CHAPTER V

The assessment of gonad inhibiting hormone levels in the giant prawn *Penaeus monodon*

Introduction

Immunological techniques using specific antibody to characterize molecular features of the molecule of interest, in this case, vitellin and vitellogenin, have been demonstrated in the two previous chapters. The present study is another aspect of using antibody for quantitative analysis of the molecules, in order to correlate the fluctuation of the vitellogenin levels in the haemolymph with ovarian development. This approach provides a benefit feature over the a determination of ovarian growth in which the prawns are not required to be sacrificed but only a small haemolymph sample is needed for each determination. Therefore, the fluctuation of haemolymph vitellogenin could be followed in the same prawn throughout the process of ovarian development. The variations risen from individual variation and from imprecise judgement of ovarian developmental stages would be minimized.

The experiment started with the determination of the correlation between haemolymph vitellogenin levels and ovarian index (OI which indicates the ovarian growth and development) by sampling the prawns with different ovarian developmental stages induced by bilateral eye-ablation. The next experiment was the determination of haemolymph vitellogenin levels of individual prawn versus the time after induction of ovarian growth.

The putative effect of gonad inhibiting hormone (GIH) on haemolymph vitellogenin levels was analyzed by injecting the eyestalk extract into the prawns with developing ovary. After the effects of eyestalk extract injections were investigated, several doses of eyestalk extracts were applied at the appropriate time to confirm the

action of the hormone. In all cases, the antibodies specific to vitellin and vitellogenin were utilized as the tools for quantitative analysis of vitellogenin in haemolymph using indirect competitive ELISA.

Materials and methods

Source, animal handling and initial preparations

Adult female *Penaeus monodon* (80-120 g) were obtained from local farms around Bangkok. They were held in 3 x 3 x 0.6 m^3, rectangular concrete tanks with natural pond water (25 $^{o}/_{oo}$) at ambient temperature (26-28 °C) and photoperiod. Squid was presented during the morning and evening and half of water was changed every two days. After three days of acclimation, the prawns were used for the experiment.

Haemolymph collection for quantitation of vitellogenin

Haemolymph was collected via arthrodial membrane of the fourth walking leg for 50 to 100 µl and stored immediately at –20 °C. Before use, it was centrifuged at 5,000 g and supernatant was used for vitellogenin analysis.

Determination of vitellogenin in haemolymph

Indirect competitive ELISA for determination of vitellogenin content in haemolymph was performed as described in Chapter III using combination of 4 monoclonal antibodies specific to all vitellin subunits (PMVS-93, 109, 140 and 158 antibodies) at the dilution of 1:3000. The intra-assay and inter-assay variations were tested using 2 haemolymph samples at a low concentration (0.8 mg/ml) and a high concentration (4.5 mg/ml).

Preparation of eyestalk extract

Eyestalks were excised from live adult female prawns with scissors, frozen immediately on dry ice and stored at –70 °C until use. Eyestalks were dissected to remove the outer shell while partially thawed out, then homogenized immediately in cold saline (Nagamine et al., 1980) with a glass homogenizer. After centrifugation at 10,000 g at 4 °C for 30 min, the pellet was re-extracted with the saline. Supernatants from both extractions were pooled and adjusted the volume with saline to 1 eyestalk/ 100 µl and divided into small aliquots before frozen and stored at –70 °C.

The relationship between haemolymph vitellogenin levels and ovarian development

Ovarian development was induced by bilateral eye-ablation. Two separate experiments were performed to determine the relationship between ovarian development and vitellogenin levels. The first experiment was performed by sampling the prawns every two days for 12 days after eye-ablation. After the haemolymph was collected for vitellogenin analysis, the prawns were sacrificed in cold water and determined the body weight and the ovarian weight for calculation of gonado-somatic index (GSI) or ovarian index (OI) as follow :

$$OI = \frac{\text{Ovary weight}}{\text{Body weight}} \times 100$$

The stages of ovarian development was determined as described by Primavera (1983) and Tan-Fermin and Pudadera (1989).

The second experiment was performed by collecting haemolymph from individual prawn every day for 12 days after eye-ablation for vitellogenin analysis. On the last day, prawns were sacrificed for determination of OI and stage of ovarian development as described above.

