_2+HCG+HCG) followed by those of treatment 2 (PGE_2). However, faster stimulation of maturation was noticed in treatment 7 (PGE_2+HCG). Among the treatments given second dosage, only group 9 showed slighter response. Meanwhile, none of them significantly improved results compared to treatments given single dose ($P>0.05$). Overall analysis of ovarian index did not show any significant difference between treatments ($P>0.05$). When injected individually or in combination with PGE_2, HCG seems to perform better than HP.

The comparison of maturation performance between ablated and non-ablated treatment is presented in table 20. There was a significant difference in average number of spawns/female and eggs/female ($P<0.05$). The average fertility and hatching percentage were not statistically different ($P>0.05$). However, there was a clear difference between treatments in the number of days between moulting and spawning.

Table 20. Moulting and maturation performance of ablated and non-ablated females during the six week experiment period

Parameter	Ablated	Non-ablated
Initial female weight (g)	38.67 ± 0.29	38.43 ± 0.32
Total number of spawns	41	24
Average spawns / female	2.73 ± 0.22[a]	1.0[b]
Average number of eggs/female	86,193 ± 1210[a]	35,462 ± 2023[b]
Average fertility (%)	81.36 ± 1.52	83.47 ± 0.84
Average hatching (%)	58.85 ± 2.33	69.59 ± 3.22
Average days between moulting and spawning	8.14 ± 0.92	11.38 ± 0.81
Average days between Ablation and moulting	9.54 ± 0.53	----

Means (± S.E.) in the same row marked with different superscript are significantly different ($P<0.05$)

The ablated females, immediately after moulting seems to under go fertilization and then for maturation. The non-ablated females, even if they go for maturation, seem to be slower in this process.

The ovarian index of matured ablated females was comparatively lesser than that of non-ablated ones. As shown in table 20 (spawns/female), most of the ablated females were in third cycle of ovary development during the end of experimental period.

DISCUSSION

Panouse (1943) was the first to recognize that removal of the X organ/sinus gland complex (Plate 3A) by eyestalk ablation often results in premature or non-seasonal gonadal hypertrophy. Now, studies have shown that the moulting and reproductive processes in shrimps are controlled by neurosecretory hormones (Yano *et al.*, 1988; Soyez *et al.*, 1987; Subramoniam, 1999). For the maturation to take place the titre of GIH should be lower or non-existent. Meanwhile, the quantity of moult inhibiting hormone should be higher such that moulting process does not happen, thus avoiding usage of metabolic reserves (Adiyodi and Adiyodi, 1970; Skinner *et al.*, 1985). As, both moulting and reproduction process are interconnected, both the processes were studied in this experiment.

Results from this study indicate the clear influence of PGE_2 on the moulting process. The mouting cycle duration was significantly reduced ($P<0.001$) when PGE_2 was included in the injection mixture. When injected individually also, PGE_2 resulted in shorter moulting duration than HCG and HP ($P>0.05$). This confirms the influence of PGE_2 over the endocrine control on moulting. Routes of PGE's action suggested is the

inhibition of neurone synthesis of serotonin or more directly, inhibiting the MIH synthesis at the X-organ-sinus gland complex. Role of serotogenic neurons in MIH release was demonstrated by Mattson and Spaziani (1985). Koskela *et al.* (1992) also predicted that if PGE_2 actively inhibits X-organ-sinus gland synthesis of MIH, it may be that GIH can also be inhibited, either by PGE_2 or by some other factor.

This study also proved the failure of HCG and HP to influence moulting, either when used individually or in combination with PGE_2. Unfortunately, except a few, most of the studies which used steroids to induce maturation in crustaceans failed to report the influence on moulting. Koskela *et al.* (1992) reported limited influence of HP and 17β-estradiol on moulting cycle of *Penaeus esculentus.* Similarly, Rodriguez *et al.* (2001) injected Spiperone, a dopaminergic receptor blocker, the terpenoid JH III (juvenile hormone III) and steroids 17α-hydroxyprogesterone, and 17β-estradiol into the red swamp crayfish, *Procambarus clarkii.* The authors reported that when animals in early vitellogenesis were injected with the above compounds, only JH III had a significant effect on moulting.

