SOME STUDIES ON

# THE REPRODUCTIVE ENDOCRINOLOGY OF TIGER PRAWN Penaeus monodon FABRICIUS

*THESIS SUBMITTED*
*IN PARTIAL FULFILMENT OF THE REQUIREMENTS*
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*BY*

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1996

Dedicated to

*My beloved*

*late GRANDFATHER*

# CERTIFICATE

This is to certify that the thesis entitled **"Some studies on the reproductive endocrinology of tiger prawn *Penaeus monodon* Fabricius"** is a bonafide record of the research work carried out by **Miss Shoji Joseph,** under my guidance and supervision and that no part thereof has been presented for the award of any other degree or diploma.

Cochin 682 014
May 1996

**Dr. A.D. Diwan, Ph.D.**
Principal Scientist
Physiology, Nutrition &
Pathology Division
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# DECLARATION

I hereby declare that this thesis entitled **"Some studies on the reproductive endocrinology of tiger prawn *Penaeus monodon* Fabricius"** is based on my own research and has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

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**Shoji Joseph**

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CONTENTS

# PREFACE

## PREFACE

Penaeid shrimp comprise one of the most economically important marine products because of its universal appeal, unique taste, high unit value, and ever increasing demand in the international market as it has been given the prime importance both domestically and world wide. This unlimited demand for shrimp in the world market resulted in the over exploitation of the natural shrimp stocks in many parts of the world including the coastal waters of India. While fisheries for shrimp have declined in the natural resources due to over exploitation, there has been a concomitant surge for the production of expoitable varieties of shrimps. In this context aquaculture of penaeids has emerged as the only viable alternative for sustaining and expanding the industry. Penaeid shrimp culture is a field to which most of the countries have attached great importance as it is highly profitable with luxury food items which command high prices and earns good amount of foreign exchange. Shrimp culture has emerged as a major frontier of modern aquaculture in recent years in India.

The giant tiger prawn P. monodon (fig 1) has been idientified as the best species for culture operations, due to its high market demands, faster growth rate, larger size, and increased tolerance capacity. In India the commercial aquaculture has been synonymous with brackishwater shrimp farming. It is surprising to note that in the total cultured prawn production P. monodon constituted the highest percentage of 75% in India and its share is around 61.00% in the total world's farmed shrimp production.

Globally the scientific community is engaged in research on the induced maturation of commercially important species of shrimps aiming to the betterment of technologies over the already available eyestalk ablation techniques especially in pond reared shrimps. In spite of all the

technological developments so far, there remains number of areas where further development would be highly desirable for optimizing commercial seed production. The shrimp P. monodon, the most preferred candidate species for culture today, is one of the shrimp species which is difficult to mature when reared in captivity. So far the attempts have however met with only limited success. The growing demand for quality prawn seed from the farmers and entrepreneurs, coupled with uncertainity of their availability from nature at the appropriate time in required quantities has prompted research on problems connected with prawn seed production. In India there is an added significance in acquiring the technology for raising the broodstock in captivity as it could solve the perennial problem of non-availability of P. monodon brooders to the hatcheries coming up along the west coast. To extend shrimp culture to areas of the world lacking native shrimp populations, to ensure seed availability at the required time and to develop a genetic programme to improve the population characteristics, it is necessary to control maturation and reproduction in captivity. Therefore, the present research programme was planned with the objective of obtaining a detailed picture of the mechanism of reproduction and its endocrine control in P. monodon through molecular investigations. The experimental evaluation of the induction of maturity by manipulating the endocrine systems were also attempted.

The results of the entire studies are embodied in the present thesis. It starts with a general introduction to the subject under study covering the present status of our knowledge on the subject. It also emphasizes the importance of the present investigation.

Subsequent to general introduction, there are five separate chapters covering different aspects of the present investigation on

reproductive endocrinology of the prawn P. monodon. Each chapter begins with an introduction, followed by materials and methods, results and discussion. Introduction of each chapter highlights the importance of the particular aspect of study and also reviews the pertaining literature. Under, the materials and methods, the materials and the techniques used, as well as the statistical procedures applied for the data analysis are described, and the data obtained, are presented in the results with necessary figures and tables. Each chapter is concluded with a discussion mentioning about the salient findings of the study.

Chapter one deals with the investigations made in the female reproductive system during the process of ovarian maturation. The results of the investigations are presented under two parts. Part A deals with the biological characterization of the gonadal maturation. This include the morphological and anatomical characters of the reproductive organ ie. the ovary in relation to the process of maturation in P. monodon. In part B through light and electron-microscopy the chain of cytoplasmic and nuclear transformations takes place inside the ovarian tissues at different maturity stages were studied and based on these changes the process of oogenesis, was classified into five different stages (stage I-V) considering the immature stage as 0- stage, viz. Immature (stage 0), Pre-vitellogenic (stage I), Early-vitellogenic (stage II), Late-vitellogenic (stage III), Mature (stage IV) and Spent (Stage V) stages. Further the mechanism of oogenesis was investigated in detail through electron-microscopy which initially deals with the histological characters of oocytes at different stages of its transformation followed by a detailed ultrastructural description of these growing oocytes. This part also deals with the ultrastructural aspects of the mechanism of vitellogenesis in the growing oocytes of P. monodon.

Second chapter is on the male reproduction. The results of the investigations are presented under three parts here. Part A deals with the morphological and anatomical characters of the male reproductive system during the process of gonadal maturation. In part B histological and ultrastructural investigations were made to study the detailed mechanism of sperm differentiation in P. monodon. It also deals with the ultrastructural investigations on the formation of the complex acrosome in the spermatozoa. Part C is on the ultrastructure of the vas deferens and the mechanism of spermatophore formation. The structural modifications in different parts of the vas deferens and the mechanism of spermatophore formation were investigated through light and electron-microscopy.

In the third chapter detailed investigations were made on the different neuroendocrine centers of P. monodon in order to study the neuroendocrine control of reproduction. The results of this chapter are presented under four parts. Part A deals with the morphology of the various neuroendocrine centers, and its anatomical details with respect to the neurosecretory cell (NSC) types. Based on the size, general shape of the cell body and presence or absence of vacuoles in the cytoplasm different cell types were tentatively identified in the entire neuroendocrine system of P. monodon. The ultrastructure of these NSCs was studied through electron-microscopy. Based on the histological and ultrastructural investigations three different phases were identified in the process of neurosecretion. The process of neurosecretion has also been described at its biomolecular level in this part. Part B is on the distribution and mapping of NSCs in different ganglionic centers of central nervous system. In part C the eyestalk neurosecretory system has investigated through light and electron-microscopy. The ultrastructure of the sinus gland and its various

types of granules has also been investigated in the penaeid prawn P. monodon under this part. Part D is on the neuroendocrine control of reproduction. Here attention has been given to unravel the histo-physiological events of the oocytes in the female gonad and the corresponding cytological localization of the regulatory neuroendocrine organs and cell types in the neuroendocrine system.

Fourth chapter deals with the investigation on the neuroendocrine manipulations and their impact on gonadal maturation. Endocrine manipulatons were carried out by adapting following methods viz. unilateral eyestalk ablation, crude ganglionic extract injections (intra-muscular), and synthetic hormone injections (intra-muscular). Three individual ganglia, the cerebral, thoracic and optic ganglia were used for the preparation of crude extracts. Two synthetic hormones, progesterone and estradiol were injected to see its effects on maturation. The results are presented in three parts. Part A is on the influence of eyestalk ablation on ovarian maturation. Part B deals with the effect of ganglionic extract injection on ovarian maturation and part C with the effect of synthetic hormone injections on ovarian maturation.

Chapter V pertains to the studies on the cryopreservation, which is again divided into two parts. Part A is on the investigations made on the reproductive quality of male P. monodon. Various parameters used for the evaluation of reproductive quality are spermatophore weight, sperm count, sperm morphology and sperm viability. Spermatophore regeneration time and effect of eyestalk ablation on the spermatophore regeneration time and reproductive quality of male P. monodon have also been investigated as a preliminary study for cryopreservation. Part B deals with the cryopreservation studies. Screening of cryoprotectants was done in two short periods of one day and ten days in order to select the best cryoprotectants

for cryopreservation of sperm of P. monodon. Three different temperatures viz. 0° C, -30° C, and -195° C were selected for the cryopreservation studies. Using the five cryoprotectants selected after screening short term and long term cryopreservation studies on spermatozoa were carried out and results are presented.

Subsequent to these chapters is the summary and conclusions which consists of the overall finding of the entire study and the condlusions drawn therof.

The references referred in the entire thesis are presented at the end.

# GENERAL INTRODUCTION

# INTRODUCTION

One of the major problems preventing optimization of the commercial culture of shrimp is the control of female reproduction, which is highly complex in penaeid shrimps (Chang et al., 1992). It appears that a number of environmental signals can influence different hormonal factors which in turn regulate reproduction. The understanding of regulatory process of reproduction is an area of intense research (Adiyodi, 1985 and Charniaux-Cotton and Payen, 1988). Each animal species uses distinct environmental cues for timing its reproduction, has a full range of neuronal structures for perception of signals, and uses a complex neuroendocrine system for transduction of the messages to the endocrine organs, which themselves produce factors regulating the activity of the organs involved in reproduction (Van Herp et al., 1992). Success depends on the optimal completion of each step in the reproductive cycle of the animals, but it is evident that the neuroendocrine factors play a predominant interactive role between the external factors such as photoperiodicity, temperature, nutrition, stress and internal organization of the animal. According to Van Herp et al. (1992), the crustacean aquaculture in the future needs the support of precise knowledge of the role and mode of action of external and internal factors controlling reproduction, in order to create a strong basis for the development of intensive aquaculture. With the increasing knowledge of endocrine activity and its control over the gonadal development in crustaceans, the various techniques of endocrine manipulations for induced maturation of gonads are receiving great attention.

In the past decade a vast amount of research has been directed towards the understanding of female reproduction in crustaceans. Studies on the breeding cycles of various penaeids have been extensively done by

Cummings (1961), Subramoniam (1965), Rao (1968), Brown and Patlan (1974), Thomas (1974), Penn (1980), Kennedy and Barba (1981), Motoh (1981, 1985), Manasveta et al. (1993), Qunitio et al. (1993), and Susan et al. (1993). Most of the investigators, especially on the female reproduction, have used morphometric characters like colour changes in the ovary (King, 1948; Cummings, 1961; Brown and Patlan, 1974; Primavera, 1980; Motoh, 1985; Tanfermin and Pudadera, 1989) or gonadosomatic index (GSI) (Pillai and Nayar, 1971; Thomas, 1974; O'connor, 1979; Quinn and Barbara, 1987; Lawrence and Castle, 1991) for the assessment of ovarian maturation. Histological investigations on the reproductive organs during the ovarian maturation in various panaeid prawns have been done by various workers like Hudinaga (1942) in Penaeus japonicus, King (1948) in P. Setiferus, Sheikhmahmud and Tambe (1958) in Parapenaeopsis stylifera, Cummings (1951) in Penaeus duorarum, Duronslet et al. (1975) in P. aztecus and P. setiferus, Anderson et al. (1984) in S. injentis, Tom et al. (1987) in P. longirostris, Yano (1988) in P. japonicus, Tanfermin and Pudadera (1989), and Qunitio et al. (1993) in P. monodon, and Mohammed and Diwan (1994) in P. indicus. However, ultrastructural investigations during the process of ovarian maturation are restricted to the studies of Duronslet et al. (1975) in P. aztecus and P. setiferus and Mohammed and Diwan (1994) in P. indicus.

Similarly vitellogenesis is the most intensely studied aspect in oogenesis in various crustaceans during the past two decades (Hinch and Cone, 1969; Lui et al., 1974; Komm and Hinch, 1985, 1987; Tom et al., 1987; Quackenbush, 1989; Young et al., 1993; Chang et al., 1994; Mohammed and Diwan, 1994; Chang and Shih, 1995 and Sagi et al., 1995). Still the site of vitellogenesis is a fact of great controversy among various crustaceans. According to Komm and Hinch (1987), an accurate evaluation of oogenesis

and vitellogenesis requires a complete ultrastructural and biochemical investigation. However ultrastructural studies on the process of vitellogenesis are still fragmentary in crustaceans, and particularly in penaeids.

Until now studies on reproduction and its control in crustaceans have dealt with the female reproduction and not much attention has been paid to the males (Van Herp, 1992). Recent studies on the male reproduction in captivity revealed a deterioration in the quality of semen (Leung-Trujillo and Lawrence, 1985, 1987; Alfaro, 1993; Alfaro and Lozano, 1993; Pratoomchat et al., 1993). In this respect full understanding of reproductive mechanism of male penaeids is essential for any manipulation of sperm. Reproductive anatomy of several penaeids have been described and figured by many investigators (Hudinaga, 1942; Eldred, 1958; Subramoniam, 1968; Tirmizi and Khan, 1970; Huq, 1980; Vasudevappa, 1992; Mohammed and Diwan, 1994). However, studies on the spermatogenesis of penaeid shrimps are less (King, 1948; Lu et al., 1973; Pochon-Masson, 1983; Adiyodi, 1983; Vasudevappa, 1992; Mohammed and Diwan, 1994) compared to other crustaceans. Many of such studies focus only on light microscopical investigations and attempts on ultrastructural studies are, however, meagre (Lu et al., 1971). Similarly though few investigators have described the mechanism of spermatophore formation in various penaeids like P. kerathurus by Malek and Bawab (1974a, b); P. indicus by Champion (1987) and Mohammed and Diwan (1994) and in M. dobsoni by Vasudevappa (1992) through light microscopy, there has been little published work at ultrastructural level (Ro et al. 1988, 1990) in P. setiferus and Chow et al. (1991) in P. setiferus and P. vannamei.

It has been demonstrated that in decapod crustaceans, reproduction is under the control of hormonal factors viz. the GIH and GSH produced from the various endocrine centres. The neuroendocrine regulation of crustacean reproduction has been reviewed by Legrand et al. (1982), Payen (1986), Fingerman (1987), Charniaux-Cotton and Payen (1988), Meusy and Payen (1988), and Van Herp and Payen (1991). The morphology and histology of crustacean endocrine system have been studied by Carlisle and Knowles (1959), Nagabhushanam et al. (1992) and Mohammed et al. (1993) and X-organ sinus gland complex by Dall (1965a), Nakamura (1974), Madhysta and Rangnekar (1977), Gyananath and Sarojini (1985), Nanda and Ghosh (1985) and Mohammed et al. (1993). Different types of neurosecretory cells and different stages of secretion have been mentioned in the reports of Durand 1956, Peryman, (1969), Diwan and Nagabhushanam et al. (1992) and Mohammed et al. (1993) and X-organ sinus gland complex by Dall (1965a), Nakamura (1974), Madhysta and Rangekar (1977), Gyananath and Sarojini (1985), Nanda and Ghosh (1985) and Mohammed et al. (1993). Different types of neurosecretory cells in different stages of secretion have been mentioned in the reports of Durand (1956), Peryman (1969), Diwan and Nagabhushanam (1974), Rao et al. (1981), Decaraman and Subramoniam (1983) and Joshi (1987). However, a detailed description of the various phases of neurosecretion in relation to reproduction has been there only in very few reports (Gyananath and Sarojini 1985 and Mohammed et al. 1993).

Similarly endocrine control of reproduction has been investigated with a wide variety of crustaceans. However, the actual mechanism and hormones' working, behind reproduction have not been properly revealed in any crustaceans (Fingerman, 1987), but eyestalk factors have been held responsible by many (Aoto and Nishida, 1956; Demeusy, 1965; Rengnekar

and Deshmukh, 1968; Diwan and Nagabhushanam, 1974; Quackenbush and Herrkind, 1983; Fingerman, 1987; Mohammed and Diwan, 1991; Van Herp, 1992) in controlling this process. However, a second reproductive hormone found in the brain and thoracic ganglia has been attributed the role of gonad stimulation by Otsu 1960, Gomez (1965), Oyama (1968), Nagabhushanam and Kulkarni (1982), Eastman-Recks and Fingerman (1984), Mohammed and Diwan (1991), Yano (1992) and Yano and Wyban (1992). In this regard cytological ultrastructural studies of gonads and neuroendocrine masses can give some clues to unravel the mechanism of reproduction. But, unfortunately such studies are meager in crustaceans and lacking in penaeids.

The most successful technique for inducing maturation in captivity has been the removal of the eyestalk which is the site of GIH and this has been applied to various penaeids by many investigators with varying degrees of success (Arnstein and Beard, 1975; Alikunhi et al., 1975; Aquacop, 1975; Muthu and Laxminarayana, 1979; Lumare, 1979; Emmerson, 1980; Kulkarni and Nagabhushanam, 1980; Primavera, 1982; Chamberlain and Gervais, 1984; Choy, 1987; Wyban, et al., 1987 and Mohammed and Diwan, 1991). Similarly advancement in the ovarian stages were demonstrated by the administration of thoracic ganglion extracts by Otsu (1960), Oyama (1968), Hinch and Bennet (1969), Nagabhushanam and Kulkarni (1982), Eastman-Recks and Fingerman (1984), Takayanagi et al., (1986) and Yano (1992), by the administration of brain extract by Nagabhushanam and Kulkarni (1982) and Yano and Wyban (1992) and by extract of central nervous organs (brain and thoracic ganglion) by Mohammed and Diwan, (1991). Junera et al. (1977) first proposed that a vitellogenic stimulating ovarian hormone was present in the ovary of amphipod, Orchestia gammarella. Since then, progesterone (Kulkarni et al., 1979; Yano, 1985) and 17-alpha Hydroxy progesterone (Nagabhushanam et al.,

1980; Yano, 1987; Tsukimura and Kamemoto, 1988 and Koskela et al., 1992) have been tried to accelerate gonad development with various degrees of success. From the foregoing literature it was found that among these various techniques for the induction of gonads in captive shrimps, only eyestalk ablation has been tried successfully in P. monodon by various investigators. Further it is needed that the percentage of animals getting matured and spawned by this technique of eyestalk ablation in captive P. monodon are still low in our country and problems are more acute especially in areas where the natural population of this species is comparatively less.

The successful cryopreservation of viable gametes would open new perspectives in any culture operations. Many attempts have been made to cryopreserve the spermatozoa of aquatic vertebrates, mainly fishes. However, less attention has been paid on invertebrates. The various attempts on cryopreservation in invertebrates include, the reports of Sawada and Chang (1964), Dunn and Mc Lachlin (1973), Zell et al. (1979); Behlmer and Brown (1984), Chow et al. (1982, 1985), Ishida et al. (1986), Anchordouguy et al. (1987, 1988), Jayalectumie and Subramoniam (1989), Renard (1992), Bury and Olive (1993) and Joshi and Diwan (1993). In spite of the fact that among invertebrates, decapod crustaceans are the most economically important group of animals, very little attention has been paid on the freezing and preservation of gametes. A few attempts made to cryopreserve the spermatozoa of decapod crustaceans including prawns and crabs are by Behlmer and Brown (1984), in Limulus polyphemus, Chow et al. (1982, 1985) in Macrobrachium rosenbergii, Anchordouguy et al. (1987, 1988) in Sicyonia injentis, Jayalectumie and Subramoniam (1989) in Scylla serrata, and Joshi and Diwan, (1992) in Macrobrachium idella. However such studies are not done with the gametes in P. monodon till now.

CHAPTER I

# FEMALE REPRODUCTIVE SYSTEM AND MECHANISM OF OOGENESIS

A *Biological characterization of the female reproductive system*

B *Architectural changes during the process of oogenesis*

# CHAPTER I

## FEMALE REPRODUCTIVE SYSTEM AND MECHANISM OF OOGENESIS

### INTRODUCTION

The extent to which any biological system can be successfully manipulated is a function of understanding the mechanisms through which that system operates. As reproductive biology is central to all biological sciences a proper understanding of it is vital for the management of the species concerned through successive generations (Adiyodi, 1982). Eventhough the breeding cycles based on the morphology of reproductive organs of crustaceans have been well studied, in-depth knowledge about the sexual biology of crustaceans is still meagre in comparison to what is known of their terrestrial equivalents, the insects and many other animal groups. Reproduction in many decapod crustaceans in general and penaeoidean shrimp in particular is significantly different from that of most aquatic invertebrates as well as vertebrates (Bauer and Martin, 1991).

The fundamental problem in shrimp mariculture industry is the lack of predictably abundant supplies of offsprings of known heritage. Development of a suitable biotechnology for controlled induction of reproduction in the candidate animal is the prime requisite to overcome this problem. Inorder to develop such a technology, basic information on morphology, anatomy, physiology, cytology and molecular biology of the reproductive systems of the species is most essential. The cytological studies correlated with the morphology during the process of ovarian maturation form a concrete framework for the assessment of maturity. Moreover, such studies are more important in the correct staging of reproductive phase of the animals in the hatcheries for their better

management. The production of egg, well equipped with the necessary reserve food for the developing embryo takes place through a dynamic as well as dramatic process within the developing germ mother cells of the ovary. Cytological as well as electronmicroscopical studies of the oocytes during the process of oogenesis reveal the clear picture of the cells during their development. Nevertheless, relatively little emphasis has been laid on the biomolecular changes in the ovaries of the penaeids during different maturity stages. In order to probe into the complicated dynamic process involved in the development of oocytes, it is evident that a detailed knowledge of the ultrastructural changes which take place during the various stages of maturation is needed. So the basic cytological as well as biomolecular studies were carried out to understand the reproduction in the penaeid shrimp Penaeus monodon.

Over the past decade a great deal of useful information on the light microscopic features of reproductive organs particularly on oogenesis has been published. Reproductive cycles of decapod crustaceans have been studied extensively by many workers. Chief among them are Knudsen (1964), Pillai and Nair (1970), Diwan and Nagabhushanam (1974), Hill (1975), Haefner (1977), Aiken and Waddy (1980), Joshi and Khanna (1982), Damrongphol et al. (1991), and Janine et al. (1991). Similarly studies on the breeding cycles of penaeid prawns were made by Cummings (1961), Subramoniam (1965), Rao (1968), Brown and Patlan (1974), Thomas (1974), Penn (1980), Kennedy and Barbara (1981), Motoh (1981) & (1985), Menasveta et al. (1993), Qunitio et al. (1993) and Susan et al. (1993). Most of the investigations especially on the female reproductive biology are restricted to the morphometric characters of the reproductive organs, spawning season and spawning areas. Morphometric characters for the assessment of maturation are based either

on the colour changes during the reproductive cycles (King, 1948; Cummings, 1961 and Brown and Patlan, 1974.) or on the gonado somatic-index (GSI) (Pillai and Nair, 1971; Thomas, 1974; O'conor, 1979; Quinn and Barbara,1987.)