The effect of eyestalk extract on haemolymph vitellogenin levels

Four days after induction of ovarian growth by eye-ablation, individual prawn was injected with eyestalk extract approximately 3 eyestalks/prawn. The haemolymph was collected at 0, 2, 4, 6, 10, 14 and 24 hr after eye-ablation and then stored at –20 °C for vitellogenin analysis. The changes of vitellogenin levels after injection of eyestalk extract were compared with vitellogenin level at 0 hr. The control experiment was performed by injecting the prawns with 300 µl of saline and treated in similar manner.

Different doses of eyestalk extract were also tested in the same fashion and the haemolymph from individual prawn was collected from 0 to 10 hr. At the end of the experiment, all the prawns were sacrificed for determination of OI and stage of ovarian development.

Results

Indirect competitive ELISA using combination of four monoclonal antibodies displayed similar result as previously shown in Chapter III experiment that used only one monoclonal antibody. The female haemolymph could replace the binding of antibodies to antigens on the plate similar to the vitellin standard while the male haemolymph had no effect (Fig. 5.1). The range of vitellogenin that can be measured by this method was 4 to 200 μg/ml. The intra-assay variation was in the range of 5 to 10% and inter-assay variation was 2 to 3 % (see appendix A: Table 1A).

There was no correlation between ovarian index and haemolymph vitellogenin because the ovarian index of ovary at stage IV showed high variation (range from 3 to 8%) and the vitellogenin levels in prawns with ovary stage II and III also showed quite high range of variations as well (Fig. 5.2. and Table 5.1). However, the correlation between vitellogenin levels and stages of ovarian development was observed. At resting stage, the haemolymph vitellogenin level was undetectable. After the ovary began to develop, the vitellogenin levels elevated to the highest concentration at stage III and dropped to a low levels at ripe stage (IV) and spent, similar to the level at stage II. The increasing in OI was associated with the increasing of haemolymph vitellogenin levels except in stage IV that the vitellogenin levels dropped before the reduction of OI (Fig. 5.3 and Table 5.1).

The vitellogenin levels of individual prawn after induction of ovarian growth show similar correlation. On the second day after eye-ablation (early development = stage II) haemolymph vitellogenin levels rose sharply and reached the maximal levels during 2-5 days after eye ablation (nearly ripe stage). When the ovary reach the maturity, the vitellogenin levels sharply dropped to a very low levels a day before spawning. After spawning the vitellogenin levels began to increase and the second cycle of ovarian development began. In the second cycle the vitellogenin levels of the

prawns seem to be higher than those in the first cycle and revealed higher variations and non synchronization (Fig. 5.4).

Four days after eye-ablation, the vitellogenin levels in the prawn injected with eyestalk extract increased sharply at 4 hr after injection, reached at the maximal levels at 10 hr, and remained high throughout 24 hr. In the control group injected with saline, the vitellogenin levels remained unchange throughout 24 hr (Fig. 5.5).

When the amount of injected eyestalk extract was decreased, the vitellogenin levels were decreased in a dose response fashion during 2-10 hr. The minimal amount of eyestalk extract that its effect could be detected was about half of an eyestalk (Figs. 5.6 and 5.7).