Though the ovary development stage of some of the injected group females were more advanced, there was no significant improvement than control. Some of the treatments gave results poorer than control. So, it is clear that none of the three (PGE_2, HP an HCG) could make any significant stimulation of maturation. Meanwhile, spawning as well as development by some of the females shows that experimental conditions were conducive for shrimp maturation. Moreover, it is vivid that none of the steroids had any inhibitory effect on maturation. This was confirmed by the absence of any significant difference between groups in ovarian index. Though the investigation

showed higher ovarian index for the ablated females, assessment of individual ablated females at the end of the experiment registered lower ovarian index.. This could be due to the repeated spawning by the same female resulting in smaller size ovary and even incomplete development of ripe ovary.

The negative results obtained with the present study contradict with a number of studies that have reported positive effects of HP and HCG on inducing vitellogenesis. Nagabushnam *et al.* (1980) first reported that injection of HP induced spawning in prawn *Parapenaeopsis stylifera.* Stimulation of oogenesis and vitellogenesis using HCG has been reported by Bomirski and Klek-Kawinska (1976) in the sand shrimp, *Crangon crangon* and the isopod *Idotea balthica* (Souty and Picaud, 1984). Takayanagi *et al.* (1986) suggested that HCG in crustaceans acts by promoting steroid hormone production by the follicle cells surrounding the developing oocyte.

Rodriguez *et al.* (2001) reported significant increase in GSI of early vitellogenic-stage red swamp crayfish *P. clarkii,* when they were injected with Spiperone, 17α-HP, and 17β-estradiol. They also reported that 17α-HP was able to significantly increase the oocyte diameter also.

The present results with both HP and HCG, corroborate with some of the negative reports documented. Koskela *et al.* (1992) reported failure of PGE_2, HP and estradiol to induce maturation of *P. esculentus.* Injection of sexually quiescent female *P. vannamei* shrimp with progesterone and estradiol did not increase hemolymph levels of yolk protein precursor. The absence of a response to these steroids was attributed to the presence of other hormones (such as the GIH) that prevent oocyte development (Tsukimura and

Kamemoto, 1988; Tsukimura, 2001). Similarly, Okumura and Sakiyama (2004) tried to establish the relationship between steroid hormone (17 β-estradiol, estriol, progesterone, testosterone, and 11-ketotestosterone) level in haemolymph and maturation. The authors found that both in natural and induced maturation, no relation existed between the hormone level and ovary development.

Brown (1998) used mammalian gonadotrophins (synthetic LH-RH, HLH and HCG) to investigate the effects of varying levels of these hormones during different stages of the reproductive cycle in *Marsupenaeus japonicus*. The author found no effects of these treatments on the previtellogenic animal, but found stimulating effect in stage III females only. Thus the suggestion was made that the effect is boosting a change that is already underway rather than instigating the change.

In this study, the shortened moulting cycle observed with ablated females (12.85 with ablated and the minimum value of 14 with injected ones) corroborate with Browdy and Samocha (1985) who reported that the moult cycle was more frequent in ablated *P. semisulcatus* females (22.1± 2.4 days) than in unablated ones (23.8 ± 2.0 days). Agreeing with the present study, higher number of spawnings and more eggs by ablated compared to unablated control has been reported in *P. semisulcatus* (Aktas and Kumlu, 1999).

Only report, where the results of induced maturation equalled results of ablated females was by Mendoza *et al.* (1997). The authors used various extracts from squid as feed supplement and found the same to trigger secondary vitellogenesis in 15-35 g female *L. vannamei*. According to them, the non-ablated showed maturations of the same order

of magnitude as the eyestalk-ablated controls. The stimulation effect was attributed to steroid-like substances.

Recently, consistent reports have been made on the positive effects of methyl farnesoate (a gonadotropic hormone produced by the mandibular organ of Crustacea) in inducing crustacean maturation (Borst *et al.,* 1987; Laufer *et al.,* 1998). But, the same also gave negative results in cray fish, *Cherax quadricarinatus* (Abdu *et al.,* 2001).

To summarize, in the present trials all three compounds resulted in lack of response from the animal, casting doubt over their role as vitellogenin stimulating hormone (VSH). The results also fail to support the report by D'Croz *et al.* (1988) about the stimulation of shrimp maturation by prostaglandins from polychaete worms.

As clear from the above discussion, the contradictory results reported even with similar compound for different crustaceans shows the complex system involved with endocrine system control over reproduction. Research on shrimp GIH isolation is still ongoing. Elucidation of the structure and function of shrimp GIH, using peptide biotechnology-based approaches shows promise for countering the reproductive inhibitory effects of GIH. Further research in this area is needed, and collaboration between researchers, shrimp farmers, and resource providers from different regions could expedite its achievement.