Many investigators studied the anatomical features of the female reproductive organs in various species of crustaceans through light microscopy. Chief contributions on the histomorphological features of the female reproductive system in various crustacean groups are by Ryan (1967) in Portunus sanguinolentus, Chandran (1968) in Charybdis variegata, Pillai and Nair (1970) in Uca annulipes, Portunus pelagicus and Metapenaeus affinis, Diwan and Nagabhushanam (1974) in Barytelphusa cunicularis, Joshi and Khanna (1982) in Potamon koolooense, Komm and Hinch (1985 & 1987) Coenobita clypeatus. Major hisological investigations on the reproductive organs during ovarian cycle in penaeid prawns are by Hudinaga (1942), King (1948), Sheikhmahmud and Tambe (1958), Cummings (1961), Duronslet et al. (1975), Anderson et al. (1984), Tom et al. (1987), Tanfermin and Pudadera (1989), Dall et al. (1990), Menasveta et al. (1993), Qunitio et al. (1993) and Mohammed and Diwan (1994). Most of these investigators describe the anatomical as well as cytological changes in the ovaries during oogenesis.

In crustacean oogenesis, as in a number of other animal taxa, one of the more conspicuous events is the gradual accumulation of nutritional and informational materials collectively referred to as yolk (Anderson, 1984). The process of yolk synthesis (vitellogenesis) is the most intensely investigated aspect in oogenesis (Hinch and Cone, 1969; Lui et al., 1974; Komm and Hinsch, 1985, 1987; Tom et al. 1987; Quackenbush, 1989; Young et al. 1993; Chang et al. 1994; Mohammed and Diwan, 1994; Chang and Sahi, 1995; and Sagi et al., 1995). Still the site of vitellogenin synthesis is an unrevealed fact in crustaceans. Studies on egg yolk protein synthesis

have been focussed on representatives from only a few of the many families of crustaceans (Charniaux-Cotton, 1985). Available information from the few crustacean species that have been studied, however, is controversial with respect to the mode of synthesis and packaging of yolk products in crustacea. Vitelloginin is the precursor to egg yolk protein and is one of the two lipoproteins known in crustaceans (Kerr, 1969; and Quackenbush, 1991) and its concentration in various tissues is correlated with yolk accumulation in the oocyte (Quckenbush, 1989; Okamura et al., 1992). The origin of yolk in crustaceans specially in decapods has been investigated through biochemical, electrophoretic, immunoflurescence, electron microscopical as well as hormone titre assays by many investigators. Biochemical and electrophoretic studies were made by Lui et al. (1974), Lui and O'connor (1976 & 1977), in crayfish Procambarus clarkii and crab, Pachygrapsus crassipes for isolation of vitelligenin to find out the site of yolk systhesis. With the same objectives Tom et al. (1987) conducted histological and immunoflurescence studies in Parapenaeus longirostris. Morphological and cytological investigations on these aspects include the studies of Hinch and Cone (1969) in Libinia emarginata, Zerbib (1980) in Orchestia gammarellus, Duronslet et al. (1975) in P. setiferus and P. azeticus, Komm and Hinch (1985, 1987) in Coenobita clypeatus, and Mohammed and Diwan (1994) in P. indicus. Yano and Chinzei (1987) studied the vitellogenesis in P. japonicus through in vitro incubation and immunoflourescence study. These studies revealed that controversy exists regarding the significance of extra ovarian source of vitellogenin systhesis in crustaceans.

Nevertheless in decapod crustaceans, several sites for yolk protein production have been proposed. These sites include the haemocytes of the

haemolymph, the hepatopancreas and sub epidermal adipose tissue of maturing female crustaceans (Kerr, 1969; Charinaux-cotton, 1978; Adiyodi and Subramoniam, 1983; Eastman Recks and Fingerman, 1985; Paulus and Laufer, 1987; Tom et al., 1987; Quackenbush and Keelay, 1988; Rankin et al., 1989; Bradfield et al., 1989; Quinitio et al., 1989&1990; Chang et al., 1993; Chang and Shih, 1995; Sagi et al., 1995). Some studies suggest that most of the vitellin in vitellogenic penaeid females is produced in the ovary and only a small amount is produced in extra-ovarian tissue (Fainzilber et al., 1992; Eastman-Recks and Fingerman, 1985, Lui and O'Connor, 1976a, 1976b). Further in vitro experiments showed a low amount of vitellogenin synthesis in the hepatopancreas of penaeid shrimp and crabs (Quackenbush and Keeley, 1988; Quackenbush, 1989a, 1989b). Other researchers have been unable to identify vitellogenin synthesis in any extraovarian tissue of penaeid shrimp (Yano and Chinzei, 1987; Rankin et al., 1989). on the other hand there is evidence that extraovarian sites do produce yolk proteins in crabs (Paulus and Laufer, 1987; Lee and Puppione, 1988). Shafir et al. (1992a) demonstrated a role for the hemolymph in transporting vitellogenin between its processing and target sites. Histological as well as electron-microscopical investigations made by Hinsch and Cone (1969) in L. emarginata, Duronslet et al. (1975) in P. aztecus and P. setiferus, and Mohammed and Diwan (1994) in P. indicus demonstrated the extra ovarian synthesis of yolk in crustaceans.

Eventhough vitellogenesis has been investigated by several investigators in various crustaceans, due to the diverse assemblage of animals, the penaeids have received little attention. Isolation and characterization of vitellin from the ovary of the prawn P. monodon have been studied by Qunitio et al. (1990) and Chang et al. (1993) and those of haemolymph have been done by Chang et al. (1994). These studies indicate

that a controversy exists regarding the significance of extra ovarian source of vitellogenin synthesis in penaeids also. In P. monodon Quinitio et al. (1990) and Chang et al. (1993) isolated the yolk precursors from two different sources and hence the exact source of vitellogenin synthesis was not clear. In this situation the biomolecular investigations can give the clear picture of yolk synthesis. Information regarding the biomolecular changes occurring in the oocytes during gonadal maturation as well as vitellogenesis, is however, scanty as far as Penaeus monodon is concerned. Hence in the present investigation of oogenesis in P. monodon was investigated in detail to learn more about the ovarian development and especially the mechanism by which ovaries accumulate their reserve food. The study includes the classification of the ovarian maturity stages based on its colour, gonadosomatic index, oocyte diameter and histological as well as ultrastructural changes in the oocyte. An elaborate mode of classification was made considering all these factors so that a better understanding of every aspect of the mechanism of reproduction at its biomolecular level is possible. The process of oogenesis has been investigated through light microscopy initially and later the same was investigated at ultrastructural level to expose the dramatic changes occurring in the penaeid ovary during its maturation.

## MATERIALS AND METHODS

### I. Collection of animals and preparation of tissues for histological studies

Wild caught prawns, P. monodon ranging in total length from 190 to 270mm in various maturity stages were used in the present study. These were captured from deeper offshore areas of Cochin and Madras. The prawns collected were transported to the laboratory in live condition and kept in 1 tonne fibre glass tanks containing seawater and provided with a sub-gravel biological filter working on an air lift mechanism. All prawns were fed daily with fresh clam meat, and maintained till sacrificed for further studies.

Prawns in different maturity stages were selected and the morphological characters of the reproductive organs were observed carefully. To study the morphological features of the reproductive system and its changes occurred during the process of gonadal maturation, the reproductive organs namely ovary oviduct, thelycum were excised from live prawns. Dissections were conducted in crustacean saline for keeping the tissues in live conditions. Ovaries and oviducts were removed from the same animals and gross features such as size, colour, nature and shape of the tissues were noted in different maturity stages. Tissues for light and electronmicroscopic studies were excised separately and fixed in appropriate fixatives. Gross morpholological studies were made using a stereoscopic dissection microscope. Different maturity stages were identified based on the morphological features such as gonadal development, colour and nature of the ovary and gonadosomatic index. The gonadosomatic index (GSI) was calculated as per the method described by Giese and Pearse (1974). The GSI was calculated as a percentage

of the weight of the ovary relative to total body weight. Since the different regions of the ovary exhibited homogenous oocyte stages (TanFermin and Pudadera, 1989) only a portion of the middle lobe was used for fixing for both histology and electron microscopy

## II. Light microscopic studies

Freshly excised tissues were immediately transferred to Bouin's fixative for a period of 24 to 48 hrs before further processing. Properly fixed tissues were washed over night in running tap water to remove the excess picric acid. Washed tissues were later dehydrated through ascending grades of alcohol series (30 to 100%) and cleared in chloroform. Dehydrated tissues were put for the cold impregnation in a wax chloroform mixture (1:1 ratio) for one day. Subsequently the tissues were transferred to molten wax (Parrafin wax with cerresin, Merck, MP. 58 to 60°C) for hot impregnation. After 2 more changes of 15 min. duration in fresh frozen wax, tissue blocks were prepared using L-blocks made of Brass.

Serial sections of the tissue blocks were taken at an approximate thickness of 5 to 7 µ using a rotary microtome. Sections were affixed on clean glass slides using fresh Meyer's egg albumen and flattened by placing on a slide warmer with few drops of distilled water. Subsequently the water was drained off and the slides were allowed to dry. Routine staining was carried out using Harri's hematoxylin stain with 1% Aqueous Eosin as the counter stain. Sections to be stained were first deparaffinized in two changes of xylene and then dehydrated through a down series of ethanol grades. Hydrated tissues were stained in Hematoxylin and blued in tap water before stained with eosin. Eosin stained slides were again dehydrated in two changes of absolute alcohol and cleared in xylene before mounting in

DPX mountant. DPX mounted slides were allowed to dry and after drying observations were made under a monocular microscope. Structural details of the developing oocytes and changes occurred during the successive stages of maturation were investigated. Since the increase of the oocyte diameter is a function of oogenesis, the micrometric measurements were taken using an occular micrometer calibrated with a stage micrometer. As oocytes strongly deviate from a spherical shape, the largest and the smallest axis of the oocyte diameter was taken and the average was used as the actual diameter.

Photo-micrographs of histological investigations of the ovarian tissues during maturation process were taken using an Olympus Universal research microscope (Vannox model PM 10 AD) equipped with an automatic exposure system. Black and white 35 mm film (Ilford, 125 ISO) was used for photography.

## III. Ultrastructural studies

The ovaries excised at different maturity stages were used for ultrastructural studies also. Initially small pieces of ovaries were bathed in cold fixative (4°C) by pipetting fixative directly on to the tissue in situ. The ovaries were then removed from the abdomen and placed in fresh primary fixative (4% gluteraldehyde), buffered with 0.2M Cacodylate buffer containing 2% tannic acid and 6% Glucose at pH 7.2 for 1-1.5 hrs at 4°C. After primary fixation, tissues were washed 3 times in cacodylate buffer, each about 15 min duration and post fixed in 1% buffered osmium tetroxide for about 1 hr at 4°C. Properly fixed tissues were again washed in cacodylate buffer and then dehydrated in a graded acetone series. The tissues were infiltrated and embedded in low viscosity resin embedding medium (Spurr, 1969) and kept at 60°C for 36 hrs.

After proper trimming semi-thin sections (0.5 to 1.0 μ) were cut from the polymerized blocks stained with toludine blue observed under light microscope in order to identify the interested area. These blocks were again trimmed for ultramicrotomy and ultra-thin sections were cut with a diamond knife on an ultramicrotome "Ultracut E". Ultra-thin sections of 300 to 600 A° collected on uncoated copper grids (300 mesh) were post stained in 10% uranyl acetate in methanol (Watson, 1968) and lead citrate (Reynolds, 1963). After drying, the grids were examined with a "Phillips CM 10" transmission electron-microscope at 60 KV. Ultrastructural details of the ovary were examined critically and interested areas were photographed using Kodak-100 ASA film.

# RESULTS

## A. BIOLOGICAL CHARACTERIZATION OF FEMALE REPRODUCTIVE SYSTEM

### A.1 Morphology of reproductive system

In P. monodon the female reproductive system consists of the internal organs such as the paired ovaries and oviducts and the external organ the single thelycum. The ovaries are partly fused bilaterally symmetrical bodies located dorsal to the hepatopancreas and ventral to the heart in the cephalothoracic region and dorsal to the gut in the abdominal region. It extends the entire length of the animal from base of the rostrum to the anterior portion of telson in the posterior region in the mature animals. Morphologically 3 main lobes were observed on each half of the ovary, anterior, middle and posterior (fig.2). The anterior lobes are situated close to the oesophagus and cardiac region and they bear a pair of somewhat elongated lobes, in the cephalic region. The six paired finger like lobules spread as a saddle surrounding the hepatopancreas constitute the middle lobe. From this a pair of lobe, one from each half of the ovary extends over the entire length of the abdomen is the posterior lobe. A pair of short narrow tubes namely the oviduct originate from the sixth lateral lobule of the middle lobe of each ovary, and descends to the gonopores on the coxae of the third pereiopods. The thelycum of P. monodon is of closed type. It is located on the ventral side between the fourth and fifth pereiopods and serves as the receptacle for spermatophores. Morphologically the thelycum consists of an anterior and two lateral lobes. Upon closer examinations a cavity was found in the thelycum behind the lateral plates for the storage of spermatophores.

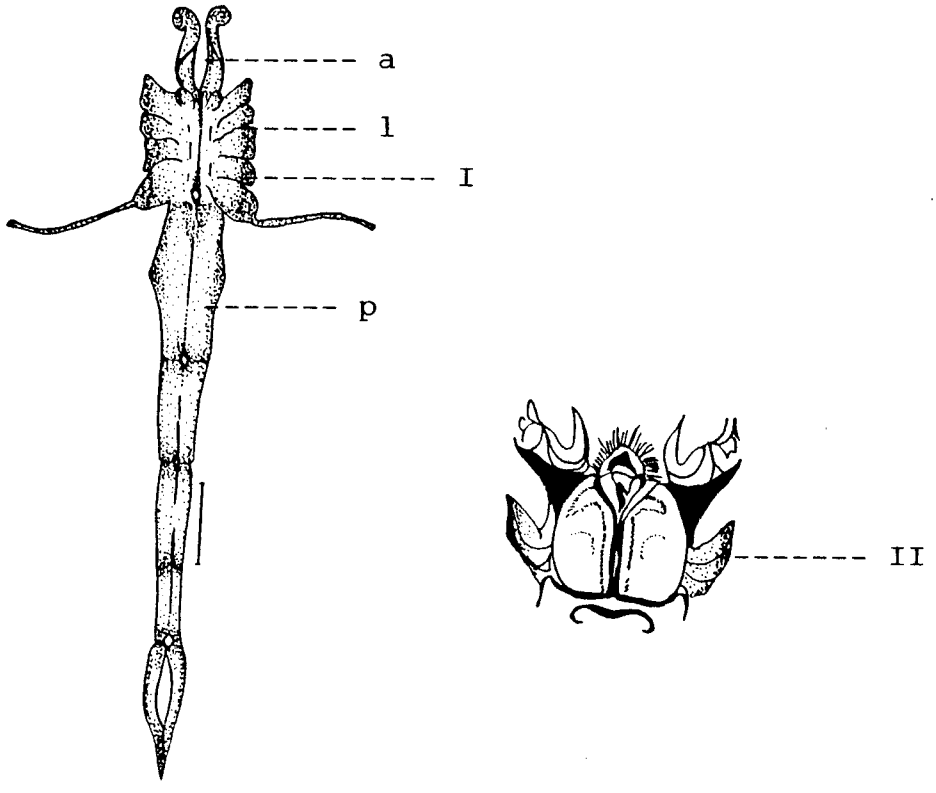


Fig. 2 Diagramatic representation of the female reproductive system of P. monodon.

I - Ovary, II - Thelycum
a - anterior lobe, l - lateral lobe, p - posterior lobe

## A.2 Evaluation of female reproductive maturity

Determination of reproductive state of a particular animal is very important for its fishery management and culture programmes. As in other penaeids, determination of ovarian maturation in P. monodon is possible through the evaluation of colour changes in the ovary during maturation process and also through the assessment of the increase in the ovarian volume viz. (GSI) during its progression towards maturation. The developmental changes in the external genital organ - the thelycum - were also examined in relation to the ovarian development in the first maturing animals. Based on these changes the ovarian development of P. monodon in the present study was classified into 5 different stages (stage I to V) considering the immature stage as 0 stage. The different maturity stages include pre-vitellogenic, early-vitellogenic, late-vitellogenic, mature, and spent ovarian stages. Quantitative parameters of these different reproductive stages are presented in the table (1).

### a. Visual assessment of ovarian development

Immature or stage 0 ovaries were not visible externally through the dorsal exoskeleton. The ovaries in this stage appeared as a thin, translucent, and unpigmented band with small finger like linear lobules in the hepatopancreatic region. The posterior lobes are found to be rudimentary. Stage I or pre-vitellogenic ovary was not visible through the exoskeleton. Upon dissection it was found that the paired ovary lost its transparency and appeared opaque. The colour of the ovarian lobules varied from white to cream and appeared a bit granular especially in the anterior and middle lobes. The anterior and middle lobes appeared larger in this stage but no increase in size was observed in the posterior lobe. Early

Table 1.

## QUANTITATIVE PARAMETERS OF PENAUS MONODOM AT DIFFERENT STAGES OF OVARIAN MATURATION

| S. No | Cryoprotectants | Conc. | | % viability of sperms through acrosome reaction | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Duration | one week | | 2 weeks | | | 1 month | | |
| | | | PF | 0°C | -30°C | -195°C | 0°C | -30°C | -195°C | 0°C | -30°C | -195°C |
| | CL(mm) | 61.3 | 63.1 | 62.4 | 64.3 | 65.2 | 63.6 | 68.5 | 65.6 | | | |
| | BL(mm) | 212 | 208 | 215 | 228 | 260 | 245 | 265 | 245 | | | |
| | BW(g) | 150 | 147 | 140 | 151 | 156 | 151 | 158 | 141 | | | |
| | GW(g) | .8 | 1.9 | 2.38 | 8.8 | 11.8 | 12.4 | 14.7 | 3.2 | | | |
| | GSI(%) | .53 | 1.3 | 1.7 | 5.8 | 7.53 | 8.17 | 9.3 | 2.2 | | | |
| | AOD(um) | 24.1 | 61.4 | 98.9 | 189.5 | 174.2 | 338.5 | 406 | 140.5 | | | |
| | AND(um) | 18.3 | 34.0 | 47.8 | 69.2 | 81.8 | 107.1 | 122.5 | 59.6 | | | |
| | Colour and appearance of ovary | Transparent unpigmented & smooth | Opaque white to cream & smooth | Cream smooth & granular | Yellow firm & granular | Greenish Yellow firm and granular | Green and higly granular | Greenish Black thick & firm | Pale Yellow granular flacid | | | |

CL - Carapace length
BL - Body length
BW - Body weight
GW - Gonad weight
GSI - Gonado somatic index
AOD - Average oocyte diameter
AND - Average nuclear diameter

vitellogenic (stage II) ovaries were faintly visible through the exoskeleton as a thick linear solid band due to its expansion in the posterior thoracic and anterior abdominal regions. The diameter of the posterior lobes increased than that of the intestine. The dissected ovaries appeared firm and granular in texture with a light yellow to greenish yellow colouration due to the accumulation of yolk. The visibility of the ovary through the exoskeleton is due to the granular nature as well as increased colouration in the outer lininig of the ovarian lobules. The late vitellogenic or stage III ovaries were characterized by the presence of a diamond shaped expansions of the ovary at the first abdominal segment. Ovaries were visible through the exoskeleton as a thick solid and dark linear band due to its further expansion in all of its lobules. The dissected ovaries appeared light to dark green in colour with a firm and granular texture. The mature (stage IV) ovaries were clearly visible through the exoskeleton as a thick band on the entire dorsal side of the animal. The fully diamond shaped or butterfly shaped expansion of the first abdominal segment was clearly visible. Upon dissection it was found that the ovarian lobes were considerably larger than the preceding ones, and these fully developed ovaries filled up all the available space in the body cavity, both in the cephalothoracic and abdominal region The olive green to dark green ovaries were found to be firm and granular in texture. Spent (stage V) ovaries were not visible through the exoskeleton and on the basis of external appearance, indistinguishable from those in the pre-vitellogenic stage. Upon dissection ovaries appeared flaccid and found greatly reduced in size. The ovaries remained opaque still, but colouration was less distinctive than the pre-vitellogenic stage. In incomplete spawns, portions of ovaries, particularly posterior lobes, retained the colouration of the mature.

**b. Gonadosomatic index (GSI)**

The GSI values of each prawn at different maturity stages were calculated and presented in table I. In P. monodon the GSI values recorded in the present study ranged from 0.54 ± 0.10 in immature females to 9.3 ± 1.1 in mature (gravid). Four fold increase in GSI values was observed during the transition stage from pre-vitellogenic stage to vitellogenic stage (stage I to II, 1.7 to 5.8). Only two fold increase was observed when it reached pre-vitellogenic stage from immature stage (0.45 to 1.1). A gradual increase in GSI values was observed during all other progressive stages of maturation. Subsequently there was a remarkable decline in GSI values in spent ovaries (2.2 to 0.1)

**c. Thelycum**

During the present investigation it was found that in the first maturing animals the development of the thelycum was also in relation with the ovarian development. It was observed that at stage 0 and sage I of maturity of the animals the lateral plates of thelycum were widely separated leaving a "V" shaped notch in the middle. The cavity between the plates appeared empty and was not impregnated with spermatophores indicating that mating has not taken place. In stage II animals the lateral plates were some what broadened than the preceding ones and had started their overlapping with the lateral flanges of the median plate. Here also the thelycum was never observed as impregnated. Further, broadened lateral plates completed their overlapping with the lateral flanges of median plates in the stage III animals. There was only a "V" shaped small slit between these lateral plates and most of animals at this stage were impregnated with spermatophores. Thickened and intersected lateral plates were observed in mature animals.

Later these plates become crescent shaped and the entire thelycum occupied almost the entire space between the sternite of the 5 th pair of pareiopods. Upon dissection a concealed hollow space was observed beneath the lateral plates where in the spermatophores were found deposited. Same was the structure observed in the thelycum of spent (stage - V) animals.