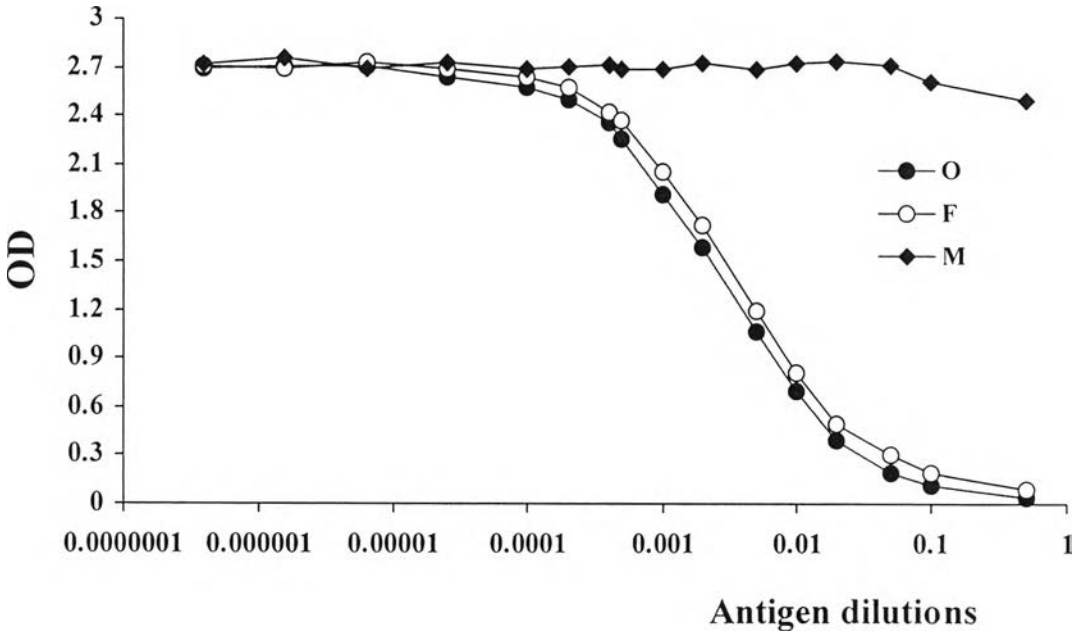

Figure 5.1 Validation of specificity and sensitivity of competitive ELISA for determination of vitellogenin using combination of four monoclonal antibodies specific to each vitellin subunits. Various dilutions of ovarian vitellin (O) with initial concentration of 10 mg/ml were compared to the similar regime of the dilutions of haemolymphs from adult male (M) and female with developing ovary (F).

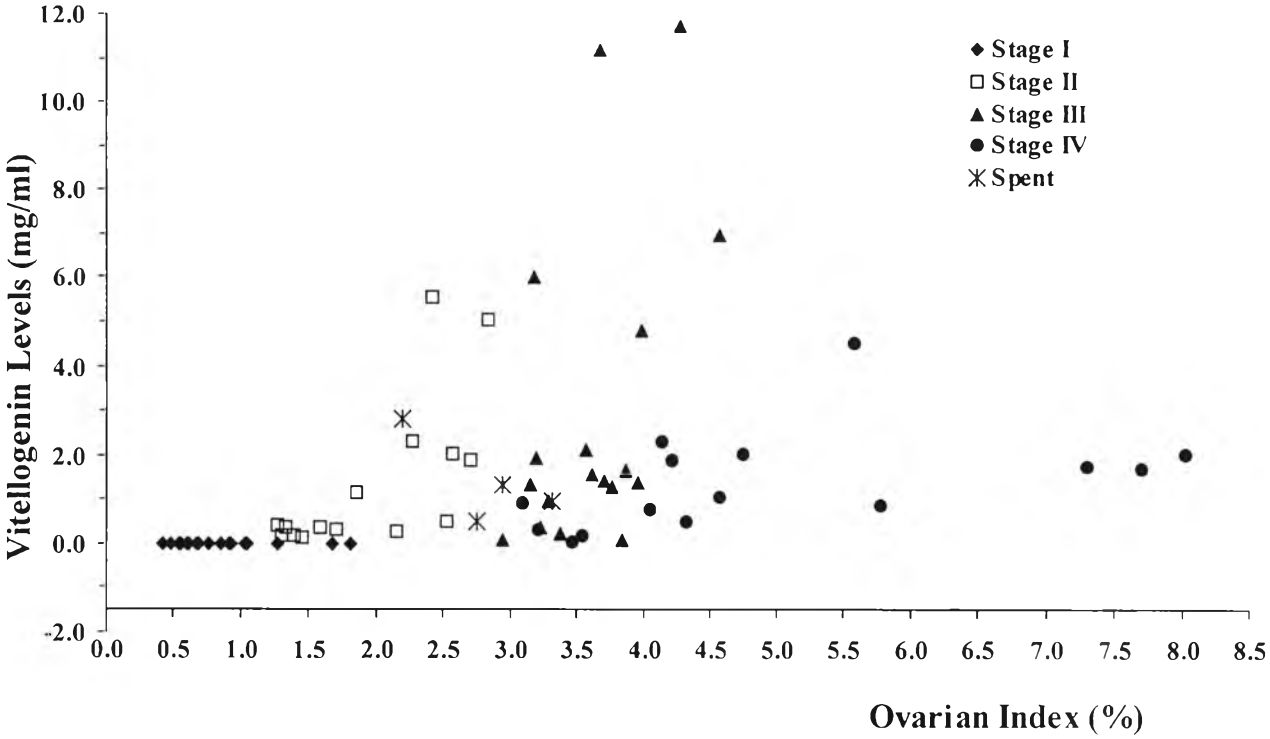

Figure 5.2 Haemolymph vitellogenin levels from individual prawn at different stages of ovarian development were compared with ovarian index. Each spot represents the data from individual prawn.

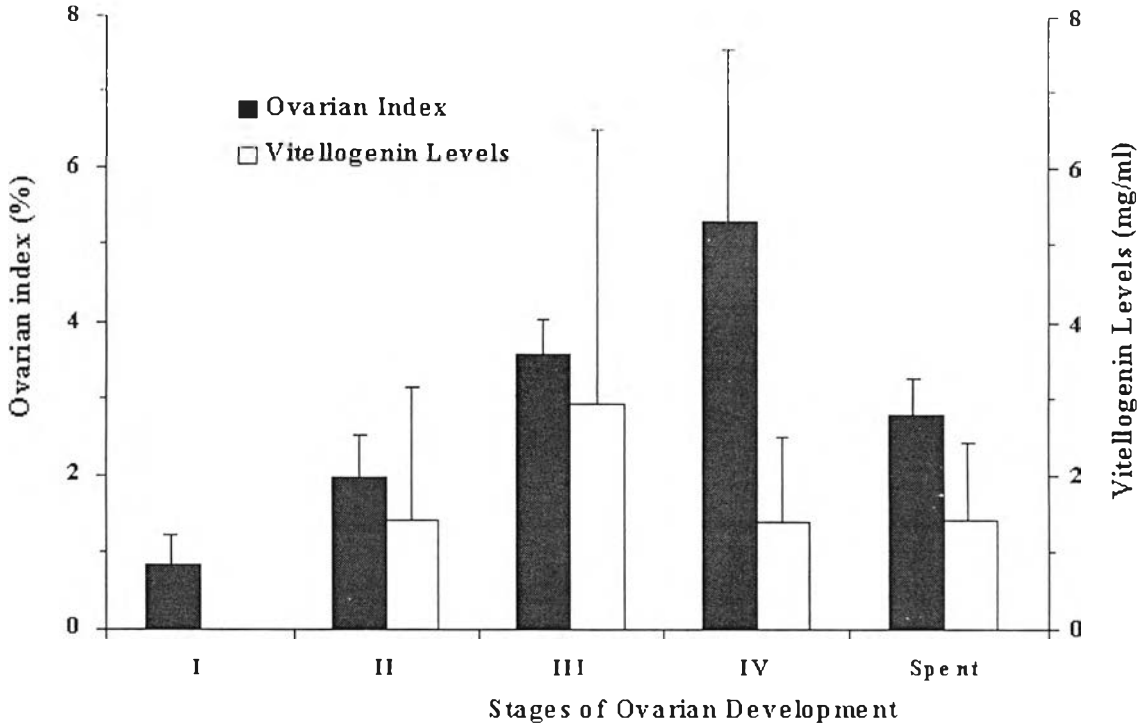

Figure 5.3 Comparison of average ovarian index and haemolymph vitellogenin levels at different stages of ovarian development. The lines above the bar represent standard deviation. All data were derived from Table 5.1.