## B. ARCHITECTURAL CHANGES DURING THE PROCESS OF OOGENESIS

Reproductive cycle of P. monodon includes a series of events starting from activation of primordial germ cells to the differentiation of highly yolk equipped ova. Staging of reproductive development based on the morphological characters is not appropriate in the light of recent studies. Correct staging of reproductive maturity requires more detailed characteristics like size of the germ cells, nature and arrangement of oogonial cells in the ovary. By microscopical investigations, it has been possible to obtain cytological information for defining the stages in the gonad development. Light microscopical examinations of the ovaries of P. monodon at different stages of their maturity revealed the chain of nuclear and cytoplasmic changes that occurred inside the developing ovary. From the investigations it was observed that the oogonial cells developed from the primordial germ cells got transformed into the mature ova with sufficient yolk for the development of the embryo. A series of dramatic as well as complicated changes were observed in the developing oocyte during its developmental phase. Based on the changes that occurrs inside the cytoplasm and nucleus of the growing oocytes, process of oogenesis was classified into six different phases, such as immature, pre-vitellogenic, early-vitellogenic, late-vitellogenic, mature or gravid and spent oocytes. These oocytes phases correspond to the stages 0 to V as earlier, based on morphological characters.

**Oogenesis**

From the light microscopical investigation of the ovarian tissue of P. monodon it was found that ovary was encompassed by a thin ovarian wall, (fig. 5) which consisted of 3 layers; a thin outer most pavement epithelium, an inner layer of germinal epithelium and a relatively thick layer of connective tissue in between. The middle layer of connective tissue was eosinophilic while the other two epithelial layers were basophilic in nature with hematoxylin & eosin. Blood capillaries were observed in the ovarian wall (fig. 4,5). A germinal zone was found in almost all sections on the ventro lateral periphery in the form of a thin band (fig. 3,4). This is the " zone of proliferation " from which the displacement of oogonial cells takes place. Invasion of this zone into the ovarian lobes was observed from the ventral portion of the ovary. It was observed from the sections that young oocytes moved farther from the germinative zone upon maturation. So the developing oocytes and ova were found towards the center of each ovarian lobe.

**I. Immature stage**

An active zone of proliferation with clusters of developing oogonial cells are the characteristic feature of immature ovary (fig. 3). It was observed in all sections that the primary and secondary oogonial cells were arranged in a graded manner in the ovary so that the growing secondary oogonial cells were shifted to the interior (fig. 4). Nuclei of the primary oogonial cells were not conspicuous The entire cell stained deeply with hematoxylin (fig.3). These primary oogonial cells had a diameter of 11 ± 1.35 $\mu$. These cells undergo mitotic division and gives rise to the secondary oogonia. It was observed that the secondary oogonial cells

possessed a conspicuous nucleus stained with hematoxylin and eosin stained very little rim of cytoplasm (fig. 4). There was an increase in the number of diffused granule like nucleolus in the nucleoplasm of these oogonia, ranging from 8 - 20 in numbers. The secondary oogonia were bigger with a cell diameter of 24.1 ± 3.5 μ and a nuclear diameter of 18.3 ± 3.9 μ.

The oogonial cells appeared round ultrastructurally and their large nuclei occupied approximately 80 % of the cell volume (fig. 6). The oolemma was smooth and without any particular morphological specializations at this stage. The diffused electron-dense chromatin materials as well as small granule like round nucleoli were not observed in the nucleolemma of these oogonial cells. Electron-loose cytoplasm in these oogonial cells contained only some small granules and filamentous materials (fig 7). Other cell organelles were not at all visible at this stage of development.

## II. Pre-vitellogenic stage (Stage I ovary)

The most striking feature of previtellogenic ovary is the presence of highly basophilic primary oocytes with much more increased cytoplasmic volume than that of the oogonial cells (fig. 5). These primary oocytes are formed through the meiotic division of the secondary oogonial cells. Upon closer examinations two phases of development were observed in this pre-vitellogenic oocytes ie. oocytes in the chromatin nucleolus stage (fig. 5) and the oocytes in the perinucleolus stage (fig. 8). Former were smaller than the latter and appeared as round to oval cells with a prominent nucleus. These nuclei contained 10 to 18 centrally located, deeply stained granule like nucleoli and prominent chromatin materials in their nucleoplasm. These oocytes with an average cell diameter of 61.4 ± 11.2 μ and a nuclear diameter of 34.0 ± 3.9 μ were devoid of individual follicle cell layers. The

perinucleolar oocytes were identified by the displacement of nucleoli towards the periphery of the nucleoplasm (fig. 8). It is only at this stage these oocytes begin their folliculogenesis around each cell. These oocytes with an average diameter of 98.9 ± 5.1 μ and nuclear diameter of 47.8 ± 3.9 μ were not fully surrounded by follicle cells. The follicle cells in this stage were rectangular or cuboidal with a highly vacuolated conspicuous nuclei (fig. 8). The homogenous agranular cytoplasm as well as the follicle cells around it were deeply basophilic.

Ultrastructurally the pre-vitellogenic oocytes also appeared round, like oogonial cells but here, the cytoplasmic volume was more compared to the oogonial cells. The nuclei was round with a clear nucleolemma which was interrupted by numerous nuclear pores through which the nuclear materials presumably passes into the perinucleolar ooplasm. The nucleoli of the pre-vitellogenic oocytes appeared as electron dense bodies along the inner periphery of the nuclear wall (fig. 9) Some membrane-free, electron-dense materials were observed at the outer side of the nuclear wall associated with the nuclear pores (fig. 9).

Ultrastructurally both phases of pre-vitellogenic oocytes ie. the oocytes at the chromatin nucleolus stage and the oocytes at the perinucleolus stage exhibited the same sub-cellular structures. It was observed that cytoplasm of the pre-vitellogenic oocyte contained ribosomes, mitochondria, Golgi elements and cisternae of the gnular endoplasmic reticulum (fig. 10,11). Variably sized vesicles filled with dense, osmiophilic granules were observed near oolemma (fig. 10). Numerous long lamellae of parallel membranes were observed throughout the cytoplasm. These lamellar congregations possessed some regular poreike structures and were identified as annulate lamellae. Membrane-free, electron-dense aggregates (nuage) appeared between dense lamellar membran and distributed throughout the

cytoplasm (fig. 11). Because of the limited cytoplasmic volume at this stage it was difficult to distinguish differences between peripheral and perinucleolar cytoplasm.

## III. Early vitellogenic stage (Stage II ovary)

Closer examinations of the early vitellogenic ovary revealed oocytes in two sub-stages ie. the oocytes at cisternal phase (fig. 12,13) and oocytes at platelet phase (fig. 18,19). The cisternal phase early vitellogenic oocytes were round to oval with an oocyte diameter of 189.5 ± 15.3 μ and a nuclear diameter of 69.2 ± 9.6 μ. Here a sudden two fold increase was noted in the size of the oocytes but not in the size of the nucleus. The nature of the cytoplasm also changed suddenly from homogenous to vesicular and little bit grannular (fig. 12,13). From the granular nature of the cytoplasm and its sudden increase in the cytoplasmic volume it was noted that during this stage onwards the oocytes started its active accumulation of yolk. This granular nature is due to the accumulation of oil globules in the cytoplasm, which is the characteristic feature of the primary vitellogenic oocytes. Consequently the nucleolar materials made a halo around the nucleus due to their circular arrangements in the peripheral karyoplasm (fig. 12,14). During this stage only, the formation of follicle cells around individual oocyte was complete. Because of the sudden increase in the cytoplasmic volume, the follicle cells stretched considerably and consequently decreased in their thickness (fig. 12,14). The hypertrophied nucleus as well as nucleolus of follicle cells was conspicuous during this stage (fig. 12,14). Some oocyte like cells but without a nucleus (Pseudo-oocytes) were also observed among the growing oocytes (fig. 14). Such cells and the oocytes in close proximity to them

was found encompassed by a common follicle cell. A reduction in the size of these pseudo-oocytes was noticed as the neighbouring oocyte increased their size (fig. 14).

Platelet phase oocytes possessed a highly vacuolated, rough and granular type cytoplasm which was wholly eosinophilic upon staining (fig.18,19). The oocytes at this phase measured 274.2 ± 8.3μ in diameter and their nuclei had diameter of 81.8 ± 4.3 μ. The centrally located round nuclei stained pale with hematoxylin and the number of nucleoli were observed to be reduced (fig. 18). Further reduction in the size of the "pseudo-oocytes" was observed as the oocytes reached their platelet phase. In the platelet phase oocytes, such cells appeared as a small bit of granular cytoplasm just nearer to it inside the same follicular encompassment (fig. 24). Due to the increase in the volume of the oocyte the follicle cells encompassing them stretched further and appeared as a narrow band of flattened cells around each oocyte (fig. 19).

In the early-vitellogenic ovary, two different types of oocytes, the cisternal phase oocytes and platelet phase oocytes were clearly distinguished through ultrastructural studies. The ultrastructural changes in the oocytes during the transition from pre-vitellogenesis to early-vitellogenesis were specially prominent in the perinuclear activity. The perikaryon of the cisternal phase oocytes contained numerous active cell organelles and developing vesicles (fig. 16,17). Membrane free granular aggregations differing in their shapes and contents appeared around the nuclear membrane (fig. 15). All these granules contained electron-opaque flocculent materials. Electron-loose membrane-bound vesicles were apparent in the perinucleolar cytoplasm (fig. 17). These extranuclear vesicles fused to form large agranular vesicles. At fine structural level, cytoplasm of these cells is found to contain numerous free ribosomes with minimal membrane association

(fig. 15,17). Only a few mitochondria were observed in these oocytes, most of them being located near the oolemma. Golgi bodies were also apparent in the peripheral cytoplasm. It was observed that the peripheral cytoplasm of the early vitellogenic oocytes appeared as smooth and electron loose with abundant supply of parallel, circular and concentric stacks of rough endoplasmic reticulum (RER) nearer to the nuclear membrane and to the oolemma (fig. 16). In addition to the RER, agranular membrane systems were also observed (fig. 17).

Ultrastructural investigations revealed an active cytoplasm in the follicle cells encompassing each oocyte at this stage. Numerous parallel stacks of RER and agranular reticular elements with intra cisternal-granules were observed in the cytoplasm of the follicle cells (fig. 25). Membrane-bound intra-cellular vesicles were also apparent in close proximity to these reticular elements. These reticular elements found were very active with some flocculant materials inside. It was observed that the reticulum in these follicle cells underwere a progressive differentiation during which the flocculant materials and the intra-cisternal granules aggregated and finally these granular vesicles made their appearance.

Ultrastructural studies revealed that the platelet-phase oocytes were characterized by their abundant supply of yolk platelets and other vesicular bodies along with their active cell organelles (fig. 20). Biomolecular investigations revealed that the platelet-phase oocytes were the transition stage oocytes between the early vitellogenic and the late-vitellogenic or cortical rod phase oocytes. It was observed that characters of both the early vitellogenic as well as cortical rod oocytes were overlapping at this particular stage. The nucleus of these oocytes appeared comparatively smaller (fig. 13,14) than the previous stage due to the increased cytoplasm to nucleus ratio occurred during the progression of vitellogenesis. Intranuclar

vesicles are still observed in these oocytes. Due to the highly porous nature of the nuclear membrane it appeared as an undulating membrane (fig. 20). Diffusion of the nucleolar materials was evident from the electron dense granules aggregated around the nucleolemma as well as in the perinucleolar cytoplasm.

The perinucleolar cytoplasm of these oocytes was highly rough and granular in appearance due to the accumulation of both electron dense as well as electron loose vesicles (fig. 20,23). Accumulation of membrane - free and membrane-bound granular bodies was continued still in these platelet phase oocytes. Masses of granules and vesicles exhibiting nucleolar like electron-density were apparent in the perinucleolar cytoplasm (fig. 20). These smaller granules fused together giving rise to larger granules. Whorls of rough endoplasmic reticulum were found in association with these larger granules (fig. 20). In addition to these granules a variety of granules differing in their size, shape and electron density were observed in the perinucleolar cytoplasm. These include membrane free extremely electron-dense large yolk bodies, rough endoplasmic reticulum bound developing yolk bodies and developing lipid vacuoles (fig. 20). Circular profiles of swollen rough endoplasmic reticular elements with an accumulation of condensing materials were also abundant in the perinucleolar cytoplasm (fig. 20). An increased number of round to elongate mitochondria with lamellar cristae of variable shape filled with electron-dense matrix, numerous free and attached ribosomes and highly active Golgi bodies were found in the cytoplasm of these oocytes (fig. 20-23).

Cytoplasm in the perinucleolar area to the oolemma contained numerous lipid vacoules. Large lipid vacoules were found in association with Golgi complexes (fig.21,23). Towards the periphery of the oocytes, RER vesicles as well as intra-cisternal granules were observed along the

forming face of the Golgi complex (fig. 23). Golgi complex were prominent, in the peripheral cytoplasm with variable number of stacked cisternae. It was noted that small moderately electron-dense vesicles were pinched off from the cisternae of the active parallel stacks of rough endoplasmic reticulum with intra-cisternal granules as observed in the cytoplasm nearer to the oolemma (fig. 23). Developing yolk bodies were abundant in these peripheral cytoplasm in association with these reticular stacks. Numerous free ribosomes also were noticed around these bodies (fig. 22). RER elements were also observed in association with these vesicles. These granules in association with the RER bodies together suggested another source of yolk constituents. It was noted that some pinocytotic vesicles were pinched off from the plasma membrane of the oolemma to the ooplasm.

Biomolecular investigations of the follicle cells around the platelet phase oocytes exhibited an increased subcellular activity. It was observed that the flat follicle cells possessed an oblong nucleus and a highly electron dense large nucleolus inside the nucleoplasm (fig. 27). Other cell organelles found in the follicle cell cytoplasm were round to oval mitochondriae, Golgi complex, smooth and rough reticular elements, ribosomes and vesicular bodies (fig. 27). There was little inter follicular space found between the oocyte and follicle cell.

## IV. Late vitellogenic stage (Stage III ovary)

Late vitellogenic oocytes were characterized by the appearance of radially arranged well developed, club shaped structures in the peripheral ooplasm which are the cortical rods (fig 28). Oocytes at this stage measured 138.5 ±9.7 $\mu$ in diameter with a nuclear diameter of 107.7 ± 7.3 $\mu$. An abundance of yolk platelets was also observed in between the cortical rods

of these oocytes. The cortical bodies measured 45.1 ± 4.4 $\mu$ in length and 20.7 ± 3.4 $\mu$ in breadth. Further flattened follicle cells were observed as a thin covering around each oocyte.

Ultrastructurally late vitellogenic oocytes were characterized by the presence of some specialized structures - the cortical rods - (fig. 28). These cortical rods were moderately electron-dense with homogenous cytoplasm. Higher magnification revealed a distinct substructure in these cortical rods. The matrix of these bodies was packed with some "feathery" structures (fig. 30). Each of these structures appeared as an electron-dense central axis upon which numerous moderately electron-dense thin fibrillar elements were arranged as in a feather. The whole cytoplasm between these cortical rods was filled with similar type of yolk spheres and platelet found in the platelet phase (fig. 29). The moderately electron-dense developing yolk bodies found in the cytoplasm of platelet phase oocytes were not apparent in this phase. Similar type of electron density of the newly appeared cortical bodies and the sudden disappearance of these yolk bodies made to assume that those yolk bodies underwent some progressive changes and finally aggregated together to form the cortical bodies. The cell organelles like ER, Golgi bodies, and mitochondria were not prominent in this stage. The oolemma at this stage appeared broken with the presence of numerous pinocytotic vesicles (fig. 31).

Some concentric layers of reticular elements encompassing developing yolk granules were noticed (fig 32,33,35) in the peripheral ooplasm closer to the follicle cells, and also in the cytoplasm of the adjacent follicle cells. It was further observed that as the granules enlarges the circular layers changed to semi-lunar shape and finally discharge the granule to the cytoplasm (fig. 32). Concentric layers of reticular elements with developing yolk precursors were also observed with abundant supply of free ribosomes

(fig. 32). These vesicles appeared in the oocyte wall in close apposition to the follicle cells (fig. 32,33). The oblong nucleus of the follicle cells at this stage was smaller than previous stage (fig. 33, 35). Developing yolk bodies in these follicle cells were also surrounded by circular or semilunar endoplasmic reticular elements. Other cytoplasmic inclusions were similar to that of the previous stage (fig. 34).

## V. Mature stage (stage IV ovary)

The mature oocytes appeared similar to those cortical rod phase oocytes, except the fact that the cortical rods moved to the extreme periphery of the ooplasm and lost its appearance (fig. 36). The fully matured oocytes appeared as more elongated than circular with a very thin rim of follicle cells around it. The nucleus now much reduced in size, started its migration to the periphery and upon full maturation many of the cells were without a nucleus as it was shifted to the cytoplasmic membrane (fig. 36). The follicle cells around each oocyte slowly got detached from the oolemma at this fully matured condition so that the ovulation became easy. Because of the more elongated appearance of the oocytes they measured up to 406.3 ± 13.4μ in diameter but their nucleii measured only 91.7 ± 4.7 μ in diameter. The greatly reduced nuclei lost their round shape due to the disintegration of the nuclear wall, but the nucleolus was prominent (fig. 36). The cytoplasm of the oocytes and the follicle cells were wholly eosinophilic upon staining. A prominent nucleus was observed in the follicle cells around each oocyte even at this full maturation.

Ultrastructural investigations revealed that mature oocytes shortly before ovulation were similar to that of cortical rod phase oocytes, except a few differences in their cytoplasm. Distinct oval cortical bodies were

# DISCUSSION

Precise staging of gonadal maturation of female prawns is essential for the selection of females in breeding, spawning and production of seed for sea farming and various other experimental purposes including manipulations of gametes for biotechnological applications like egg and embryo preservation. A detailed knowledge regarding the oocyte maturation is essential for the better understanding of the reproduction, which is the inevitable part of any culture operations. Moreover as gametogenesis is the core of reproduction in any animal, a thorough investigation on such aspects is unwarranted for evolving biotechnological methods, which may be helpful in acquiring reproduction in captivity. From the perusal of the literature cited earlier it was found that an ultrastructural study of gonad, pariticularly ovary is unavoidable for the indepth investigations of the various dynamic processes involved during the gamete development. Once the morphological development of gonad correlated with its cytological characteristics is well understood, one can easily predict the reproductive quality of the brooder by observing its morphological characters without disturbing the animal more. Understanding of shrimp reproduction requires combined knowledge from all the aspects viz. the changes in the morphological characters of the gonads, and corresponding cytological figures of the normal animal which are essential for the better management of brooders for seed production. Considering all these factors, a detailed investigation in the various aspects of female reproduction was carried out in a penaeid prawn P. monodon during the present study.

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The present investigation in P. monodon revealed that the female genital organ consisted of paired ovaries, which are partly fused, bilaterally symmetrical bodies extending from the cardiac region of the anterior portion

to the telson, paired oviducts and the thelycum in the ventral side. Similar type of ovarian morphology has been described by Motoh (1981) and Solis (1988) in the same species from the seas of Philippines. Morphologically it was similar to the observations in other penaeids like Penaeus setiferus (King, 1948), P. duorarum (Cummings, 1961), P. indicus (Subramoniam 1965, and Mohammed and Diwan, 1994), P. merguiensis (Tuma, 1967), P. setiferus and P. aztecus (Duronslet et al., 1975).

In penaeid shrimp gonadal growth can be evaluated using a number of parameters. Visual assessment of gonadal development is one of the effective methods of studying reproductive state of the marine organisms to determine the degree of gonadal growth (Castle and Lawrence, 1991). For females the common mehod has been the visusl assessment of the ovaries on the basis of egg size and colour (King, 1948; Cummings, 1961; Subramoniam, 1965; Tuma, 1967; Rao, 1968; Villaluz et al., 1969; Primavera, 1980; Motoh, 1981; Yano, 1985; Tanfermin and Pudadera, 1989). The gonadal maturation in female penaeids categorized by the external staging system developed by King (1948) for P. setiferus and Tuma (1967) for P. merguiensis based on the colour, size and nature of developing ovary was used for the classification of maturity stages in P. monodon during the present study. Another simple indicator of the reproductive state of the marine organism is the gonado somatic index (GSI) (Grant and Taylor, 1983). In this method of assessing gonadal growth, changes in the ratio between gonadal weight and body weight is taken into account. This has been variously used in penaeids (Lawrence, et al., 1979; Joshi, 1980; Lawrence and Castle, 1991).

Histological characteristics are more reliable conrete evidence for the assessment of gonadal maturation. Macroscopic and histological characterization of oocyte development in several penaeid species have been carried out by different investigators like Subramanyam (1965) and Rao (1968)

in P. indicus, Brown and Patlan (1974) in P. aztecus, P. duorarum, and P. setiferus, Duronslet et al. (1975) in P. aztecus and P. setiferus, Tom et al. (1987) in Parapenaeus longirostris, Yano (1988) in P. japonicus and Mohammed and Diwan (1994) in P. indicus. Although the evaluation criteria for the classification of gonadal development and number of ovarian developmental stages are arbitrary and vary in literature, the most commonly used system divides the ovarian maturation into five stages. On the basis of ovum size, gonad expansion and colouration, the maturation of ovary in P. monodon has been categorized into 5 stages by Villaluz et al. (1969), Santiago (1977), Primavera (1980), Motoh (1981) and Solis (1988). But later Tan Fermin and Pudadera (1989) revised this classification into four stages based on the histological and histochemical studies for an easy identification of the maturity stages of spawners. Qunitio et al. (1993) added 2 more stages and described the ovarian maturation of P. monodon in 6 stages for the practical use of brooders in the hatcheries. From these reports the classification was found confusing and therefore in the present study based on the morphological characters and histological and ultrastructural changes in oocytes, the maturity stages of P. monodon were classified into five main ovarian stages. Detailed investigations through light and electron-microscope revealed different sub-stages particularly during the vitellogenic phases of the ovarian development. It was found that these sub-stages were more relevant in the process of vitellogenesis. So the five main ovarian stages taken for the classification of maturity stages are Immature (stage 0), pre-vitellogenic (stage I), early-vitellogenic (stage II), late-vitellogenic (stage III) mature (stage IV) and spent (stage,V).