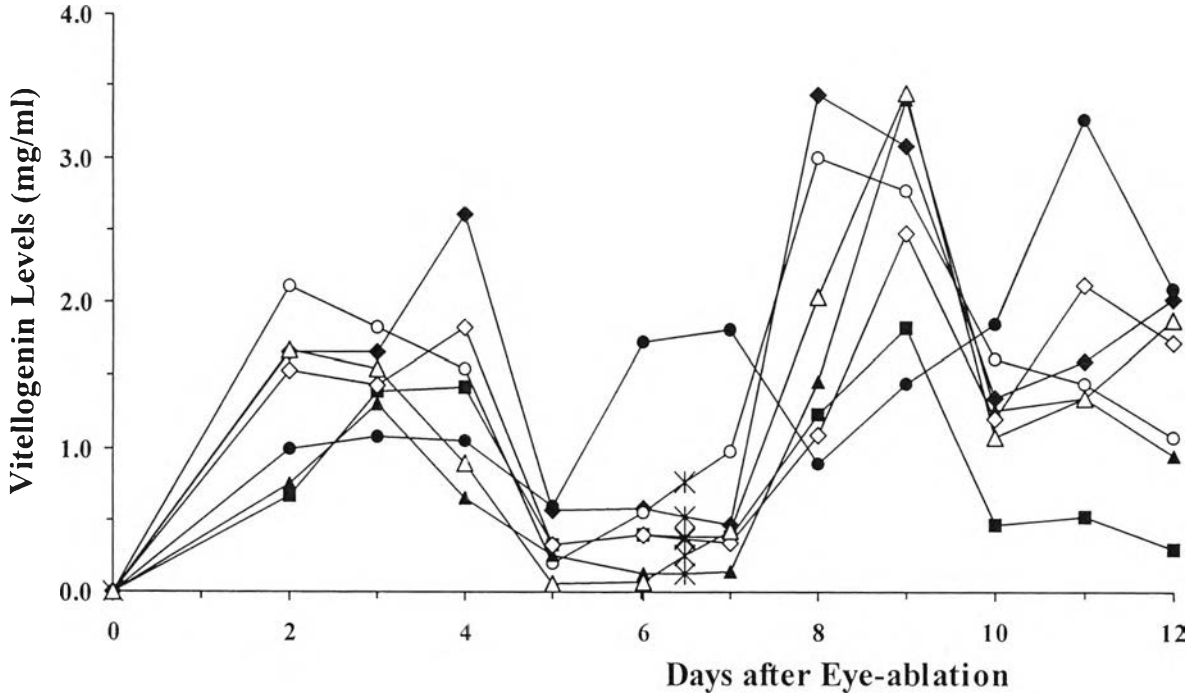

Figure 5.4 The relationship between haemolymph vitellogenin levels and ovarian development indicated by time after eye-ablation. The asterisk represents observable spawning. Each line represents data from individual prawn (n=7).

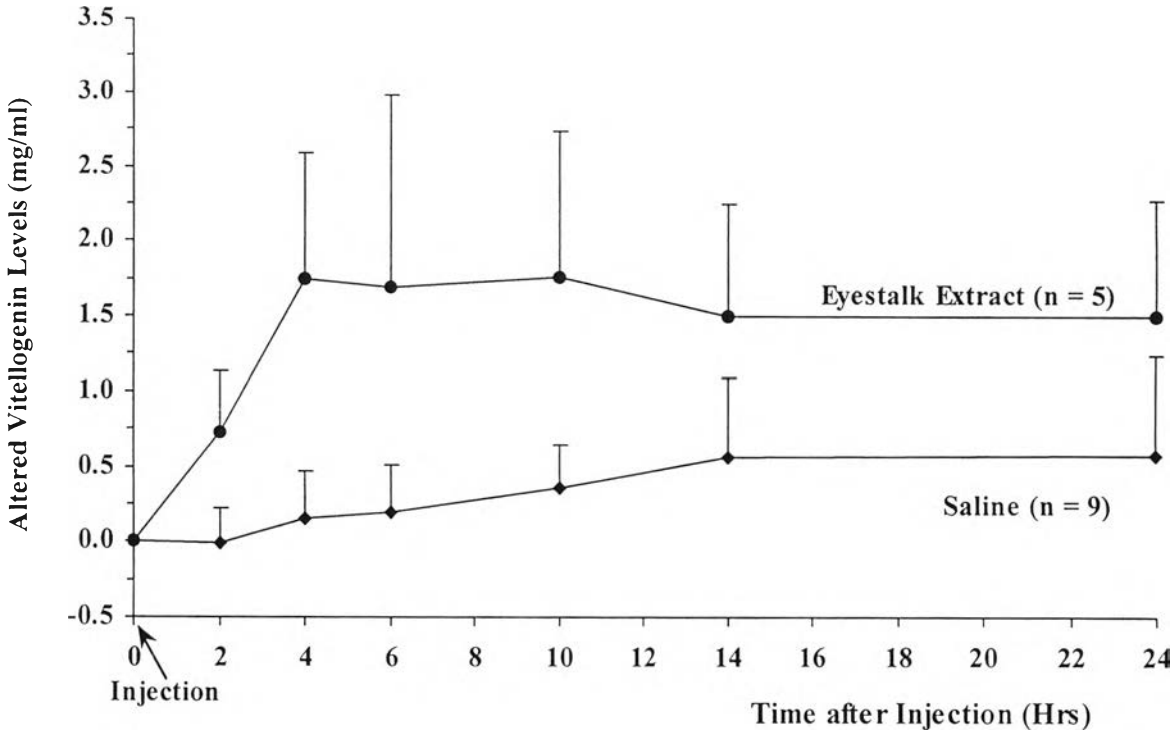

Figure 5.5 Alteration of haemolymph vitellogenin levels during 0 to 24 hr after injection of eyestalk extract (3 eyestalks/prawn) or saline (n = the number of sample).