In the present study it was observed that the thin ovarian wall is composed of 3 layers. King (1948) in P. setiferus and Subramanyam (1965) in P. indicus described three layers in the wall of the ovary, a thin outer

most layer of pavement epithelium, an inner layer of germinal epithelium and a layer of connective tissue in between. Mohammed and Diwan (1994) however, described only 2 layers in the ovarian wall of P. indicus. A zone of proliferation or germinal zone was observed in the periphery of the ventro-lateral wall in P. monodon during the present study in almost all maturity stages. Invasion of this zone into the ovarian lobes are noted during early developmental stages. Similar type of observations were reported in the ovary of P. setiferus, by King (1948), P. indicus by Subramanyam (1965), and Mohammed and Diwan (1994) and P. japonicus by Yano (1988).

Oogenesis in crustaceans involves two distinct processes; proliferative and differentiative. During the proliferative process in the germinal zone the number of oogonial cells increases by mitotic multiplication. The primary oocytes derived from the secondary oogonial cells are transformed into typical egg cells by differentiative process (Adiyodi and Subramoniam, 1983). It was observed in the present study that the process of oogenesis in P. monodon also get completed through these two phases. The proliferative phase of multiplication was observed in the immature as well as maturing ovaries. The primary oogonial cells derived from the germinal zone undergoe rapid multiplication and form the secondary oogonial cells. During differentiative phase it was found that these oocytes accumulated its reserve yolk and finally developed into fully matured oocytes. Yano (1988) in P. japonicus observed a similar pattern of oocyte formation. The stages of mitotic division of primary oogonial cells were difficult to observe and recorded as they occurred in rapid succession (Adiyodi and Subramoniam, 1983). Description of the primary oogonial cells in crustaceans, especially in penaeids are scanty. Instead the secondary oogonial cells in the immature ovary as well as in their resting phase in maturing ovary have been often described in other crustaceans but still are rare in penaeids.

In the present investigation the primary oogonial cells appeared as small round deeply stained cells around the germinal zone. The secondary oogonial cells of P. monodon are also observed as round cells with a prominent nucleus and a thin layer of cytoplasm around the nucleus. Diffused chromatin materials as well as small granular nucleoli are observed in the karyoplasm. Weitzman, (1966) described the secondary oogonial cells in Gecarcinus lateralis as "germ nests" having large, round vesicular nuclei with deeply stained chromatin materials and a thin rim of cytoplasm. In P. indicus Mohammed and Diwan (1994) reported that the oogonial cells possessed a large, conspicuous nucleus and weakly eosinophilic cytoplasm. Qunitio et al. (1993) reported that the secondary oogonial cells in P. monodon had a large nucleus with granular nucleoli which fill up the entire cytoplasm. Biomolecular investigations of these oogonial cells revealed that the large round nucleus with diffused chromatin materials and granular nucleolus occupied the 80% of the cell diameter. The thin outer lining of cytoplasm was devoid of granular materials and cell organelles. Trentini and Scanabissi, (1978) described similar types of observations in the oogonial cells of crab Tripos cancriformis but furhter they described the oogonial cells as four-celled groups (one oogonia and 3 nurse cells) resulted from incomplete cytokinesis.

In the present study, it was observed that after the proliferative divisions, the oocyte entered the differentiative phase during which a chain of nuclear and cytoplasmic changes and an appreciable increase in the size of the oocyte occurred. These changes associated with the vitellogenesis are included here in the classification of different maturity stages of the ovary. The first differentiated oocytes are considered as the pre-vitellogenic oocytes in P. monodon in this study. Two sub stages, viz. the chromatin nucleolus stage and perinucleolus stage based on the arrangement of nucleolus in the karyoplasm were described by Tanfermin and Pudadera (1984) in the pre-vitellogenic oocytes of P. monodon. Again Qunitio

et al. (1993) described these two sub-stages in the stage III pre-vitellogenic ovary of P. monodon. The same two sub-stages were identified in the pre-vitellogenic oocytes of the P. monodon during the present study.

Ultrastructural studies of previtellogenic oocytes of crustaceans has been over looked because most of the investigators have been primarily concerned with vitellogenic rather than pre-vitellogenic oocytes. Fine structural investigations on pre-vitellogenic oocytes of P. monodon revealed that these oocytes are characterized by a large nuclear cytoplasm ratio and are immediately recognizable by the dense nucleolus located on the peripheral nucleoplasm. The oocytes at this stage of pre-vitellogenesis were found to be accumulated with precursors which are required for the complicated process of vitellogenesis in the developing ovary. The cytoplasmic organelles in the oocyte became active and recognizable in contrast to the oogonial cells. The nuclear wall at this stage appeared interrupted by numerous gaps or nuclear pores through which the electron dense nucleolar materials diffused into the cytoplasm. In the present investigation it was also observed that the cytoplasm especially around the nucleus was supplied with abundant number of ribosomes, mitochondria, Golgi elements and RER in the pre-vitellogenic oocytes of P. monodon. Similar type of observations have been made by Hinch (1969) in L. emarginata, Duronslet et al. (1975) in P. setiferus, Komm and Hinch (1985) in Coenobita clypeatus and Mohammed and Diwan (1994) in P. indicus. In the present study it was found that along with these organelles the cytoplasm of these pre-vitellogenic oocytes possessed an agranular membrane system (annulate lamellae) and nuages. This type of annulate lamellae and nuage have been reported in the gametes of many animals (review by Kessel, 1983). In decapod crustaceans occurrence of these annulate lamellae were first reported by Komm and Hinch (1985) in the pre-vitellogenic oocytes of Coenobita clypeatus.

Another characteristic feature observed in the pre-vitellogenic oocytes of P. monodon was the granule filled vesicles accumulated in the cytoplasm around the nucleus. The similar electron-density of these vesicles and nucleolar materials and its appearance near the nuclear pores indicated that these vesicles originated in the nucleus. Similar type of granules were reported by Hinch (1980) and Komm and Hinch (1985) in Coenobita clypeatus.

Though the vitellogenesis is a largely explored area in crustaceans over the past two decades, the site of synthesis of crustacean yolk is still not yet known. So the detailed investigations carried out in the early-vitellogenic oocytes of P. monodon revealed two distinct type of oocytes, oocytes at cisternal phase and oocytes at platelet phase. Duronslet et al. (1975) described these two phases of oocytes at two stages of maturity in P. setiferus and P. aztecus. Early-vitellogenic oocytes in decapod have been investigated through electron-microscope by Hinch (1969) in Libinia emarginata; Duronslet et al. (1975) in P. setifeurs and P. aztecus, Komm and Hinch (1987) in Coenobita clypeatus and Mohammed and Diwan, (1994) in P. indicus. Ultrastructural studies in the cisternal phase oocytes of P. monodon showed an intense perinucleolar activity. The vesicles transferred from nucleus acted as the prelude for further vitellogenesis inside the oocyte cytoplasm. Such nuclear emissions were reported in many early-vitellogenic crustacean oocytes by Adiyodi and Subramoniam (1983). Dark staining cytoplasmic bodies around the nuclear envelope and in the perinucleolar cytoplasm were reported in the cisternal phase oocytes of P. setiferus and P. azeticus by Duronslet et al. (1975) and in the early vitellogenic oocytes of P. indicus by Mohammed and Diwan (1994).

As the oocyte of P. monodon reaches the cisternal phase, an increase in the concentration of free ribosomes and RER occurred. Zerbib, (1980) attributed the growth of Orchestia oocytes along with increased ribosome concentration. The abundant RER in growing oocytes has been

demonstrated by many crustacean investigators namely Beams and Kessel (1962 & 1963) in crayfish oocytes, Hinch and Cone (1969) in Libinia emarginata, Zerbib (1980) in Orchestia gammaralla and Komm and Hinch (1987) in Coenobita clypeatus. In penaeids the abudant supply of both RER and free ribosomes has been reported by Duronslet et al. (1975) and Mohammed and Diwan (1994). During the present investigation the ultrastructure of early-vitellogenic oocytes showed well developed Golgi bodies in association with some RER. It was observed that the vesicles blebed off from the ER fused with the cisternae of these Golgi bodies, condensed into yolk bodies and accumulated within the confines of the oocyte. Such metabolic co-ordination has been extensively investigated and demonstrated by Caro and Palade (1964), Tartakoff and Vassalli (1978) and Komm and Hinch (1987). Micropinocytotic vesicles also made their appearence in the plasma-membrane of these oocytes at the end of this phase.

Platelet phase oocytes of P. monodon were be packed with different types of granule filled vesicles and yolk platelets. The vesicles observed at this phase included membrane free electron-dense yolk bodies, RER bound developing yolk bodies and developing lipid vacuoles. Another characteristic feature observed was swollen circular profiles of RER with condensing materials which were blebbed off from the reticular elements. This type of vesicles and endoplasmic reticular profiles were typically demonstrated by Beams and Kessel (1962 & 1963) in crayfish, Hinch and Cone (1969) in Libinia emarginata, Eurenius (1973) in Cancer pagurus, Zebib (1980) in Orchestia gammarellus and Komm and Hinch (1987) in Coenobita clypeatus. Ultrastructural studies of Duronslet et al. (1975) in P. aztecus and P. setiferus and Mohammed and Diwan (1994) in P. indicus documented this type of ER elements and yolk bodies. The cytoplsm of these platelet phase oocytes contained an increased number of active cell organelles. Very active Golgi

complex at this stage were associated with developing vesicles as well as large lipid vacuoles. Hinch and Cone (1969) in Libinia emarginata and Komm and Hinch (1987) in Coenobita clypeatus reported this type of developing yolk bodies and lipid vacuoles associated with active Golgi bodies.

The ultrastructural observations on platelet phase oocytes in P. monodon revealed an irregular oocyte wall at this phase due to the dense accumulation of micropinocytotic vesicles. It was found that these vesicles gradually blebbed off into the peripheral cytoplasm of the oocytes. These vesicles together with the associated RER elements suggested a new source of yolk materials in these oocytes. In the oocytes of P. monodon it was found that these smaller granule filled vesicles originated in the ovary fused together, underwent some progressive modifications and finally transformed into the larger yolk spheres. An intra-oocytic yolk synthesis with an extra oocytic yolk supplimentation was observed in these platelet phase oocytes. Ultrastructural studies by Hinch and Cone (1969) in Libinia emarginata, Eurenius (1973) in Cancer pagurus, Zerbib (1980) in Orchestia gammarellus, Beams and Kessel (1980) in Oniscus asellus, Komm and Hinch (1987) in Coenobita clypeatus all proposed an extra-oocytic source of yolk constituents, complementing the intra-oocytic source. In penaeids like P. setiferus and P. aztecus studies by Duronslet et al. (1975) and in P. indicus by Mohammed and Diwan (1994) demonstrated a similar type of micropinocytic vesicles and extra-ovarian uptake of yolk materials in the vitellogenic oocytes.

Late-vitellogenic oocytes in P. monodon are characterized by the presence of a distinct sub-structure the cortical bodies. Tanfermin and Pudadera (1989) and Qunitio et al. (1993) described these cortical rods in the cortical rod phase ovary of P. monodon. This is a unique feature in penaeids studied so far. Different investigators described the same structure in different terminologies. In P. japonicus Hudinaga (1942)

described these structures as jelly sub-stances, King (1948) in P. setiferus and Rao (1968) in penaeids as peripheral bodies, Subramoniam (1965) in P. indicus as marginal bodies, Duronslet et al. (1975) in P. setiferus and P. aztecus as cortical bodies, Anderson et al. (1984) in Sicyonia injentis as cortical specializations and Yano (1988) in P. japonicus as cortical crypts. Shaikhmahmud and Tambe (1958) did not find the cortical rods or related structures in Parapenaeopsis stylifera. Ultrastructural investigations of Duronslet et al. (1975) in P. setiferus and P. aztecus and Mohammed and Diwan (1994) in P. indicus revealed that the matrix of these oocytes were packed with small feathery structures. The cortical bodies found in P. monodon exhibited a similar type of ultrastructure. The matrix was packed with small "feather" like structures and each of these structures possessed a thick electron-dense axis and thin fibrillar elements. The cytoplasm between these bodies appeared similar to that of platelet phase, with full of lipid bodies and yolk platelets. Micropinocytotic yolk uptake continued in these late-vitellogenic oocytes. Cell organelles like ER, Golgi bodies and mitochondria were not apparent. Consequently the intra-oocytic yolk production was not found in these oocytes. In P. indicus, Mohammed and Diwan, (1994) reported a similar type shift of in yolk synthesis, from intra-oocytic to extra-oocytic as the oocytes reached in the late-vitellogenic phase. Hinch and Cone (1969) in Libinia emarginata and Komm and Hinch (1987) in Coenobita clypaetus has been described the similar type of shift during the late-vitellogenesis.

In P. monodon fully matured oocytes (late-vitellogenic) oocytes are apparent shortly before ovulation as enlarged cells with eosinophilic cytoplasm. The distinct cortical rods were not apparent in these oocytes. In the histological investigations Tanfermin and Pudadera, (1989) observed similar type of oocytes in 5% of the fully matured P. monodon samples. They

concluded that these oocytes with vacuolated cytoplasm were in their another step of vitellogenesis/ artesia. Ultrastructurally it was found that highly enlarged cortical bodies shifted further to the periphery and because of their larger size and shift to the periphery they were not visible at light microscopical level. Similar type of cortical rod movement to the peripheral cytoplasm has been reported by Duronslet et al. (1975) in the mature oocytes of P. setiferus and P. aztecus. In the present study it was observed that the highly expanded cortical rods filled the entire space of the ooplasm of these fully matured oocyte. Ultrastructurally the whole ooplasm of the mature oocytes had an appearence which is similar to that of the matrix of the cortical bodies. Cytoplasm in restricted areas in between these cortical rods contained electron-dense lipid globules, lipid vacuoles and few yolk platelets as found in the late vitellogenic oocytes. It was found that the remaining yolk platelets also emptied their contents to the matrix of the cortical rods. From these observations it is concluded that these cortical bodies are formed by the fusion of the abundant yolk platelets found in the preceding stages.

Folliculogenesis or the investment of follicular cells around the oocytes in P. monodon was found to be initiated only at the end of pre-vitellogenic oocytes. During this phase the rectangular or cuboidal follicle cells were thick and possessed a highly vacuolated cytoplasm and conspicuous nucleus. Similar type of follicle cells were reported by Tanfermin and Pudadera, (1989) in P. monodon and by Mohammed and Diwan (1994) in P. indicus. The flattening of the follicle cells was observed during the progressive growth of the oocyte due to its increase in cell volume. The complete encompassment of individual oocytes with the follicular cells was observed in the early vitellogenic phase of the ovary. The follicle cells at this stage possessed a hypertrophied nucleus with prominent nucleolus. According to Charinaux-cotton (1975) folliculogenesis in the early

vitellogenic oocytes is a pre-requisite for the uptake of yolk proteins form external sources. Yano and Chinezei (1987) suggested that the follicular cells which surround the oocytes are the site of exogenous egg yolk protein production in P. japonicus. So far the cytological changes in the follicle cells encompassing the oocytes of penaeids has not been a subject of detailed investigation and consequently very little is known about such changes.

The ultrastructural observations in the follicle cells of early vitellogenic oocytes of P. monodon showed an active cytoplasm, with numerous RER and agranular membrane systems. These active organelles in the follicle cells suggested a role in yolk protein synthesis in these cells. Follicle cells around the platelet phase oocytes showed an active protein synthesis than the preceding stage. At this phase along with the active cell organelles like Golgi bodies, RER, and mitochondria, granule filled vesicles were also apparent. The vesicular bodies found in the follicle cell cytoplasm include membrane-free electron-dense yolk bodies, developing membrane-bound yolk platelets and lipid vacuoles. According to Rateau and Zerbib (1978) protein synthetic activity in the vitellogenic oocytes of Orchestia gammarella is evident in the ergoplasmic and Golgi complex region of the follicle cells. Highly active organelles in the follicle cells of vitellogenic oocytes have been reported in by Griffond and Gomot, (1979) and Shoenmakers et al. (1981). The follicle cells surrounding the late-vitellogenic oocytes in P. monodon are characterized by the presence of concentric layers of RER elements with developing yolk granules. Similar type of yolk synthesis inside the concentric layers of smooth endoplasmic reticulum has been reported in the 4 celled follicle cells of Triops cancriformes by Trentini and Scanabicci, (1978). Abundant pinocytotic vesicles found apparent in the oolemma of these oocytes in close apposition to the follicle cells give added evidence for the protein synthetic activity of these cells during this phase. Similar type of pinocytotic vesicles have been described in P. indicus by Mohammed

and Diwan (1994). A dramatic change was observed in the follicle cells around the fully matured oocytes. Nucleus as well as other cell organelles were not at all observed in the follicle cells. A clear space was apparent between the plasma membrane of the follicle cell as well as the oolemma. According to Bray and Lawerence (1990) the final flush of colour change during ovarian maturation seems to occur within hours of spawning coupled with the breakdown of follicle cells surrounding the ova in preparation for spawning.

The light microscopical and ultrastructural studies presented here indicated that in P. monodon oocyte development is a relatively complex combination of events involving an intricate oocyte organellar system and extra-oocytic sources through micropinocytosis. Present investigation through electron-microscope revealed that sub-synthetic machinery of the growing oocytes is the major site of yolk synthesis in P. monodon. However, the pinocytotic vesicles observed in the later oocytes indicated that the auto-synthesized intra-ovarian yolk is supplemented at the later stages with a small fraction of the yolk synthesized in an extra-ovarian site through micropinocytosis. According to Komm and Hinch, (1987) an accurate evaluation of oogenesis requires a complete ultrastructural and biochemical investigation. Qunitio et al. (1990) and Chang et al. (1993) isolated vitellin from the ovary of P. monodon and suggested that the site of synthesis of vitellin is located in the ovary itself. But Chang et al. (1994) extracted vitellogenin from the haemolymph and concluded that resource is out side the ovarian tissue. From the results of the present investigation as well as from the results of the above studies it was concluded that the vitellogenesis of P. monodon is mostly intra-oocytic eventhough a small fraction of extra-ovarian yolk is also supplemented during the later stages of vitellogenesis in P. monodon.

CHAPTER II

# MALE REPRODUCTIVE SYSTEM SPERMATOGENESIS AND SPERMATOPHORE FORMATION

A *Biological characterization of the male reproductive system*

B *Mechanism of spermatogenesis*

C *Mechanism of spermatophore formation*

# CHAPTER II

# MALE REPRODUCTIVE SYSTEM

## INTRODUCTION

Achievement of full economic potential of the shrimp mariculture industry depends on the successful domestication of the species concerned, along with genetic selection for desired traits; such as rapid growth, larger size, and high tolerance capacity. The key to domestication lies in enhanced controlled reproduction by the broodstock animals. It can be achieved only through a better understanding of specific biological aspects, particularly related to the reproductive biology of the animal, to ensure that adequate numbers of laboratory reared post larvae can be produced at higher efficiency levels.

Although significant achievements in controlled reproduction of the penaeids have been made in different species in the past 10 to 15 years, much of the interest has been focused on female maturation alone (Primavera, 1985 and Chamberlain, 1987a). And knowledge about the male maturation, semen quality and maximum utilization of the high quality sperm etc. are still fragmentary (Leung-Trujillo and Lawrence, 1987). Therefore, for the selection of better strains and production of offsprings with the desired quality, not only require the knowledge about the reproductive biology of females but also of males. Recently, condition of the male gonad also has been observed to be an important variable in captive reproduction due to the higher number of unsuccessful attempts in captive maturation. Studies dealt are mainly on the quality of the semen stored in the spermatophores and its deterioration in captive animals (Leung-Trujillo and Lawrence, 1985, 1987; Brey et al., 1985; Talbot, et al., 1989; Alfaro, 1993; Alfaro and Lozano,

1993; Pratoomchat et al., 1993; Heitzman et al., 1993, Gomez and Primavera, 1994). These reports reveal that the reproductive quality of the males decrease during captivity in penaeids. The exact reason for this is not clear and therefore it is emphasized to gain full understanding of the male reproductive mechanism, for the development of sound captive breeding techniques in penaeids. Therefore, during the present study, attempts were made to understand the physiology of male reproduction at its fine structural level by investigating the different aspects, like spermatogenesis and spermatophore formation in the penaeid shrimp P. monodon.

Reproductive anatomy of male reproductive system in several penaeids has been described and figured by (Hudinaga, 1942; King, 1948; Eldred, 1958; Subramanyam, 1965 & 1968; Tirmizi and Khan, 1970; Huq, 1980; Mohammed and Diwan, 1994) Similarly, there is voluminous information available on the spermatogenesis in various other crustaceans (King, 1948; Montrose, 1961b; Langreth, 1969; Koebler, 1979; Hinsch, 1980; Jesperson, 1983; Sagi et al., 1988; Mohammed and Diwan, 1994). However, studies on the spermatogenesis of penaeids are less compared to other crustaceans (King, 1948; Pochon-Masson, 1983; Adiyodi, 1985; Vasudevappa, 1992 and Mohammed and Diwan, 1994). Many of such studies focus only on light microscopical investigations while ultrastructural studies on spermatogenesis of penaeids are, however, meagre. The male reproductive system of shrimps of the genus penaeus, consists of paired testes and paired vas deferentia (King, 1948; Subramoniam, 1965; Tuma, 1967; Malek and Bawab, 1974a; Tirmizi and Javed, 1976; Motoh, 1978; Motoh and Buri, 1980; Vasudevappa, 1992 and Mohammed and Diwan, 1994).

Spermatogenesis begins in the peripheral germinative layer of the testicular lobes, when spermatogonia enter into the prophase of meiosis (King, 1948). These spermatogonia after repeated divisions and transformations

develop into spermatozoa in the testicular acini (Hinch, 1980). Most of these studies, particularly in the earlier stages of spermatogenesis, in various crustaceans are only through light-microscopy. However spermatid differentiation has been studied electron-microscopically in P. setiferus by Lu et al. (1973) and in Sicyonidae by Shigekawa and Clark (1986). The spermatozoan in Decapoda is considerably modified and usually consists of a number of non-motile arms surrounding a central nuclear region (Pochon-Masson, 1965). Its ultrastructure has been studied by Clark et al. (1973) in P. aztecus; Ogawa and Kakuda (1987) in P. japonicus; and and Mohammed and Diwan (1994) in P. indicus.

Several studies have dealt with the formation and/or structure of the spermatophores in decapods (Hudinaga, 1942; King, 1948; Eldred, 1958; Tirmizi, 1958; Subrahmoniam, 1965; Tirmizi & Khan, 1970; Malek and Bawab, 1974a & b; Farfante, 1975; Haley, 1984; Champion, 1987; Ro et al., 1990; Chow et al., 1991; Vasudevappa, 1992 and Mohammed and Diwan, 1994). The spermatophore formation has been described histologically in detail, in P. kerathurus by Malek and Bawab (1974a & b), in P. indicus by Champion (1987), and Mohammed and Diwan (1994) and in Metapenaeus dobsonii by Vasudevappa (1992).

Despite the importance of the spermatophore in decapod reproduction, little is known about its formation and packaging at the ultrastructural level. Hinch and Walker (1974) have described the basic ultrastructure of the secretory cells in vas deferens and also stages of spermatophore wall production in the vas deferens of Libinia emarginata. Kooda-Cisco and Talbot (1986) described the production of the primary and intermediate spermatophore layers in the proximal vas deferens of lobster (Homarus). Talbot and Beach (1989) used light microscopy and transmission electron-microscopy to examine

the structure of the vas deferens and its role in the formation of the spermatophore wall in the cray-fish Cherax albidius. In P. indicus Mohammed and Diwan (1994) have given a detailed description of male reproductive system, spermatogenesis and spermatophore formation through light microscopy.

However, there has been little published work on the mechanism of spermatophore development in penaeid shrimps at ultrastructural level. Ro et al. (1988 & 1990) have described the role of segments 1 and 2 in the spermatophore production of penaeid shrimp P. setiferus. Similarly the structure of vas deferens, terminal ampoule and compound spermatophore, the process of spermatophore matrix deposition and the role of vas deferens in spermatophore formation etc. were studied through electron-microscopy in two American white shrimps P. setiferus and P. vannamei by Chow et al. (1991). The spermatophore not only functions as a vehicle for sperm transportation but also its acellular layers also protect the sperm during their transfer, and storage by female. Therefore, it is very much essential to study their development and structure for the fruitful manipulations of their gametes during biotechnological applications for augmenting shrimp seed production.

# MATERIALS AND METHODS

## I. Collection of animals and preparation of tissues for hislological studies

Wild caught prawns P. monodon, ranging in size from 180 to 220 mm in total length, in various maturity stages were used in the present study. Prawns were captured from deeper offshore areas of Cochin and Madras. The prawns were transported to the laboratory in live condition and kept in 1 tonne capacity fibre-glass tanks, in seawater provided with a sub-gravel biological filter working on an air lift mechanism. Prawns were fed daily with clam meat and maintained till their sacrifice for further studies.

Prawns in different maturity stages were selected to study the morphological features of the male reproductive system and its changes occurring during the process of gonadal maturation, and reproductive organs like testis, different parts of the vas deferens viz. the proximal vas deferens (PVD), median vas deferens (MVD), the distal vas deferens (DVD) and the terminal ampoule (TA) etc. were excised from the live prawns. Tissues for light and electron-microscopic studies excised separately, from the same animal and fixed in appropriate fixatives. Dissections were conducted in crustacean saline to keep the tissues in live condition. Testis and vas deferens were removed from the same animal and gross features like size, colour, nature and shape of the tissues etc. were noted in different maturity stages. Gross morphological studies were made using a sterioscopic dissection microscope. Different maturity stages were identified based on the morphological features, like gonadal development, nature and colour of the testis. Since different regions of the testis exhibited homogenous characters only a portion of the middle lobe was used for fixation of both histology and electron-microscopy.

## II. Light microscopic studies

Freshly excised tissues were immediately transferred to Bouin's fixative for a period of 24 to 48 hrs before further processing. Properly fixed tissues were washed over night in running tap water to remove the excess picric acid. Washed tissues were later dehydrated through an ascending grades of alcohol series (30 to 100%) and cleared in chloroform. Dehydrated tissues were put for the cold impregnation in a wax chloroform mixture (1:1 ratio) for one day. Subsequently the tissues were transferred to molten wax (Parrafin wax with cerresin, Merck, MP. 58 to 60°C) for hot impregnation. After 2 more changes of duration, 15 min. in fresh frozen wax, tissue blocks were prepared using L-blocks made of Brass.

Serial sections of the tissue blocks were taken at an approximate thickness of 5 to 7 µ using a rotary microtome. Sections were affixed on clean glass slides using fresh Meyer's egg albumen and flattened by placing on a slide warmer with few drops of distilled water. Subsequently the water was drained off and the slides were allowed to dry. These slides were then used for further histological investigations.

Routine staining was carried out for the gross morphological studies, using Harris hematoxylin stain with 1% Aqueous Eosin as the counter stain. Sections to be stained were first deparaffinized in two changes of xylene and then dehydrated through a down series of ethanol grades. Hydrated tissues were staining in Hematoxylin and blued in tap water before stained with Eosin. Eosin stained slides were again dehydrated in two changes of absolute alcohol and cleared in xylene before mounting in DPX mountant. DPX mounted slides were allowed to dry and after drying, observations were made under a monocular microscope. Structural details of the developing germ cells and their changes during the successive stages

were investigated. Micrometric measurements of the developing spermatogonial cells, spermatocytes and spermatids were taken using an occular micrometer calibrated with a stage micrometer. As spermatocytes and spermatids strongly deviate from a spherical shape, the largest and the smallest axis of the cell diameter was taken and the average was used as the actual cell diameter. Structural modifications in different parts of the vas deferens and the role of each part in the formation of spermatophore was studied, by taking serial sections of the entire vas deferens of fully matured animals. Histological observations of the vas deferens were made in animals at different stages of maturity, to study developmental pattern of the reproductive system.

Photomicrographs of histological investigations in the testes as well as vas deferens were taken using an Olympus Universal Research Microscope (Vannox model PM 10 AD) equipped with an automatic exposure system. Black and white 35 mm film (Ilford, 125 ISO) was used for photography.

## III. Ultrastructural studies

The gonadal tissues excised at different maturity stages were used for the ultrastructural studies. Initially small pieces of testes and different parts of vas deferens were bathed in cold fixative (4°C) by pipetting fixative directly on to the tissue in situ. The tissues were then removed from the abdomen and placed in fresh primary fixative which is 4% gluteraldehyde, buffered with 0.2 M Cacodylate buffer containing 2% tannic acid and 6% glucose at pH 7.2 for 1 - 1.5 hrs at 4 °C. After primary fixation, tissues were washed 3 times in cacodylate buffer, each about 15 min. duration and post fixed in 1% buffered osmium tetroxide for about 1 hr. at 4°C. Properly fixed tissues again washed in cacodylate buffer

and then dehydrated in a graded acetone series. The tissues were infiltrated and embedded in low viscosity resin embedding medium (Spurr, 1969) and kept at 60°C for 36 hrs.

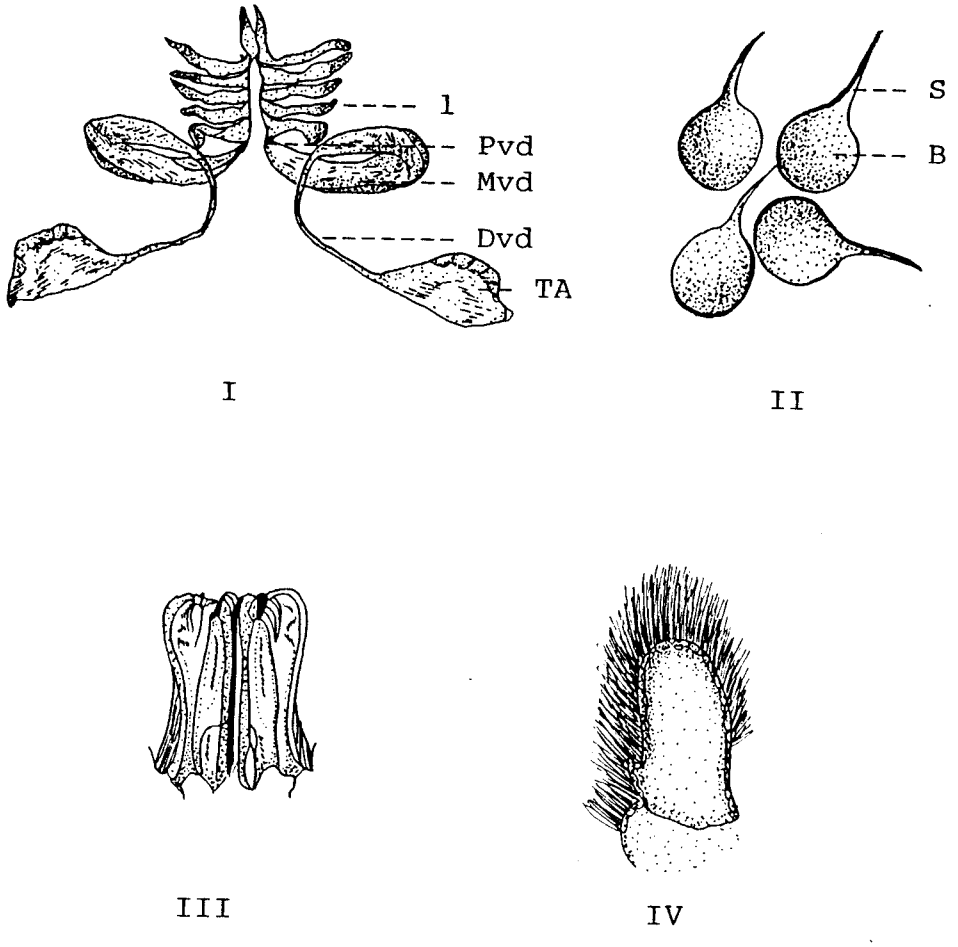
After proper trimming semi-thin sections (0.5 to 1.0 u) were cut from the polymerized blocks and stained with toludine blue and observed under light microscope in order to identify the interested area. These blocks were again trimmed for ultramicrotomy, and ultrathin sections were cut with a diamond knife on an ultra-microtome "Ultracut E". Ultra-thin sections (300-600 A°) collected on uncoated copper grids (300 mesh), were post stained in 10% uranyl acetate in methanol (Watson, 1968) and lead citrate (Reynold, 1963). After drying, the grids were examined with a "Phillips CM 10" transmission electron-microscope at 60 KV. Ultrastructural details of the testes as well as vas deferens were examined and interested areas were photographed using Kodak-100 ASA film.

# RESULTS

## A. BIOLOGICAL CHARACTERIZATION OF THE MALE REPRODUCTIVE SYSTEM

The male reproductive system of P. monodon is composed of both internal and external organs. The internal organs consisted of paired testis and paired vas deferentia terminating in ampoules, containing spermatophores or sperm packets, and opens out through the gonopores, at the fifth walking leg (fig. 41). The external organs are the petasma, formed by the modified endopods of the 1st pleopod and a pair of appendix masculina, the modified endopods of the 2nd pleopod. The six lobed translucent testes are located in the cardiac region. These testicular lobes are connected to each other at their inner ends and lead to the vasa deferentia (fig. 41).

Vas deferens or testicular duct is the collecting tubule of the fully matured spermatozoa from the testes to the exterior, as spermatophores or sperm packets through gonopores. The paired vas deferentia of P. monodon are found originating from the posterior margin of the main axis of the testicular lobes, and descending ventro-laterally to terminate in the gonopore at the coxopodite of the 5th pareiopod. Each vas deferens consisted of four portions, the short and narrow proximal vas deferens (PVD) located nearer to the testes, the thickened and long median vas deferens (MVD) located in the middle region which consists of a blind pouch at the junction of PVD and MVD, an ascending limb and a descending limb, the long narrow distal vas deferens (DVD) located at the farther end of the testicular duct just prior to the terminal ampoule, and the greatly dilated muscular terminal ampoule (TA) which is found embedded in the coxal muscles of 5th pereiopod. The fully developed sperm packets or spermatophores are found inside this muscular organ. It opens to the outside through the gonopores situated on the coxae of the 5th pereiopod.




41 Diagramatic representation of the male reproductive system of P. monodon.

I - Testes, II - Sperm, III - Petasma, IV - Appendix musculina
B - body, l - lobules, S - spike, Pvd - Proximal vas deferens, Mvd - Median vas deferens, Dvd - Distal vas deferens, TA - Terminal ampoule

## A.1 Development of male reproductive organs - Morphological characterization

Based on the morphological as well as histological investigations, the maturity stages of male P. monodon was classified into three stages, viz. immature, maturing and matured. In immature males, the testis is a thin translucent and extremely delicate organ in the cardiac region. It was found that, the under developed testicular lobes are not distinguishable in immature animals and the anterior most lobe was not at all developed. The extremely delicate vas deferens appeared as a thin translucent thread. The complicated structures in the lumen of the tube were not developed and the entire tube showed the same morphology. Different parts were not distinguishable morphologically. The terminal ampoule appeared as a thin delicate and membraneous bag in immature males. The secondary sexual characters were not developed and the endopods of the 1st pleopods were two simple, elongated and flattened structures.

Even though the testis is found to be transparent and delicate in maturing animals it was much more developed than the immature animals. The testicular lobes are found to be somewhat larger and distinguishable, and the vas deferens was much more thicker and tubular in structure. Different parts of the vas deferens could be distinguishable but not well defined. The terminal ampoule was found as a small slightly thickened bag like structure, in the cavity at the base of the 5th pereiopods. Here the entire vas deferens including the TA was translucent and without any sperm or spermatophore. The endopods of the 1st pleopods were modified and got partially united to form the petasma in maturing males. Appendix masculina also appeared as small buds at the base of the 2nd pleopods.

However, in fully matured males all the primary as well as the secondary reproductive organs were well developed and fully functional. The fully developed testis now appeared as opaque to cream in colour. The testicular

lobes were thick short and stumpy in appearance and the well developed vas deferens was easily distinguishable externally. The fully developed terminal ampoule was thick and muscular and it appeared as white due to the presence of fully developed spermatophores inside. The white colour at the coxae of the 5th pereiopod, due to the fully developed spermatophores present inside the TA, can be used as a character for identifying mature males. The endopods of the 1st pleopod became more elaborated and closely linked by a series of minute hook like structure in the mature males. Well developed appendix musculina was clearly visible.

**A. 2 Development of male reproductive organs - Histological characterization**

**a. Testis**

Histological studies of the testes at different maturity stages revealed that each testicular lobe was in the form of a moderately convoluted collecting tubule, held together by connective tissue, about which are arrayed multiple follicles (acini) which are spermeogenic (fig. 42). Transverse sections revealed that bulk of the testicular volume was occupied by these follicles. Each follicle was found to be a collecting tubule lined by an epithelium. It was also noted that the entire testis is covered by a thin wall of outer epithelium (fig. 42). Structural details of which was not clear through light microscopy.

Light microscopical investigations in testis revealed that the testicular follicles were empty in immature animals. The follicular wall was found thicker and it contained a small germinal area with non differentiated cells at one side of it. Whereas, the cross sections of the testes from maturing animals, showed a more pronounced germinal area with differentiating primordial germ cells (fig. 43). Most of the follicular area was found filled with germinal zone. The follicular wall was still found thicker and a few

numbers of differentiating spermatogonial cells were found among the developing primordial germ cells. In cross sections of the testes of fully matured males also, the testicular follicles were lined by germinal epithelium however, the germinal zone was found shifted to one of its side andit contained a few developing spermatogonial cells (fig. 44). Transitions through which the spermatogonial cells passes, are found in graded manner towards the centre of the acini. Fully developed spermatozoa are found in the central most region of each follicle.

## b. Vas deferens

Histological investigations of the testicular duct during different maturity stages, revealed the developmental stages of the highly specialized testicular duct in this species. It was found that the vas deferens of immature animals was a simple tube throughout its entire length. Whereas the same in the fully matured animals were highly modified and specialized. Therefore, histological investigations were made in different parts of the testicular ducts taken from animals at different maturity stages. Different morphological divisions of the vas deferens were apparent in maturing as well as fully matured animals, but was not identifiable in immature animals. Hence the corresponding stages found in the maturing and matured animals were considered for the identification of different parts. The proximal portion of the testicular duct taken as the PVD, middle part as the MVD and the distal part as the DVD respectively.

### 1. Proximal vas deferens (PVD)

The proximal vas deferens is a short slender tube leaving the testes. From the investigations, it was found that, the testicular duct is a simple tube comprised of outer connective tissue sheath and inner circular muscle

layer. However, the testicular duct of fully matured animals showed a highly modified internal anatomy and therefore it has been explained with light micrographs. Light microscopically, it was observed that the PVD was the site of completion of spermatogenesis, as well as the beginning of encapsulation of the sperm into spermatophores and the production of seminal fluid. PVD in general appeared as a short tube in immature as well as matured animals. Cross-sections revealed that in immature animals, it is composed of an outer connective tissue layer and an inner layer of circular muscle fibers. The lumen of the PVD was found empty while, in maturing animals along with the two above said outer connective tissue layer and inner circular muscle layer, an inner most layer of epithelial cells were also encountered. An amorphous matrix was found to be secreted by these low epithelial cells lining the inner lumen of the PVD. Sperm cells were not encountered in any of the sections of PVD from immature or maturing animals.

The inner lining of low columnar epithelial cells was fully developed in the inner lumen of the PVD of fully matured animals (fig. 64,65). Even though the tube was found empty most of the times, aggregations of fully matured sperms bathed in an amorphous matrix secreted by these epithelial cells were encountered occasionally (fig. 65). The sperm supporting matrix is found to be stained in eosin while sperm cells in hematoxylin.

## 2. Median vas deferens

The proximal vas deferens is dilated posteriorly to form the median vas deferens (MVD). Histological investigations of the MVD in different maturity stages, exhibited entirely different pictures. In immature animals MVD was not distinguishable and therefore the middle portion of the sperm duct was considered as the MVD during investigations. Cross-sections revealed that as in the case of PVD, in immature animals, MVD also composed of two

layers viz. the outer connective tissue layer and inner circular muscle layer. Histological investigations did not show any structural modifications in and the inner lumen of MVD was found empty in immature animals.

Whereas, the MVD of maturing animals was more developed and contained different morphologically distinct areas viz. blind pouch, ascending limb and descending limb. Blind pouch was the junction of PVD and the ascending limb of the MVD. A thin layer of columnar epithelial cells lined the inner lumen of the blind pouch in maturing animals. Even though sperm cells were absent, the lumen contained an amorphous material secreted by the epithelial cells lining the inner lumen. In cross-sections, the blind pouch of fully matured animals exhibited structural features of PVD and MVD (fig. 72). Though the blind pouch contained only a single lumen as in the case of PVD, the width of the lumen was more in this part like MVD. The inner layer of columnar epithelial cells was well developed and more regularly arranged (fig. 72). Its lumen contained thickly packed sperm cells in a sticky amorphous material (fig. 72). The circular muscle layer of this blind pouch was thicker than that of PVD.

The blind pouch dilated into wider ascending limb of the MVD. In immature animals, MVD contained a single lumen and its ascending and descending limbs did not show any structural differences and its empty lumen did not possess an epithelial lining. However histological studies of the MVD of maturing animals revealed that its lumen was partially compartmentalized and contained well developed columnar epithelial cells throughout its length. As it enters the ascending limb of the MVD, elongated as well as stunted typhlosoles are found inside its lumen, in the maturing animals. Typhlosoles, are nothing but out growths of the epithelial cells into the lumen of the duct, in order to facilitate the increased secretory activity of these cells. The typhlosoles possessed more elongated epithelial cells than that of the other area. These elongated typhlosoles made the

partial division of the inner lumen and are composed of an inner layer of connective tissue lined by epithelial cells on its two sides. Sperm cells are not found in the ascending limb of the maturing animals and the lumen was found empty in most of the times. However, some acellular basophilic secretions were found occassionally in the lumen of this duct. The ascending limb is continued as the descending limb which also exhibited more or less a similar structure. Elongated typhlosoles are found in this descending limb of the MVD in maturing animals. Sperm cells were not encountered but the lumen contained an amorphous secretory material. The descending limb leads to the narrower and elongated distal vas deferens.

Histological investigations in the MVD of fully matured animals revealed the most complex internal architecture. The distinction of MVD into blind pouch, ascending and descending limbs is seen clearly particularly in fully matured males. Unlike the previous cases, blind pouch of the matured animals contain a densely packed sperm mass in a sticky fibrillar supporting matrix. Epithelial cells lining this area probably released the matrix which gave the sperm supporting matrix in this region its cohesive property.

The blind pouch continued as the ascending limb of the MVD and was highly modified throughout its length in fully matured animals. It enclosed a some what enlarged lumen which was divided into two unequal independent ducts (fig. 76,77). The wider one was found in continuation with the cavity of the blind pouch, and the sperm mass passes forward through this duct. It was the 'spermatophoric duct' (fig. 76) as observed to deposit the different spermatophoric layers, around the sperm mass during its forward movement towards the terminal ampoule. The other narrower duct was called 'wing duct' (fig. 77) due to its role in the wing formation. Walls of these ducts are found to be highly secretory in nature. All along the two ducts, innumerable numbers of glands were found, and more over they were in a state of supreme activity. The wall of the spermatophoric duct was found to be

thinner than the preceding parts, even though it was composed of the above said three layers viz. outer connective tissue layer, middle circular muscle layer and the innermost columnar epithelial lining. Highly enlarged and secretory typhlosoles were particularly distinct in the duct. The sperm mass inside the lumen was found surrounded by amorphous materials of different nature. The wing duct was also composed of the same three layers. Highly active epithelial cells are apparent also in this small duct. It was found that amorphous materials of different natures were secreted by the secretory epithelial cells lining this duct. Only a small typhlosole was present in this wing duct.

The intervening septum separating the spermatophoric and wing duct was made of a thin strand of connective tissue with glandular epithelial cells on either of its side (fig. 86). Invading this strand, were few muscle fibers scattered here and there and of more importance, blood islands for conveying the metabolites of these highly active epithelial cells. It was noted that despite the differences in size or thickness, both the spermatophoric duct and the wing duct consists of the same layers as already described. It was further observed that massive secretions, discharged by the epithelial cells, occupied the cavities of both the spermatophoric as well as the wing ducts. These materials appeared to form discrete layers around the sperm mass. The sperm mass along with its discrete acellular secretions moved towards the descending limb of the MVD.