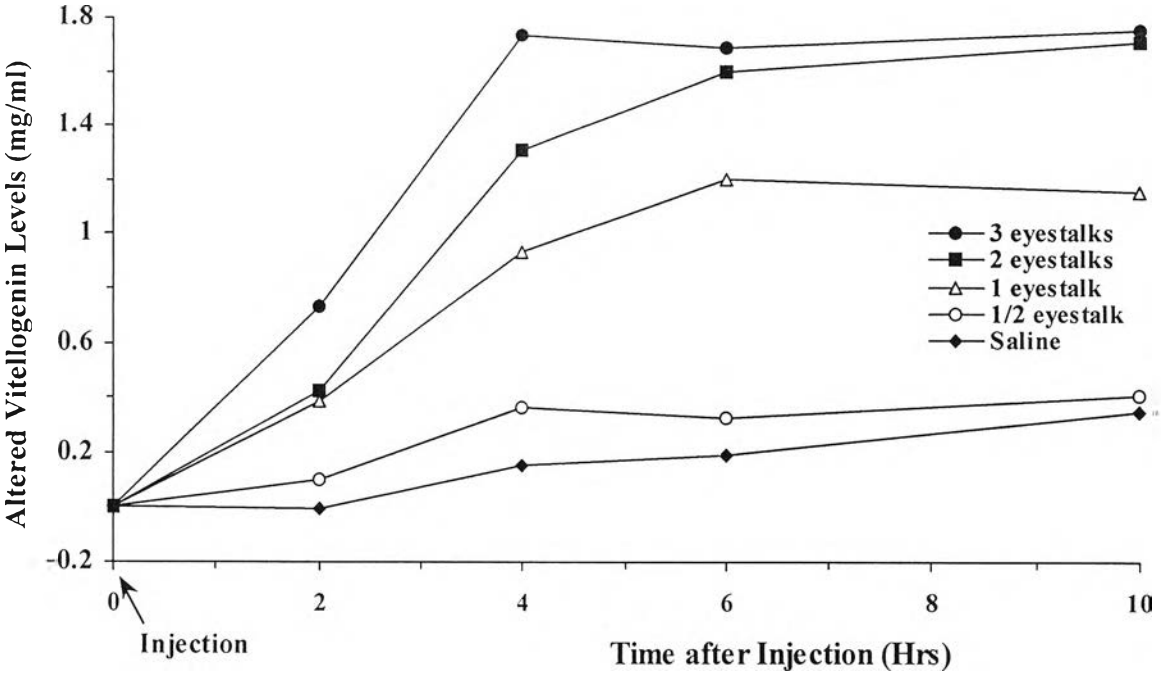

Figure 5.6 Alteration of haemolymph vitellogenin levels during 0 to 10 hrs after injection of various doses of eyestalk extract (1/2 to 3 eyestalks/prawn) or saline.