Cross-sections of the descending limb revealed that, the same layers already identified in the ascending limb are found here also. Almost similar structural configurations are observed in the descending limb of the MVD through light microscopy. The division of the lumen into the spermatophoric and wing duct persisted to some distance and then gradually opens at one side and finally it became a common lumen before reaching the DVD. Light microscopical observations revealed that compactly arranged sperm mass in

the descending limb was packed in different pouches inside the spermatophoric layer. Elongated and branched typhlosoles with long and highly secretory cells are found in side its lumen. The entire lumen was found packed with amorphous secretions of the epithelial cells. Descending limb of the MVD gets constricted and becomes narrower and continues as distal vas deferens.

**3. Distal vas deferens (DVD)**

In immature animals, distal vas deferens is a slender tube with a diameter of 0.15 to 0.2 mm. It was constituted by the same two layers viz. the outer connective tissue layer and the inner circular muscle layer of the preceding parts and the inner most epithelial lining was absent. The duct was found empty. However, in maturing animals the distal vas deferens contained all the three layers as in the MVD of this stage. The inner most epithelial lining was fully developed and contained well developed typhlosoles. The diameter of the lumen was comparatively lesser than that of the MVD. This duct contained an amorphous material in a very low concentration. The diameter of DVD varied between 0.5 - 0.8 mm in these maturing animals.

In matured animals the distal vas deferens was an elongated slender tube ending in the terminal ampoule. Highly elongated and branched typhlosoles were found in the lumen (fig. 87). The epithelial cells in the typhlosoles and other areas were particularly active in the secretion of the amorphous materials. Cross-sections revealed that the sperm mass in the DVD was encompassed in a covering made of these amorphous secretory materials. Different layers were observed in this covering in the Hematoxylin-Eosin stained sections. Amorphous materials secreted by the epithelial cells in the DVD were found deposited over the spermatophore (fig. 88). The distal vas deferens ended in a pear shaped muscular organ viz. the terminal ampoule.

**4. Terminal ampoule**

The terminal portion of the vas deferens is a muscular organ in P. monodon. It is observed within the musculature of the fifth pereiopodal coxopodite. In immature animals it is not well developed and it appeared as a small elongated bag like organ at the terminal end of the vas deferens. Cross sections revealed that it is composed of an outer thin layer of connective tissue and inner thick layer of circular muscle fibers. Typhlosole or epithelial cell lining was not at all developed in the inner lumen of the terminal ampoule of the immature animal and the lumen was found empty. No secretory material was observed in these swollen bag like structure in the immature animal.

Terminal ampoule of the maturing animals did not exhibit any conspicuous developments except in size which has increased considerably than that of the immature animals. Its musculature was well developed, with distinct inner longitudinal and outer circular muscle layers. Epithelial lining was not well developed in the inner lumen. The lumen was found empty even in these maturing animals.

TA of the matured animals was well developed and highly enlarged. Light microscopical investigations revealed a well developed musculature, with a distinct inner longitudinal and outer circular layer (fig. 91,92). Highly developed and branched typhlosoles were present in the inner lumen of the terminal ampoule. However, the epithelial cells lining the inner lumen and on the typhlosoles are found to be low columnar and a lightly staining matrix was found secreted by these epithelial cells. The basophilic epithelium was found thrown into longitudinal folds throughout its length.

## B. MECHANISM OF SPERMATOGENESIS

Spermeogenesis or production of mature spermatozoa takes place inside the lumen of the testicular acini. It was noted during the present study that spermatogenesis in each testicular follicle is cyclic, characterized by a distinct set of cytological transitions. In cross-section of the testis a strand like germinal zone was apparent in each follicular wall at one of its sides (fig. 43,44). Germinal zone contained primary gonial cells and nurse cells, and the process of spermatogenesis is found to be initiated with the development of primary gonial cells in the germinal zone. The follicular epithelium always contained a few numbers of developing spermatogonial cells. Spermatogenesis was observed to progress towards the central lumen of the acini and therefore subsequent developmental stages ware found towards the centre in a graded manner. Light microscopically, it was observed that at the on set of spermatogenesis, the primary spermatogonial cells derived from the germinal zone underwent rapid mitotic divisions, followed by periods of quick growth and formation of secondary spermatogonia (fig. 45). As spermatogenesis continued it was observed that this layer of highly proliferated secondary spermatogonia became stratified and formed a new layer between the follicular cells and the basal lamina. These secondary oogonial cells after mitotic division get differentiated into the primary spermatocytes. The primary spermatocytes underwent reduction division and formed two secondary spermatocytes (fig. 47), which again divided mitotically into two and thus resulted in the formation of four spermatids (fig. 48), from each of the primary spermatocyte. These spermatids without further division get modified into spermatozoa (fig.51).

The secondary spermatocytes, spermatids and spermatozoa are found in the center of each acini in a sequence from the primary spermatocyte, which inturn were developed from spermatogonial cells in the germinal area.

Since spermatogenesis involves the progressive reduction of cytoplasm and condensation of chromatin to produce the spermatozoa, the spermatogonial cells are observed to be the largest of all the cell types, followed by spermatocytes, which inturn were larger than spermatids and spermatozoa. The spermatogonial cells possessed a large round vesicular nucleus with diffused chromatin materials weakly stained with hematoxylin (fig. 44,46). However, the cytoplasm around the nucleus was found to be eosinophilic. The cell boundaries of these cells could not be distinguished and therefore the measurement of cell diameter was not possible, however, the nuclear diameter of these cells varied between 6-9 u. Small elongated nurse cells are found in close association with the primary gonial cells. The nurse cells are seen to have a prominent nuclei. By virtue of their close association with the spermatogonial cells, it was assumed that they have nutritive and supportive role.

Cell boundaries of spermatocytes, spermatids and spermatozoa are distinct. The primary spermatocytes showed a cell diameter of 6-8 $\mu$ and a nuclear diameter of 4-6 $\mu$. The nuclei of primary spermatocytes had an over all dense appearance, with hetero-chromatin material spread over homogeneously throughout (fig. 46,47). The thin rim of cytoplasm around the nucleus was eosinophilic and not prominent. The secondary spermatocytes are observed to be smaller in size than the primary spermatocytes (fig. 47). The cytoplasm in them was poorly stainable, but cell boundaries were clearly visible. These spermatocytes are usually the dividing cells in the testis, and diakinetic stages characteristic of dividing cells were frequently

noticed among them (fig. 47). The spermatids are round structures with a very dense cluster of chromatin in their nucleus (fig. 48) and are much smaller than the spermatocytes due to the reduction division and condensation of the chromatin materials. The diameter of the spermatid varied between 5-7 µ and its nuclei between 4-5 µ.

The spermatids were found to undergo further structural modifications and later get differentiated into spermatozoa without anymore divisions. Fully matured spermatozoa are found to be located at the central part of each follicle (fig. 50). The fully matured spermatozoa of P. monodon are found to be almost spherical in out line, with condensed chromatin materials (fig. 51). A short spike was apparent at the anterior region of the main body. The size of the body varied between 4-5 µ.

Ultrastructural investigations of the testis revealed that the testicular wall is comprised of three layers, an outer layer of squamous epithelium, a middle layer of connective tissue and an inner layer of germinal epithelium. Ultrastructurally, the spermatogonial cells are found to be roughly spherical to oval in outline (fig. 52). Small radial arms of the cytoplasm are apparent at the boundary of these gonial cells. Radial cytoplasmic materials are present in the cell body. The centrally located large nucleus occupied nearly one third of the cell volume. The vacuolar nucleus contained highly electron dense condensed chromatin materials in their finely granular nucleoplasm (fig. 52). The nuclear boundary was not clearly separated from the cytoplasmic materials. Even though distinct nuclear membrane was not apparent in the spermatogonial cells, the highly electron-dense chromatin materials present at the outer boundary of the nucleus, separated the nucleus from the cytoplasm. Numerous cytoplasmic vesicles are found in the granular cytoplasm. The cytoplasm of the spermatogonial cells contained numerous

mitochondria and stacks of RER in the cytoplasm around the nucleus. Ribosomes are found in association with the RER as well as free in the cytoplasm. Large numbers of small elongated non-germinative cells were apparent around each spermatogonial cells. These non-germinative cells also possessed cytoplasmic extensions, which extended out around and between the developing spermatogonial cells. The nucleus of these cells is not well developed, however some of these cells contained electron-dense chromatin materials (fig.53). Nuclear membrane was not distinguishable and the cytoplasm was more electron-dense than the adjoining germinal cells Fig.53).

Primary spermatocytes are formed from the spermatogonial cells through mitotic division (fig. 54). The nucleus of such cells was observed to occupy almost 80% of the cell volume (fig. 55). The primary spermatocytes exhibited almost similar characteristics of late spermatogonial cells, except the fact that the nucleus is surprisingly larger in size and it contained highly electron-dense chromatin materials (fig 55). Nucleolus was not apparent in the nucleus, however, patches of condensed chromatin materials distributed in the finely granular nucleoplasm was prominent in close association with the nuclear envelope (fig. 55). The nuclear membrane of spermatocytes was found to be interrupted by nuclear pores (fig. 55). The secondary spermatocytes are formed through the reduction division of the primary spermatocytes. During this phase, blebbing of the nuclear membrane was apparent in these dividing spermatocytes (fig. 57). Cytoplasm of the secondary spermatocytes contained sub-cellular organelles like RER, free and attached ribosomes, mitochondria, multivesicular bodies and clear vesicles (fig. 56). RER was found in different forms, like circular layers around the nucleus and near the plasma membrane of cells, segments of cisternae richly endowed with ribosomes containing little secretion products (fig 57)

and swollen round to oval bodies with secretory products of medium electron density. Smooth ER was also seen in very less numbers. A few numbers of mitochondria are found in the outer cytoplasm, though their cristae were not clearly seen. Their outer profile varied from elongated to oval. Variably shaped multivesicular bodies were visible in the cytoplasm around the nucleus. A few clear vesicles have been observed in association with the RER (fig. 56). The early spermatids are found to originate from the secondary spermatocytes through simple mitotic division and get transffered into spermatozoa (fig. 58). In the spermatids polarization of the cell contents get initiated. The nucleus migrates to one side of the cell while the cytoplasmic organelle move towards the other side. The shifted nucleus contained a prominent nucleolus. RER was abundant in circles around the nuclear membrane and also parallel to the plasma membrane of the cells. Vacuoles and granules filled vesicles were apparent throughout the cytoplasm.

Spermatids are found to be very much reduced in size due to the repeated divisions than the preceding stages of spermatogenesis. The large elongated nuclei of the spermatocytes became half in size and roughly spherical in shape during the early stages of spermatid differentiation. The nuclear envelope became increasingly convoluted and irregular (fig. 58). The most striking characteristic noticed in the early spermatids, were the blebbing of the nuclear surface, which is more pronounced in this stage (fig. 59). It was noted that as the blebbing progresses, streamers of small vesicles and dense granules became closely associated with these blebs(fig. 59). Clusters of small vesicles and dense granules found, shifted towards the nucleus and aligned themselves more or less perpendicularly with the nuclear surface and those which were closely associated with the nuclear blebs fused

to the nuclear envelope (fig. 59). Generally outer membrane of the nucleus was involved in the blebbing, though both of them may also get involved occassionally.

The cytoplasm of the early spermatids contained numerous small granules similar to those observed around the nucleus, freely and also on the outer surfaces of the granular endoplasmic reticulum (fig. 58). In addition to this, the cytoplasm contained numerous mitochondria, rough and smooth endoplasmic reticular elements, ribosomes and multivesicular bodies (fig. 60). Ribosomes are found in association with the endoplasmic reticulum as well as free in the cytoplasm (fig. 60). However, Golgi complex have not been discerned in these early spermatids of P. monodon during the present investigation. The mitochondria of these early spermatids are found to be varying somewhat in their outer profile. Transformation of the filamentous type of mitochondria to the oval spheroid, was noticed in the spermatids, during the earlier stages. Vesicles similar to those seen in the secondary spermatocytes were also observed in these spermatids (fig. 59). Some of these vesicles had smooth membranes, while some others are found to be lined with ribosomes and a number of these vesicles contained faintly electron-opaque materials. It was also observed that these vesicles fused to form homogenous large vesicles surrounded by a single membrane in the sub-acrosomal region. Consequently, it was noted that the acrosome also started its development as the vesicular fusion takes place. It was observed that the acrosome has formed from the fused product of the small granule filled vesicles, which were found throughout the cytoplasm, especially around the nuclear membrane. Structures resembling lipid bodies sometimes appeared between the acrosomal area and the nucleus of the spermatids. Spermatids during their intermediary stages of development, possessed a comparatively

electron dense round to oval nucleus (fig. 58). Concomitantly, a reduction in the total cell volume was also noted. The blebs of the nuclear membrane was found receded, but the surface of the nucleus still appeared irregular. During this stage, the outer nuclear membrane has an intented appeareance, caused by the infoldings of the nuclear membrane. Concentric layers of highly active RER was found encompassing the nucleus (fig. 58). Small mitochondria were apparent in the indentations of the nuclear membrane, as well as around these active RER (fig. 60). Areas of increased cytoplasmic activity were found between the cisternae of these RER. A few projections were noticed in the nuclear envelope and this out pocketing of the membrane collapsed, leaving, short flat sheets of closely apposed envelope membrane, (fig. 60). In other areas, the inner nuclear membrane had receded, while the outer membrane projects for a short distance, where it was found usually associated with chains of vesicles, apparent during the preceding stages, some of which appeared to have fused and collapsed.

In the later stages of spermatid differentiation it was noted that the active RER elements produced numerous electron-dense granular materials. Masses of the small vesicles and granules appeared to have been excluded from the vicinity of the nucleus and are found packed near the cell periphery (fig. 61). Stacks of two to six annulate lamellae are also noticed in association with these vesicle-masses usually on the pole, away from the nucleus, near the clear area (fig 61). These vacuoles and granules coalesce gradually and form the acrosomal vesicle. Condensation of the sub-acrosomal substance appeared between the vesicle and the nucleus (fig. 61). The acrosomal vesicle gradually found filled with these condensed material formed the acrosomal complex. The vesicle appeared cup shaped and the sub-acrosomal material underlined the acrosomal complex.

The nucleus has remained free of intrusion of vesicular and granular materials from the cytoplasm and is filled with clusters of fibrillar particles (fig. 61). During the final stages of spermatid differentiation it was observed that, the acrosomal vesicle along with the attached annular lamellae moved inwards and formed the acrosomal core, and it became a part of the acrosomal complex. In later spermatids, presumably by further invagination of the acrosomal vesicle along with the cell membrane of the spermatid and fusion with the small vesicles, formation of the limiting membrane is found initiated. The part of the spermatid membrane lining the base of the acrosomal complex was dense and thickened.

During the transition of the late spermatids to the spermatozoa, it was observed that the granular materials of the acrosomal vesicle completely set off from the rest of the cell and incorporated into the acrosomal complex (fig. 62). The acrosomal granule has been replaced by a dense rim or cap which lines the surface of the vesicle proximal to the nucleus. A dense material from the cytoplasm, accumulated at the distal part of the acrosomal vesicle and joined with the acrosomal cap region, appearing as a highly condensed structure (fig. 62). At this stage the entire acrosomal complex protruded out from the spermatid and differentiated into a highly condensed and integrated area at the anterior most region of the spermatozoa. The formation of the anterior spike was also apparent in these developing spermatozoans. A small projection of the acrosomal membrane began, to project across the periacrosomal space and became closely associated with plasma membrane. Extensions of the dense materials from the cap region was found projected to some distance anteriorly in a slender and elongated form (fig. 61). This extension of the cap region developed into the anterior spike. The spike also contained the dense granules that accumulated from

the cytoplasm of the spermatids, which formed the main component of the acrosomal vesicle. As maturation continued, it was found that an amorphous material originated from the cytoplasm at the periacrosomal area, accumulated and filled the space between the acrosomal vesicle and plasma membranes, and along the sides of the acrosome to the middle of the spike (fig. 62). The acrosomal membrane which surrounds the spike at the proximal part of this developing spermatozoa did not appear to cover the outer edge, at the point where it approaches the plasma membrane. The acrosomal membrane, however, can be seen along the sides of this projection.

The fully matured spermatozoan of P. monodon was unistellate but non-motile. Ultrastructural observations revealed that the sperm was composed of a spherical main body, partially encompassed by a morphologically diverse cap region, containing acrosomal complex from which extended a single spike (fig. 63). The sperm's main body is found be an irregular sphere, one half of which encompassed by the cap region or the acrosomal cap and composed of structurally different components like the cytoplasmic band, small vesicles and the fibrillar mass (fig. 63). The cytoplasmic band which contained an amorphous material, surrounds the posterior and lateral margins of the main body. A few small membrane bound vesicles were apparent around the periphery of this band. Loosely packed fibrillar material constituted the majority of the cell body and it is composed of an electron loose matrix containing a reticulum of fine putative electron-dense DNA fibrils. The fibrillar materials was not membrane bound at its outer region but the cytoplasmic band bordered its lateral sides, (fig. 63) and the acrosomal vesicle at its anterior side. The nuclear material is in direct contact with the cell membrane, as a discrete nuclear membrane is not present.

The middle cap region is the most structurally complex region of the spermatozoa. It consisted of distinct components viz. the central core of the acrosome, sub-acrosomal materials and pro-acrosomal granule (fig. 62). In cross-sections, the acrosomal cap region appeared cup shaped with posterior surface partially encompassing the sperm nucleus. The components of the acrosomal complex, except the pro-acrosomal granule were radially symmetrical around the sperms central axis. The sub-acrosomal material underlined the acrosome and at the posterior end this sub-acrosomal materials separated the cap region from the nucleus. The fibrillar nuclear materials are found often inserted into the sub-acrosomal materials. The pro-acrosomal granule was found inside this sub-acrosomal material, usually in one side, in close apposition to the plasma-membrane. The thick saucer shaped acrosomal membrane separated the sub-acrosomal materials from the central core of the cap region (fig. 62). Extensions of this central core was found towards the anterior spike region also.

A common membrane was found covering the anterior regions of the sperm ie. the acrosomal cap region and the spike. A group of some what thick and elongated filaments are found at the widest part of the dense acrosomal core between the two arms which formed the cup. These elongated fine filaments are found extended anteriorly and constituted the inner core of the spike (fig. 62). The structure of this anterior spike was comparatively simple, and consisted of two distinct structural components viz. a limiting membrane and the internal spike material (fig. 62). Fine elongated tubules extending from the central core of the acrosomal vesicle constituted majority of the spike materials.

## MECHANISM OF SPERMATOPHORE FORMATION

Light microscopical as well as electron microscopical investigations in the vas deferens of fully matured male P. monodon showed that, each part of this highly modified testicular duct had specific roles in the production of the highly evolved spermatophores. Serial sections of the entire vas deferens revealed the mechanism of spermatophore formation in this species. The entire process of spermatophore formation was found inside this duct with the aid of glandular epithelial cells lining its entire lumen.

The formation of the spermatophore starts at the PVD and the process continues throughout the duct in a step wise manner. Light microscopical observations revealed that, the irregularly distributed multicellular glands in the inner lumen of the PVD was highly secretory in nature. It was noticed that many of the secretory epithelial cells found packed with highly refractile granules, secreting copious materials, spread over rather unevenly among the individually separated sperm cells. Tentatively it was found from the staining properties that similar copious materials were encountered both inside and outside of the epithelial cells. The same copious material is found to convert the individual sperm cells into a coherent mass, inside the lumen of the PVD.

Electron-microscopical investigations of different parts of the vas deferens showed its significant role in the spermatophore production. Cross-sections of the PVD revealed that it is the collecting area for the spermatozoans (fig. 64). It was found that PVD was the site of completion of spermatogenesis, as it received the sperm cells which were individually released from the testis. At the same time, it is the site for initiation of the spermatophore formation, as the acellular spermatophoric matrix is

found to be secreted in this area by the glandular epithelial cells (fig. 65). Ultrastructural observations revealed that the low columnar epithelial cells were responsible for the secretion of the amorphous materials inside the duct (fig. 67), and these cells were supported by a thick basal lamina of circular striated muscular fibers (fig. 68). A holocrine mode of secretion was noted in these epithelial cells lining the proximal part of the vas deferens. The columnar epithelial cells contained numerous mitochondria and cell organelles associated with protein synthesis and secretion (fig. 68). The basal regions of the adjacent secretary epithelial cells were highly inter-digitated and contained numerous mitochondria. The nucleus was comparatively large and possessed rich supplies of euchromatin materials. In most sections, numerous large vesicles were found between the nucleus and basal plasma membrane. These vesicles contained numerous lamellar zones and low density materials. RER occupied a large fraction of the cell volume (fig. 68). Ribosomes and polyribosomes were observed free in the cytoplasm. Numerous small Golgi bodies were found distributed throughout the cell, but most abundant in the perinuclear zone. Many small vesicles appeared to originate from the transfuse of the Golgi bodies. A relatively thick layer of connective tissue covered the muscular layer at its outside.

In concomintance with the release of the sperm cells from the testis, the epithelial cells described above, secrete a flocculant moderately electron -dense matrix, which forms the sperm supporting matrix in the PVD. Detailed investigations revealed that sperm supporting matrix is comprised of low electron density components and linear strands of dense fibers which together get converted with the individual sperm cells into a mass (fig. 69). As the sperm passes forward, it was found that, apart from these low electron

in the lumen as small droplets here and there. These droplets fused to form large droplets and in certain areas the larger droplets are found diffused. These materials are found to constitute the secondary spermatophoric layer which get deposited over the primary spermatophoric layer.

At the same time, the wing duct also showed heightened secretory activity. Like the spermatophoric duct, wing duct also contained a typhlosole with apparently active epithelial cells (fig. 77). A frothy secretion was observed at the proximal most region of the wing duct. This secretion formed the third spermatophoric layer. It is found that a finely granular material was first secreted in wing duct, and later on, more and more materials of different characters were laid on these granular materials and finally get converted into a thick accumulation in the wing duct (fig. 86). Meanwhile, the capacity of the wing duct has been observed to increase at the expense of the delimiting septum towards the latter duct. Still it was found that the materials for the III layer continued to add up as it passed forward. Apart from the materials of the III layer, another homogeneous material constituted the IV layer of the spermatophore, found secreted from the glandular cells of the wing duct at the farther most end of the ascending limb. It was observed that immediately after this secretion, the materials are found to set around the free borders of the III layer and appeared as a sandwich. The III layer sandwiched on either of its two flattened sides with the newly developed IV layer. Secretion of the IVth layer was found to be continuing through the descending limb of the MVD to the terminal portions of the testicular duct.

Serial sections of the descending limb of the MVD showed, separation of the lumen into spermatophoric duct and wing duct, as observed in the ascending limb, at its proximal region for a short distance. Immediately

after this area, it was observed that the septum interveinig between the two duct, detached at a point on the opposite end (fig. 88). This occurred when the newly formed material in the wing duct started its flow into the spermatophoric duct, inorder to establish its connection to the spermatophore. Cross-sections of the middle and terminal portions of this descending limb revealed that, the opening of the wing duct to the sperm duct became wider by the degeneration of the intervening septum. Epithelial cells observed in this region are found to be similar to that of the preceding region but the typhlosole was highly branched. Cross sections of the ascending limb at its farthest end and in the proximal parts of the descending limb revealed the presence of a third layer in the enlarged wing duct. It was observed that these materials get fixed around the IV layer in the same manner as the IV layer deposited around the IIIrd layer. This newly formed layer constituted the V and the outermost layer of the spermatophore. Epithelial cells in the sperm duct of the descending limb also secreted the materials responsible for this V layer. It was noted that the fifth layer was comparatively thinner during its formation at the descending limb of the MVD and got thicker during its passage through the distal vas deferens.

Electron-microscopical studies revealed that the MVD differed from the PVD in many ways. It was found that, it possessed a sperm filled highly secretory lumen wider than the preceding tube. Here both the spermatophoric and wing duct are found to be lined by tall columnar epithelial cells (fig. 82,83). The surrounding muscle layer was thicker in this part than the PVD. Cross-sections showed that the wider spermatophoric duct contained portions of the sperm cord and materials of the secondary spermatophoric layer. The secondary spermatophoric layer comprised of a matrix of low density, irregular zones of high electron-density and small and large electron

dense granules (fig. 85). The basal plasma membrane of these epithelial cells was highly infolded and contained numerous mitochondria and small cisterns of RER, consistent with protein synthesis (fig. 81). At the apex of these cells, the plasma membrane was found projected into blebs to the inner lumen (fig. 81). These blebs of the epithelial cells contained materials, which appeared similar to the secretion present inside the duct. It was noted that the microvilli extending off the basal portion of the bleb were smooth. Free ribosomes are noted throughout the cytoplasm of these cells (fig. 82). These free ribosomes intact presumed to synthesize the secretory materials, remained in the cytoplasm and subsequently appeared in the apical most region of the bleb. The portion of the bleb contained a distinct flocculent matrix which was almost completely devoid of organelles (fig. 80). The boundary between the organelle free and organelle containing zone of the bleb was often surrounded by an electron-dense material, which made the plasma membrane difficult to trace. However, at high magnifications it was clear that the apical most region of the bleb was devoid of the plasma membrane covering. The contents of the bleb and secondary spermatophoric layer appeared to be continuous in these regions. Furthermore, it appeared that the highly electron-dense contents of the bleb gave rise by way of apocrine secretion to the low density flocculent material of the secondary spermatophore layer. In some sections, the apical portion of the bleb was found pinched off the cell, (fig. 84) and released its contents to the neighbouring secondary spermatophoric materials found inside the lumen. Here the plasma membrane found immediately fused and maintained the integrity of the cell (fig. 84).

The remainder of the secondary spermatophoric layer appeared to be derived from the distal end of the ascending limb and descending limb of

the MVD. The blebs of the epithelial cells contained electron-dense materials which appeared similar to the secretions present inside the duct. It was noted that these cells released their contents from the blebs to the inner lumen by exocytosis. In addition to this, secretory materials processed through the Golgi apparatus and released from the microvillar portions of the epithelial cells are also found added to this secondary spermatophore layer. In this region two types secretory vesicles are observed near the Golgi bodies. Small vesicles with homogeneous material of moderate electron -density forms the first type and the second type includes few small electron dense granules. Both types of vesicles are frequently observed just beneath the plasma membrane, although they are not necessarily attached to each other. Contents of both of these granules are released to the space between microvilli by exocytosis. At the tips of the microvilli, the dense material appeared to break up and form the secondary spermatophore layer.

Electron-microscopical observations revealed that, the relatively smaller lumen of the wing duct in the ascending limb of the MVD, contained a thick core of moderately electron dense material, secreted by the epithelial cells lining the inner most area (fig. 82). This material appeared as extremely granular and moderately electron-dense during the time of its formation. Later it became electron-dense and homogeneous at the terminal portions of ascending limb after some structural modifications. Similar type of secretory pattern found in the spermatophoric duct of the ascending limb was encountered also in the MVD. Blebs are apparent at the apical most regions of the epithelial cells lining the inner lumen of this wing duct. The same apocrine mode of secretion was observed in these epithelial cells. But in certain cases, the blebs were found pinched off from the apical portion and joined the secretory materials present in the lumen. Secretion of the III layer

was found in the entire lumen of the wing duct. However, towards the terminal end of the ascending limb along with the IIIrd layer, secretory granules of another consistency are also observed. This highly granular electron -lucent layer formed the IV layer of the spermatophore. It was observed that the epithelial cells in the proximal parts of the wing duct secreted the morphologically changed materials of the IV layer. The basal plasma membrane of these epithelial cells are found to be highly infolded (fig. 83). The cytoplasm of these cells contained numerous active subcellular organelles like mitochondria, RER, free and attached ribosomes and Golgi materials consistent with protein synthesis. Wing duct at the distal part of the ascending limb and the proximal part of the descending limb secretes an electron-dense and homogeneous matrix, which was found deposited over the IV layer, forming the V layer. Formation of this layer leads to the detachment of the septum intervening the two ducts at one point. It was observed that the materials from the wing started its displacement to the larger spermatophoric duct immediately after the detachment of this intervening septum. Excess materials of the V layer made its entry first in the sperm duct and got deposited over the spermatophore on either of its sides to some extent. Secretion of the V layer is found till the terminal end of the descending limb.

Cross-sections of the long distal vas deferens (DVD) showed that, it had a thick layer of muscle and highly infolded epithelium, which has been found to create compartments, that conveyed the spermatophores to the terminal ampoule (fig. 87). These folds did not form complete partitions between compartments. Lumen of the duct was found empty in many sections, however, spermatophores are observed occasionally. Even though the typhlosoles are found to be lined with large accumulations of secretory epithelial cells,

They did not contain secretory materials as in the case of the preceding parts (MVD) of the vas deferens. The comparatively thick connective tissue layer found in the centre of the wall of this duct indicated its role of conveyance of the spermatophores. It was noticed that the size of the spermatophore decreased as it passed through the slender DVD. The different layers get attached firmly against the sperm mass and distinction of different layers are well identifiable at this stage (fig 88). Cross-sections showed that the III layer was electron dense and homogeneous where as the relatively wider fourth layer was granular with moderate electron-density. However, the outermost V layer was homogeneous and electron-dense with almost similar characters of the III layer.

Ultrastructural studies revealed that, the epithelial cells lining the inner lumen of the DVD became low columnar and the cell organelles like RER and Golgi bodies are less abundant, than the preceding parts of the vas deferens (fig. 89). Large moderately dense fibers of circular muscles are apparent in this part of the vas deferens. Acellular materials morphologically similar to that of the V layer are observed in the lumen occasionally. Numerous apical blebs are also noticed in certain sections of the epithelial cells, though it was not observed them being released from the epithelium. Very rarely, these vesicles appeared to be pinched off from the epithelial cells into the lumen. The basal region of these epithelial cells are found to be highly infolded and numerous mitochondria are found among these folds (fig. 89). As the distal vas deferens approaches the TA a reduction in epithelial area was noticed and the cells did not contain any apical blebs.

Cross-sections of the terminal ampoule of P. monodon revealed the presence of single lumen, which is found to be divided into four to five inter connecting chambers or lumina. It was observed that these

## DISCUSSION

A perusal of the available literature on the reproductive biology of penaeid prawns revealed that the process of gametogenesis has not been studied in male as thoroughly as that of the females. However, the morphology of the male reproductive system has been described in various penaeid shrimps like Penaeus setiferus (King, 1948); P. stylifera (Shaikhmahmud and Tambe, 1958; Rao, 1969); P. indicus (Subramoniam, 1965; Mohammed and Diwan, 1994); P. merguensis (Tuma, 1967); Metapenaeus stebbinal (Tirmizi and Javed, 1976) P. monodon (Motoh, 1978 & 1981 and Solis, 1988) and M. dobsonii (Vasudevappa, 1992). Present study has indicated that the general morphology of the reproductive system of P. monodon is also more or less similar to that of the other penaeids described by these above mentioned workers. During the present study in P. monodon it was noted that each testis has five pairs of lateral lobes and one pair of anterior lobe. This observation closely matches to that described by Motoh, (1981) and Solis, (1988) in the same species. Even though the general morphology of male reproductive system is similar to other penaeids, there are some variations in the number of testis lobes and the nature of fusion of the two halves of the testis. For instance in P. indicus it is reported to have only four lobes on either side of the testis (Subramoniam, 1965; Mohammed and Diwan, 1994). In P. stylifera testis has only three lobes on both the sides (Shaikhmahmud and Tambe, 1958). However, (King, 1948) in P. setiferus and (Vasudevappa, 1992) in M. dobsoni it has been reported that the testis of these shrimps possessed an anterior lobe, six lateral lobes and one posterior lobe. Similarly an accessory gland was noticed by Shaikhmahmud and Tambe, (1958) and Rao, (1969) in P. stylifera. No such gland was recognized in P. monodon during the present investigations or by any of the earlier workers in the same species.

The vas deferens of penaeids was commonly distinguished into four regions viz. Proximal vas deferens, Median vas deferens, Distal vas deferens and the terminating bulbous Terminal ampoule by various investigators like King, (1948); Subramoniam, (1965); Malek and Bawab, (1974a, b); Motoh, (1982); Champion, (1987); Vasudevappa, (1992) and Mohammed and Diwan, (1994). Present investigations revealed that vas deferens of P. monodon also contained similar morphological regions with characteristic size and shape. However, slight variations of this general pattern were reported by some workers in certain species of the same genera. According to Subramoniam, (1965) the median vas deferens was the tubular portion of the testis in P. indicus. Similarly Tirmizi and Khan, (1970) described a membraneous bag filled with spermatic cones attached to the middle portion of the vas deferens. These types of modifications were not observed in the vas deferens of P. monodon during the present study.

Unlike female P. monodon males did not possess any clearly visible characteristics for the visual assessment of gonadal maturity. Therefore, less emphasis has been placed so far for the assessment of gonadal development in male penaeid shrimps. Moreover in terms of the productions of spermatozoa, males mature at a smaller size and earlier than females and information regarding the maturity stages in male shrimps are scanty. Based on the smaller changes in opacity and size of the testes in relation to the size of the animal, Subramoniam, (1965) had described five maturity stages in P. indicus. Where as Castille and Lawrence, (1991) divided the maturity stages of the male penaeid shrimps P. aztecus and P. setiferus into three viz. Immature, Developing and Mature based on the size of the terminal ampoule. In the present study, depending upon the entire morphological characters of the testis as well as the testicular duct or vas deferens,

the gonadal maturation of male P. monodon was classified into three stages viz. Immature, Maturing and Mature. From the observations, it was found that morphological changes were more pronounced in the vas deferens or testicular duct, rather than the testes during these transitional stages. Based on the morphological differences, histological characters of different regions of the reproductive system have been studied, and noticeable differences were identified during these maturity stages. Though structural variations were present in both the testes as well as testicular duct it was more pronounced in the highly modified vas deference than the testis. In contrast to female reproductive system, there is a paucity of information in male reproductive system about the structural modifications that occur in different regions during its development. However, the complete structure of the fully matured vas deferens as well as its role during the process of spermatophore formation has been studied by many in the past two decades.

Four different regions Proximal vas deferens, Median vas deferens, Distal vas deferens and Terminal ampoule could be recognized in the vas deferens of P. monodon as in other penaeid prawns like Penaeus kerathurus (Malek & Bawab, 1974a,b); P. setiferus (Chow et al., 1991a); P. vannamei (Chow et al., 1991a) M. dobsoni (Vasudevappa, 1992); P. indicus (Mohammed and Diwan, 1994). Among the four regions, the median vas deferens is again divided into three functional subregions as blind pouch, ascending limb and descendig limb, as in many of the above mentioned prawns. These regions are distinct in maturing and fully matured males of P. monodon, while it is not so distinct in immature animals. Histologically, the structural modifications of the vas deferens has been studied in other penaeids by different investigators in connection with the spermatophore formation in P. kerathurus (Malek and Bawab, 1974a,b); P. setiferus (Ro et al., 1990); P. setiferus and P. vannamei

(Chow et al., 1991a); M. dobsoni (Vasudevappa, 1992) P. indicus (Mohammed and Diwan, 1994). However, none of them had studied the structural variations in this duct during the process of maturation.

Histologically the proximal vas deferens of P. monodon was a short unmodified tube composed of an outer connective tissue layer and inner circular muscle layer. Low columnar epithelial cells lining the inner lumen of PVD as in other penaeids like P. setiferus (King, 1948; Chow et al., 1991b; Ro et al., 1990); P. vannamei (Chow et al., 1991b) and M. dobsoni (Vasudevappa, 1992) were apparent in the maturing and mature animals but not in the immature animals. In the above mentioned prawns it is further reported that the columnar cells are responsible for the secretion of sperm supporting matrix present in the PVD. However, some investigators like Mohammed and Diwan, (1994) in P. indicus has not observed any glandular epithelial cells as well as sperm supporting matrix in the PVD.

The MVD, the highly complicated region has been divided into three sub-regions in P. monodon based on their internal anatomical modifications, as in other penaeids. Histological investigations of the MVD in different maturity stages showed that this structural modifications were apparent only in the mature animals. In immature animals the MVD is a simple tube, the intermediatery stages can be seen in maturing animals and the exact structure of each part is visible only in fully matured animals. The blind pouch of the MVD, in the fully matured P. monodon enclosed a single cavity lined by low columnar epithelial cells and always contained thickly packed sperm cells indicating its storage function. This blind pouch dilates into the ascending limb of the MVD, the inner lumen of which is divided into two unequal compartments, the wing duct and the sperm duct as in other penaeids. The lumen of the third portion of the MVD, the descending limb

was also compartmentalized for a short distance and the separation was found gradually disappearing in the distal end of the descending limb. The separation of this duct is not complete in maturing animals, while there is no such separation in immature animals. Similar characters were described for the MVD of penaeids like, P. setiferus (King, 1948; Ro et al., 1990, Chow et al., 1991a); P. kerathurus (Malek and Bawab, 1974a,b); P. vannamei (Chow et al. 1991b); M. dobsoni (Vasudevappa, 1992) and P. indicus (Mohammed and Diwan, 1994).

The blind pouch of P. monodon contained a branched typhlosole in contrast to the previous reports. Presence of two to three typhlosoles in the sperm duct and one typhlosole in the wing duct are found in the MVD of P. monodon as in M. dobsoni. Two typhlosoles are found on the wall separating the sperm duct and wing duct which is not at all reported in any other penaeids. In all other penaeids studied, usually one typhlosole in each of the sperm duct and wing duct is reported (Malek and Bawab, 1974a,b; Mohammed and Diwan, 1994). The three typhlosoles of the sperm duct developed in the ascending limb of the MVD disappeared in the distal part of the descending limb of the MVD. The presence of rich blood supply to the typhlosoles indicated high metabolic rate of these cells.

The distal vas deferens of fully matured male P. monodon was a highly complicated tube to which the MVD opens. Unlikely in MVD, it possessed only a single lumen throughout its length. However, the highly developed typhlosoles partially divide the lumen into different compartments. In immature animals, the DVD is a long simple tube but in maturing animals the lumen contained elongated columnar epithelial cells but the typhlosoles were not fully developed. Almost similar structure has been reported in other penaeids (King, 1948; Malek and Bawab, 1974a,b; Chow et al., 1991a;

Vasudevappa, 1992; Mohammed and Diwan, 1994). In these penaeids it has been reported that the DVD opens into a pear shaped muscular terminal ampoule, as also found in P. monodon. The musculature of the terminal ampoule is found to be fully developed in the matured animals. In immature animals it was a small sac like structure without well developed musculature and secretory cells. Outgrowths of columnar epithelial cells as typhlosoles as described in many penaeids were found in P. monodon during the present investigation. In P. monodon, small bud like typhlosoles are found from the distal end of the PVD onwards.

The process of spermeogenesis in P. monodon appeared to be similar to that reported in other decapod crustaceans (Pochon- Masson, 1983). There is a volume of information on spermatogenesis of various decapod crustaceans (King, 1948; Montrose, 1961b; Langrenth, 1969; Koebler, 1979; Hinch, 1980; Jesperson, 1983; Sagi et al., 1988; Vasudevappa, 1992 and Mohammed and Diwan, 1994). However, studies on the spermatogenesis of penaeids are less compared to other decapods. The process of spermatogenesis in penaeids through light microscopy has been reported by few investigators like King, (1948); Joshi et al. (1982); Pochon- Masson, (1983); Adiyodi, (1985); Vasudevappa, (1992) and Mohammed and Diwan, (1994).

Spermatogenesis begins in the peripheral germinative layer of the testicular tubules, when spermatogonia enter into the prophase of meiosis (King, 1948). A general feature of the process is that, these spermatogonial cells originated from the germinal area after passing through a period of quick growth, will transform into primary spermatocytes and then undergo reduction division to become the secondary spermatocytes. These secondary spermatocytes divide mitotically and form the spermatid, which without further division will transform into the spermatozoa. This pattern of

spermatogenesis has been described in P. setiferus by King (1948) through light microscopy and by Lu et al. (1973) through electron-microscopy. In P. indicus the whole process of spermatid differentiation has been studied by Rao (1968) and Mohammed and Diwan (1994) through light microscopy. Joshi et al. (1982) studied the gametogenesis of Prapenaeopsis stylifera. and Vasudevappa, (1992) studied the spermatogenesis in M. dobsoni through light microscopy. There is no much difference in the spermatogenesis in these penaeids according to them. All these previous reports as well as the present study revealed that the pattern of sperm differentiation is more or less similar in all the natantian shrimps.

Apart from these the process of spermatogenesis had been investigated through electron-microscopy in many other decapod crustaceans like crabs Eriocheir japonicus (Yasuzumi, 1959); crayfishes, Procambarus clarkii (Moses, 1961a & 1961b); cancer crabs (Langreth, 1969); Coenobita clypaetus (Hinch, 1980); Scyllarus chacei (Mc Knight and Hinch, 1986) and in Penaeids, Sicyonia injentis (Kleve et al., 1980 and Shigikawa and Clark, 1986). However information on the ultrastructural details of the spermatogenesis in natantian shrimps are restricted to Sicyonia injentis (Kleve et al., 1980 and Shigikawa and Clark, 1986) and P. setiferus (Lu et al., 1973). However, these reports as well as the present investigation revealed that during spermatogenesis the cytoplasm of the developing spermatogonial cells follows basically the same pattern of changes seen in other crustaceans ie. general reduction in organellar content. Early spermatogonial cells were located in the germinal zone and it is found towards the periphery of the follicular tubule in P. monodon during the present investigation. Similarly spermatogonia are grouped along the periphery of the tubules in P. setiferus (Lu et al., 1973). They were large cells with a centrally located spherical nucleus and during the

early stages, the nuclei are not easily distinguishable. Its cytoplasm contained numerous cell organelles like aggregates of ribosome and lamellae of endoplasmic reticulum. Similar type of spermatogonial cells were described in P. setiferus by Lu et al. (1973). Another interesting feature noticed during the early stages was the non-germinative cells observed in large numbers among the germinal components. Even though Lu et al. (1973) did not mention about these type of cells, there are other reports of crustacean testis which mention the presence of "nurse" or nutritive cells among the germinal components (Pillai, 1960, Pochon- Masson, 1968a,b and Hinch, 1980). These cells function in providing nourishment, support and possibly hormones during spermiogenesis (Hinch, 1980). From the characteristics it is assumed that a more or less similar function was performed by these non germinative cells, found among the spermatogonial cells in the testes of P. monodon during early stages of spermiogenesis.

From earlier reports it was found that, during the ultrastructural investigations on spermatogenesis in decapod crustaceans, investigators have given more emphasize on the later stages of spermatogenesis ie. the transition of spermatids to the spermatozoa, which is more complicated and dramatical than earlier stages. Therefore the information regarding the ultrastructural changes in the early stages of spermatogenesis is very limited in decapod crustaceans and it is lacking in penaeids. Results of the present investigation in the early stages of spermatogenesis in P. monodon as well as the limited reports in the spermatogenesis of other crustaceans revealed a more or less common pattern of changes during the early stages of their development. As Pochon-Mason (Review-1983) reported in other penaeids, in P. monodon also spermatogenesis was found synchronous in each of the testicular lobule.

Secondary spermatogonia which are transformed into spermatocytes have similar characters to the former. However the spermocytes were distinguishable by certain characters. During the present study it was observed that the nuclear wall of the spermatocytes became more prominent and was easily demarcated from the surrounding cytoplasm. Highly electron dense chromatin materials were also apparent in the nucleoplasm and the cytoplsam contained organized ergastoplasm. Similar type of characteristics were noticed in other crustaceans by Meusy (1972) and Lu et al., (1973).

The cytoplasm of secondary spermatocytes was characterized by the abundant supply of membrane system around the nuclear wall. Lu et al. (1973) described similar type of secondary spermatocytes in P. setiferus. The cell organelles noticed in the cytoplasm were the abundant randomly distributed cisternae, rough endoplasmic reticulum (RER) and the granular bodies originating from the cisternae. Membrane complex comprised of mainly 3 types were described in the early spermatids of Cancer crabs by Langreth, (1969) as well as in the cray fish Procambarus clarkii by Moses (1958). Apart from the membrane systems oval to elongated mitochondria with feeble cristae were apparent in the secondary spermatocytes of P. monodon. Other prominent cell organelles found during the present investigation are, numerous ribosomes and vesicular systems. Transformation from the common filamentous type of mitochondria to the oblated spheroid were noticed as the meiosis progresses in the spermatocytes of the crayfish P. clarkii (Moses 1961b). In the same animal at the telophase of the meiotic division the cytoplasm contained numerous vesicles of varying diameters at the pole ward side. Similarly, the role of mitochondria in the membrane complex of dividing spermatids was described in Cancer crabs by Langrenth (1969) in Anaspides tasmaniae by Jesperson (1983) and in S. chacei by Mc Knight and Hinch (1986).