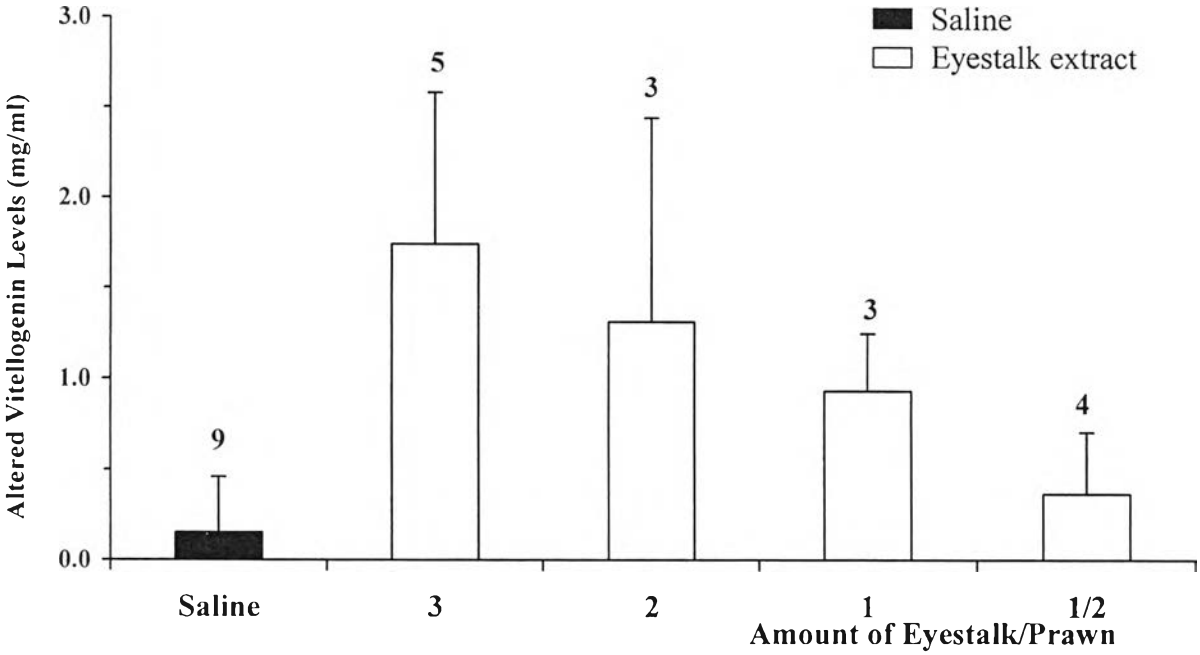

Figure 5.7 Alteration of haemolymph vitellogenin levels at 4 hr after injection of various doses of eyestalk extract (1/2 to 3 eyestalks/prawn) or saline (the number above each bar represents the number of samples used in each experiment).

Table 5.1 Haemolymph vitellogenin concentrations and ovarian index of *P. monodon* at various stages of ovarian development. The values are means ± SD.

Stage of Ovarian Development	Body Weight (g)	Ovarian Weight (g)	Ovarian Index (%)	Vitellogenin Levels (mg/ml)	Number of Sample (n)
I	91.51 ± 20.23	0.77 ± 0.50	0.82 ± 0.39	0	20
II	126.29 ± 38.40	2.39 ± 0.80	1.96 ± 0.55	1.427 ± 1.701	16
III	103.93 ± 25.41	3.71 ± 1.04	3.57 ± 0.47	2.910 ± 3.601	19
IV	121.67 ± 21.90	6.62 ± 3.43	5.32 ± 2.26	1.384 ± 1.105	16
Spent	113.84 ± 34.81	3.41 ± 0.88	2.80 ± 0.47	1.409 ± 1.011	4

Discussion

Quantitative analysis of vitellogenin in the haemolymph using indirect competitive ELISA with combination of four monoclonal antibodies yields high fidelity results since the intra-assay and inter-assay variations were relatively low. Even though the sensitivity was not different from using one monoclonal antibody (Longyant et al., 1999), four monoclonal antibodies were used to insure the detection of all vitellogenin subunits present in the haemolymph.

The correlation between haemolymph vitellogenin levels and ovarian index (OI) at various stages of ovarian development was difficult to elucidate (Fig. 5.2) since the variations of OI and haemolymph vitellogenin levels at each stage of ovarian development from individual prawn were very high especially at stage II, III and IV due to the fact that ovarian developmental processes are a continuous process, a clear line between each stage can not be identified accurately. Similar results were reported in *Macrobrachium rosenbergii* (Lee and Chang, 1997). However, the relationship between haemolymph vitellogenin levels and stages of ovarian development was clearly observed. The haemolymph vitellogenin levels elevated and highly fluctuated during ovarian development and sharply dropped when the ovary was mature and ready to spawn (Fig. 5.3 and 5.4). This relationship is clearly established when the haemolymph was sampled from individual prawn at different periods after eye-ablation. Even though the variation among individual prawn was still very high, all of the tested prawns were undergone development into two reproductive cycles (Fig. 5.4). Similar experiments were conducted in *M. rosenbergii* and similar results were obtained (Derelle et al., 1986; Chang and Shih, 1995). Since the prawn was subjected to bleed every day, such a bleeding regime may cause stress and spiking of vitellogenin levels (Chang and Shih, 1995).

Injection of eyestalk extract into eye-ablated prawn with developing ovary caused a sharp elevation of haemolymph vitellogenin levels within 2 hr and remained at high levels up to 24 hr. This result seem to be unusual since in many studies demonstrated that eyestalk extract exhibited inhibitory effect on incorporation of radioactive labeled amino acid into vitellin in ovaries of many shrimps and crabs (Aguilar et al., 1992; Quackenbush and Keeley, 1988). A study in *M. rosenbergii* demonstrated that injection of eyestalk extract induced slow and continuous reduction of vitellogenin in eye-ablated female with developing ovary (Suktangman et al., 1996). However, in most penaeid species, it has been well known that when the intact female prawns with developing ovary are reared in captivity, the developing ovary is usually undergone degeneration. In contrast, the normal development of ovary of *M. rosenbergii* is unaffected by captivity. Therefore, it is possible that the additional inhibitory effect of eyestalk extract in penaeid species is to induce degeneration of ovary and resorption of vitellin back into haemolymph. The supporting evidence is that when the prawn was eye-ablated the degeneration process stop and the progress of ovarian development is continue. Similar observation was also seen in *Metapenaeus affinis* in a similar experiment (Longyant, et al., 1996; Sithigorngul et al., 1996). Moreover, the vitellogenin levels of intact *M. affinis* with developing ovary sharply increased to unusually high levels after taken from the sea and reared in captivity for only one day (Longyant et al., 1994).

This study indicated that haemolymph vitellogenin levels could be used for determination of GIH activity. This method has several advantages over other methods in previous reports such as suppression of *in vitro* protein synthesis by determination of incorporated radioactive amino acid into ovarian tissue (Quackenbush, 1989; Quackenbush and Keeley, 1988; Aguilar et al., 1992) or an inhibition of *in vivo* ovarian development using gonado-somatic index (GSI) (Quackenbush and Herrnkind, 1983; Soyez et al., 1987) (Table 5.2). This assay

requires neither high amount of eyestalk extract nor radioactive substance. Moreover, it can be performed in less time, about 4 hr after injection of half eyestalk extract, for determining the GIH activity. Whereas, the suppression of protein synthesis requires 1-2 days and radioactive amino acid as a marker for immunoassay using highly specific anti-vitellin antiserum. In case of determination of GSI, a large amount and several injections of eyestalk extracts over long period of time (6-15 days) are required. The high variation of GSI among individual prawn usually contributes to incorrect interpretation of the assay. Therefore, the determination of GIH by measuring haemolymph vitellogenin levels is an alternative method for further characterization of this hormone.

The sites of vitellogenin synthesis in shrimp and crab are still on debate. Two possible sites are intra-ovary such as in *Procambarus sp.* (Lui and O'Connor, 1976), *Penaeus japonicus* (Yano and Chinzei, 1987), *Penaeus vannamei* (Quackenbush, 1989a), and *Penaeus semisalcatus* (Browdy et al., 1990) or extra-ovary such as in the crabs, *Carcinus maenas* and *Libinia emerginata*. In these crabs, the vitellogenin was localized in hepatopancreas (Paulus and Laufer, 1987) and in *Parapenaeus longirostris*, the vitellin synthesis was identified in fat bodies (Tom et al., 1987). The vitellin could also be synthesized in both intra-oocyte or extra-oocyte as suggested by Byard and Aiken (1984); and Quackenbush (1989a, b). Ovarian follicular cells have been suggested as major cells for exogenous vitellogenesis in *P. japonicus* (Yano and Chinzei, 1987). Therefore, utilizing these monoclonal antibodies to localize the vitellin could clarify these questions in the future. By knowing the synthesis site of vitellin would be another step to identify the main target of GIH which is in turn benefit to the study of the inhibitory mechanisms of the hormone.

In conclusion, cyclic pattern of ovarian development in *P. monodon* can be demonstrated by determination of haemolymph vitellogenin levels using competitive ELISA. Application of the eyestalk extract caused a direct affect on elevation of

haemolymph vitellogenin levels which could be detected during 2-10 hr in dose dependent fashion. Therefore, the GIH assay could be established from determination of the haemolymph vitellogenin in prawn with developing ovary.

Table 5.2 Comparison of three different methods for determination of GIH activity.

	Haemolymph Vitellogenin Levels	Inhibition of Ovarian Development	Suppression of Protein Synthesis
1. Author	This study	Quackenbush and Herrnkind, 1983.[1] Soyez et al., 1987.[2]	Quackenbush and Keeley, 1988. [3] Quackenbush, 1989a. [4] Aguilar et al., 1992. [5]
2. Eyestalk/assay (Percent inhibition)	1/2	1/1000 (30 %) [1] 1/10 (50 %) [2]	1/2000 (30 %) [3] 1/40 (40 %) [4] 5 (35 %) [5]
3. Prawn/assay	2	> 5	1
4. Number of eyestalk application	1	Every two days	1
5. Duration of assay	4-10 hr.	15 [1] and 6 [2] days	1-2 days [3, 4, 5]
6. Stage of ovarian development	Developing ovary	Resting stage	Ovarian stage II (Secondary vitellogenesis)
7. Control prawn	Same individual Negative control	Different individual + control - control	Same individual
8. Antibody specific to vitellin	Required	Not required	Required
9. Tissue culture facilities	Not required	Not required	Required
10. Radioactive labeling	Not required	Not required	Required
11. Method	*In vivo*	*In vivo*	*In vitro*