In the present study, another factor noticed in the secondary spermatocytes of P. monodon at its final stages was, the out pocketings of the nuclear membrane as vesicles of varying size and shape. In crayfish P. clarkii, Moses, (1961b) has described similar pattern of changes in nucleus as well as nuclear membrane during the transition of the spermatocytes to the spermatids.

As the secondary spermatocytes transform into the spermatids by the 2nd division of the meiosis the size of the cells became considerably less. Drastic ultrastructural changes were noticeable in the developing spermatids from these stages onwards during the process of sperm differentiation. The cell organelles became highly active and secretory. The most striking feature noticed in the early spermatids of P. monodon was the polarization of the cellular materials and the nucleus. Displacement of the ER elements to one side of the cells as well as the nucleus to the other pole has been described in P. setiferus by Lu et al. (1973). Whereas such polarization of the cellular materials was not reported in any of the Brachiuran crabs. Instead in Cancer crabs Langrenth, (1969) reported that the cytoplasmic materials encircles the centrally located nucleus in early spermatids. A partial polarization of the cell contents along with the multivesicular bodies to one end of the cells, was reported in crayfish P. clarkii (Moses, 1961b). Mc Knight and Hinch, (1986) reported that in Scyllarus chacei polarization of the cell components started in Stage 1 spermatids, as the nucleus moves to one side of the cell while the cytoplasmic organelles move to the other side. Here the RER was often found in concentric rings around the nucleus. Similarly, in the early spermatids of P. monodon also the RER was found in concentric layers around the nucleus at one side. While other cell organelles were found shifted towards the opposite end of the cell.

On the contrary, the polarization of the cells was found only in later spermatids in Anaspides tasmaniae (Jesperson, 1983).

The decondensation of the nucleus as well as the blebbing of the highly convoluted nuclear wall was more pronounced in developing spermatids of P. monodon during the present study. Clusters of small vesicles and dense granules appeared around the nucleus in close proximity to the nuclear blebs, indicated the highly active condition of the spermatid nuclei during its development as a pre-requisite for acrosome formation. Decondensation of the spermatid nucleus has been reported in many decapod crustaceans including penaeids. In P. setiferus Lu et al. (1973) reported that the size of the nucleus diminishes in the spermatids due to the condensation of the nuclear materials. During the transformation of the spermatids to the spermatozoa in P. clarkii, the nucleus underwent considerable shrinkage and decrease in volume by two thirds to three fourths and towards the end of the stage it represents only one eighth of the total volume of the cell (Moses, 1961b). Mc Knight and Hinch, (1986) reported that the nuclear decondensation in Scyllarus chacei begins at the stage 1 spermatid and gets completed in the stage III spermatids. A reduction in the size of the nucleus was also reported by Langrenth, (1969) in Cancer crabs and in Coenobita clypeatus by Hinch, (1980).

Another noticeable feature of the early spermatids in P. monodon was the blebbing of nuclear membrane and the highly active perinuclear cytoplasm. In P. setiferus, Lu et al. (1973) reported that, the distinguishing structures of early spermatid are the dilated vesicles, which may have originated from the nuclear membrane and subsequently fuse to form a structure which caps the nucleus at one pole. Blebbing of the nuclear wall and a high perinuclear activity was also reported in the early stages of

spermatids of P. clarkii, and blebs have been found receded during the later stages, but the surface of the nucleus was still irregular (Moses, 1961b). The vesicular formation as well as its fusion was observed throughout the spermatids in S. chacei (Mc Knight and Hinch, 1986). Similarly, smooth and roud vesicles containing faintly electron opaque filamentous materials were described in the early spermatids of Cancer crabs by Langrenth (1969), which were presumed to be the acrosome precursors because of their nature and subsequent fusion.

The most important event in the spermatid differentiation is the acrosome formation. In P. monodon it was observed that the acrosome formation started in the early spermatids and completed only in the final stages of differentiation of the spermatozoa. The classical literature on decapod spermatogenesis is not clear in its descriptions of the origin of acrosome. Its formation is attributed to Golgi elements by some earlier workers (Nath, 1937 and 1965) and to mitochondria by Mc Croan, (1940) and Nath, (1956, 1965). Some other investigators are of opinion that it is formed from the vesicle or vacuole appearing in the cytoplasm (Binford, 1913 and Fasten, 1926). However, electron microscope studies of Yasuzumi, et al. (1960) revealed that certain dense granules appeared in the interzonal spindle region, as the acrosomal precursor. Pochon-Masson, (1965,1968) concluded that the acrosome in crustaceans is derived from the fusion of dilated ergastoplasmic cisternae, and the nuclear envelop derivatives may also contribute to the acrosome. From the ultrastructural investigations of Moses, (1961b) in P. clarkii and Langreth, (1969) in Cancer crabs, it was found that the acrosome forms from the fusion of the cytoplasmic vesicles, which appeared to be derived from the rough ER. Results of the present study closely resembles that of the observations of Yasuzumi, (1960) in P. clarkii and

Langrenth, (1969) in Cancer crabs. The small granule filled vesicles derived from the cytoplasm and the nuclear blebs formed the precursors of acrosomal materials in the spermatids of P. monodon. Other structures involved mainly in the acrosome formation are smooth ER elements and nuclear envelops.

In P. monodon the acrosomal core forms by an inpocketing of the acrosomal vesicle in the regions closest to the nucleus. Fibrous and granular materials were noted within the acrosomal core during the present investigation in the early spermatozoa of P. monodon. A similar mode of formation of the acrosomal core is described in other decapods (Moses, 1961a,b; Yasuzumi et al., 1960; Pochon-Masson, 1965 and Langrenth, 1969). These investigators also noted similar type of fibrous tubular materials in connection with the acrosomal complex. As in the present study, most of these investigators could not relate these structures to any previously existing body in the spermatids.

Morphological studies by earlier investigators has resulted in the crustacean decapod gametes being divided into two classes, the unistellate sperm of the natantians (shrimps) and multistellate sperm of the reptantians (Lynn and Clark, 1983). Studies on the fine structure of the sperm in P. monodon revealed that, it is more or less round and unistellate as in other natantians and superficially it can be distinguished into three regions viz. 1. a posterior main body, 2. a central cap region and 3. an anterior spike. Ultrastructure of other natantians has been studied by investigators like Clark et al., (1973) in P. aztecus; Lu et al., (1973) in P. setiferus; Kleve et al. (1980) in S. injentis; Mohammed and Diwan, (1994) in P. indicus described more or less similar type of spermatozoa in these shrimps.

The nucleus of spermatozoa in P. monodon is decondensed as is typical of the decapod crustacean sperm, and a nuclear envelop is absent as in other

natantians (Pochon- Masson, 1969; Clark et al., 1973; Lu et al., 1973; Kleve et al., 1980; Mohammed and Diwan, 1994). The nucleus of the natantian sperm was found confined to the main body (Pochon- Masson, 1969; Clark et al., 1973; Brown et al., 1974 and Mohammed and Diwan, 1994). The sperm nuclei of P. monodon confirm this typical natantian pattern. The nucleus extends to the plasma membrane at its outer region with no intervening region of cytoplasm as shown in P. aztecus (Clark et al., 1973) and in M. rosenbergii (Lynn and Clark, 1983). Loosely packed fibrillar materials constituted the majority of the cell body as in other shrimps like P. aztecus (Clark et al., 1973; Kleve et al., 1980) P. indicus (Mohammed and Diwan, 1994) and P. setiferus (Lu et al., 1973). As in other shrimps, the nucleus of the sperm of P. monodon is surrounded partially by an amorphous cytoplasmic band, that contains small sized vesicles whose functions are unknown.

Morphologically complex cap region represents the acrosomal complex in P. monodon sperm. This complex cap region of the sperm exhibit structures similar to those described in other penaeids like P, aztecus (Clark et al., 1973); P. setiferus (Lu et al., 1973); Sicyonia injentis (Kleve et al., 1980) and P. indicus (Mohammed and Diwan, 1994) with slight modifications. The granular central core observed in P. monodon was also reported in S. injentis (Kleve et al., 1980) and in P. indicus (Mohammed and Diwan, 1994). The central core was found along with the spike in P. aztecus by Clark et al., (1973). The acrosomal materials found in the sperm of P. monodon separating the nuclei and the central core of the acrosomal complex were described in P. indicus (Mohammed and Diwan, 1994) and as granule in P. aztecus (Clark et al., 1973). Crystalline lattice of S. injentis (Kleve et al., 1980) also occupied the same position of the sub-acrosomal materials. The single pro-acrosomal granule was located in one side of this sub-acrosomal

granule in P. monodon. In P. indicus (Mohammed and Diwan, 1994) had noticed one or two pro-acrosomal granules, either on one or on both the sides.

The spike in the sperm of P. monodon was an anterior extension of the acrosomal complex, as both were found encompassed in a single membrane. Similarly in S. injentis (Kleve et al., 1980) reported that a continuous membrane encloses the acrosomal cap region and its anterior extension, the spike. In other penaeids also, the spike was considered as an anterior extension of the acrosomal complex (Clark et al., 1973; Lu et al., 1973; Kleve et al., 1980 and Mohammed and Diwan, 1994). Surprisingly no organelles were observed in the fully matured spermatozoa of P. monodon, except the small vacuoles and vesicles found in the cytoplasmic band on both the lateral sides. The degeneration of organelles were reported in many decapod crustaceans (Moses, 1961a,b; Langrenth, 1969; Clark et al., 1973; Lu et al., 1973; Kleve, 1980; Hinch, 1980; Jesperson, 1983; Mc Knight and Hinch, 1986 and Mohammed and Diwan, 1994). It is not only in decapods, among Malacostraca itself, the spermatozoa show considerable modification and loss of organelles (Adiyodi, 1985). From all these characteristics, it is concluded that the sperm of P. monodon belongs to an altered vesicular type, which is different from other flagellate and non flagellate gametes in other crustaceans (Pochon- Masson, 1983).

The vas deferens of decapod crustaceans functions as a site of sperm maturation, encapsulation of sperm into spermatophores, production of seminal fluids and their storage (Hinch and Mc Knight, 1986). Many studies have described the structure of the vas deferens and its role in the formation of the spermatophore in crustacea through light and electronmicroscopy (Cronin, 1947; Mathews, 1954; Hinch and Walker, 1974; Malek and Bawab, 1974a,b; Kooda-Cisco and Talbot, 1986; Hinch and Mc Knight, 1986; Talbot

and Beach, 1989; Chow et al. 1991; Ro et al., 1990; Vasudevappa, 1992; Mohammed and Diwan, 1994). However, such studies in penaeids are limited to P. kerathurus (Malek and Bawab 1974a;b); P. setiferus (Ro et al., 1990 and Chow et al. 1991) P. vannamei (Chow et al. 1991); Metapenaeus dobsoni (Vasudevappa 1992) and P. indicus (Mohammed and Diwan 1994). Ultrastructural investigations in penaeids on the spermatophore formation have been more limited ( Ro et al., 1990; Chow et al., 1991).

The vas deferens of decapod crustaceans conveys sperm from the testes to the exterior in the form of a spermatophore. The spermatophore is laid down by the glandular epithelial cells lining the vas deferens during the passage of sperm. The mechanism of spermatophore formation in P. monodon observed through light microscopy during the present study, agrees with the earlier reports of other penaeids like P. kerathurus (Malek and Bawab, 1974a,b); P. setiferus (Ro et al., 1990, Chow et al., 1991); P. vannamei (Chow et al., 1991) P. indicus (Mohammed and Diwan, 1994) and M. dobsoni (Vasudevappa, 1992). The process in which the different layers of the spermatophore wall are laid appeared to be similar to other penaeids like P. kerathurus (Malek and Bawab, 1974a,b). As in P. kerathurus, spermatophore of P. monodon is composed of five acellular layers. Different layers are found secreted in different parts of the vas deferens by glandular epithelial cells lining the corresponding parts.

Its formation starts at the PVD and is continued till the terminal ampoule. The PVD secretes an amorphous material which forms the sperm supporting matrix. The first two layers of the spermatophore wall are found to be secreted by the epithelial cells in the sperm duct of MVD in its ascending limb. The sperm mass was found completely encompassed in these two layers in P. monodon as in P. kerathurus (Malek and Bawab, 1974a,b)

and P. indicus (Mohammed and Diwan, 1994). The third and the fourth layer are found to be secreted in the wing duct, whereas the secretion of the fifth layer was found done by the epithelial cells of wing duct at the descending limb of MVD, as well as sperm duct of the same area. The three incomplete layers were found out side the two complete layers. In M. dobsoni, Vasudevappa (1992) has noticed two complete layers for its spermatophore. The first three layers are incomplete layers, while the fourth and fifth layers completely surrounds the spermatophore of M. dobosoni. Kooda- Cisco and Talbot (1982) observed three complete layers in H. americanus. The morphological and histological structure of the wing of the spermatophore in P. monodon is found to be similar to that described in M. dobsoni by (Vasudevappa, 1992) and in P. indicus by (Mohammed and Diwan, 1994).

The ultrastructure of the vas deferens of P. monodon is found to be similar to that of other decapod crustaceans, such as Libinia (Hinsch and Walker, 1974); Homarus (Kooda- Cisco and Talbot, 1986) and P. setiferus (Ro et al., 1990; Chow et al., 1991). It was found that the entire lumen of the vas deferens in P. monodon is lined by secretory epithelium and that in turn is surrounded by a basal lamina, and connective tissue containing blood vessels and striated muscle. As in other decapods that have been studied so far the vas deferens of P. monodon is divided into several morphological and functional regions, while the secretory cells throughout the tracts are ultrastructurally similar. These secretory cells functions in formation of the acellular components of the spermatophore. It was observed that, the epithelial cells in the proximal vas deferens are specialized for secretion of sperm supporting matrix, and those in the rest of the tract, release other components that form different layers of the spermatophore. In P. monodon secretion of spermatophoric layers was found

to start in the PVD and continued till the TA. In P. kerathurus (Malek and Bawab, 1974a,b) and in P. setiferus (Ro et al. 1990 and Chow et al., 1991) reported that the secretion of the primary spermatophoric layers starts only in the sperm duct of MVD. The PVD as well as the blind pouch secrete the sperm supporting matrix and the primary spermatophoric layer. In contrast, Mohammed and Diwan, (1991) have reported that in P. indicus the PVD is devoid of secretory cells and the spermatophore formation starts in MVD.

In P. monodon the low columnar epithelial cells of the PVD secretes an amorphous material of low electron density and linear strands of dense fibers which constitute the sperm supporting matrix. At the distal end of the PVD these secretory epithelial cells secrete an electron-dense material, which forms the primary spermatophoric layer along with the other secretions of sperm supporting matrix. Similar observations have been reported in P. setiferus (King, 1948; Ro et al., 1990 and Chow et al., 1991); P. kerathurus (Malek and Bawab, 1974a) P. vannamei (Chow, et al., 1991). Whereas Mohammed and Diwan, (1994) reported that there is no secretory activity in the PVD as well as blind pouch of P. indicus. It has been reported that the corresponding regions of the vas deferens is lined by a simple secretory epithelium as in other decapods like Homarus (Kooda-Cisco and Talbot, 1986) and Crayfishes (Talbot and Beach, 1939) and probably this epithelium contributes material to the matrix surrounding the sperm mass.

The blind pouch of P. monodon is lined by low columnar epithelial cells as reported in other penaeids. The small but branched typhlosole projecting into the lumen of the blind pouch is characteristic of P. monodon. Malek and Bawab (1974a,b) reported numerous regularly arranged glandular cells but no typhlosole. Highly secretory epithelial cells were also reported in the blind pouch of P. setiferus (Ro et al., 1990). These epithelial cells

lining the blind pouch as well as the typhlosole in this area secrete an electron-dense material which gave a cohesive property to the sperm supporting matrix. "Filamentous materials" in the sperm supporting matrix, which keep the sperm cells together has been reported in P. kerathurus (Malek and Bawab, 1974a,b). Filaments which are probably collagen, have been observed in the sperm supporting matrices of other decapods such as lobsters (Talbot et al.,1976; Talbot and Chanmanon, 1980) and Crayfishes (Talbot and Beach, 1989). However such filaments have not been reported in the sperm supporting matrix of P. vannamei (Ro et al. 1990). Thus the presence or absence and the structure of filaments when present in the matrix seem to be variable among decapods. In P. monodon the first evidence of orientation of the sperm occurs in the blind pouch as in other penaeids like P. kerathurus (Malek and Bawab, 1974a,b); P. setiferus (Ro et al., 1990) and P. indicus (Mohammed and Diwan, 1994).

The ascending limb of MVD of P. monodon contained two ducts. Heldt (1989) first described the double duct structure of the vas deferens in P. trisulcatus, and this character has been shown to be common in the genus penaeus (King, 1948; Malek and Bawab, 1974b; Champion, 1987; Ro et al., 1990; Chow et al., 1991; Vasudevappa, 1992 and Mohammed and Diwan, 1994). The present observations in P. monodon agree with these findings. The two ducts in the ascending limb of the MVD termed, spermatophoric and wing ducts by Malek and Bawab (1974b) in P. kerathurus, were also used in the present study. Tall epithelial cells with increased secretory activity lines the inner lumen of both the spermatophoric and wing duct of P. monodon. Out growths of these epithelial cells as typhlosoles were present in the lumen of both of these ducts. The wing duct contained only a single typhlosole whereas the spermatophoric duct contained three to four

typhlosoles. Secretion of fine granular materials of the third spermatophoric layer was found in the wing duct, while the spermatophoric duct contained the materials of the secondary spermatophoric layer. Mohammed and Diwan, (1994) reported in P. indicus, that the wing duct secretes the materials of the wing. A similar type of secretion was reported in P. setiferus by Ro et al. (1990). In M. dobsoni Vasudevappa (1992) described the structure as well as formation of wing material in the wing duct. Bell and Lightner, (1988) mistakenly presumed that, the accessory duct deposited the primary spermatophore layer in P. stylirostris. In contrary, Chow et al., (1991) did not find any wing materials in the wing duct of P. vannamei and so they reterned the "wing duct" as the "accessory duct". Secretion of the materials of the fourth spermatophoric layer was found in the wing duct, along with the third layer in the distal end of the ascending limb and also in the descending limb in P. monodon. The fifth layer was found to be secreted in both the wing duct at its terminal end, as well as in the sperm duct and continued till the terminal ampoule.

In P. monodon the primary spermatophoric layer was heterogeneous and fibrillar in appearance. The region closer to the sperm matrix is only moderately electron dense but the region outside is more electron dense. Whereas the secondary spermatophoric layer comprised of a matrix of low density, irregular zones of high density and small and large electron dense granules. These primary and secondary spermatophoric layers are secreted by both, an apocrine mechanism and by exocytosis. Similar type of secretion has been reported in the segment III of the vas deferens of P. setiferus by Ro et al., (1990) and in crayfish Cherax by Talbot and Beach, (1989). This is in contrast to Homarus (Kooda- Cisco and Talbot, 1986); Libinia, (Hinsch and Walker, 1974). From the ultrastructural observations during

the present study, it is presumed that the numerous polysomes observed in the cytoplasm of the secretory epithelium are generally responsible for synthesizing the secretory products, that become sequestered in the apical blebs; while those products released by exocytosis are synthesized on the RER and processed through the Golgi bodies. One remarkable feature of the apocrine blebs was the absence of organelles within the bleb. The secretory epithelium of P. monodon is similar to that of Homarus (Kooda- Cisco and Talbot, 1986); crayfish Cherax (Talbot and Beach, 1989) and P. setiferus (Ro et al., 1990). The third spermatophoric layer present in the spermatophore of P. monodon was found secreted in the wing duct. Similar type of apocrine as well as exocytotoic secretory pattern has been noticed both in the spermatophoric duct and the wing duct during the present investigation. The electron lucent fourth, as well as the highly electron dense fifth layer was secreted by the epithelial cells of the wing duct by the same mode of apocrine secretion and exocytosis.

CHAPTER III

# NEUROENDOCRINE SYSTEM AND ITS ROLE IN REPRODUCTION

A. *Neurosecretory cells (NSCs) and their secretory cycle*

B. *Distribution and mapping of NSCs in different ganglia of central nervous system*

C. *Eyestalk neurosecretory system*

D. *Neuroendocrine control of reproduction*

# CHAPTER III

## NEUROENDOCRINE CELLS AND THEIR ROLE IN REPRODUCTION

### INTRODUCTION

In the culture of some groups of crustaceans such as penaeid shrimps, the production of healthy seed of a particular organism in sufficient quantity has hindered the potential farming of the species. Controlled reproductive maturation is the major problem in the development of commercial aquaculture programmes in penaeid shrimps. Even though some achievement have already been made in the captive breeding of penaeids, the full control of the process is yet to be been revealed. Similarly due to the lack of understanding of the entire maturation process, constraints of selective breeding in these animals still remain. In this regard, the basic knowledge of female reproduction and its endocrine control is of prime importance in crustacean aquaculture. A better understanding of the mechanism that regulates the reproduction at its ultrastructural levels is fundamental to the successful aquaculture programmes. Controlling reproduction in captivity could help to provide a reliable year supply of juveniles, in developing selective breeding programmes, and will be generally useful for obtaining disease free spawners.

Any attempt in captive seed production calls for a better understanding of the endocrine mechanism involved in the control of reproduction (Fingerman, 1987). During the past two decades our understanding of crustacean reproductive endocrinology, especially that of females, has grown steadily. Neuroendocrine regulation of reproduction has been reviewed by Legrand (1982), Payen (1986), Fingerman (1987), Charniaux-Cotton and Payen (1988), Meusy and Payen (1988) and Van Herp and Payen (1991). These literature showed that the classical manipulations being practiced are extirpation and

reimplantation of the suspected endocrine tissues. Eyestalk ablation has been used to mature female shrimp in captivity in conjunction with the management of water quality parameters such as water temperature, water salinity, photoperiod, light intensity, sex ratio, and nutrition (Caillouet, 1972, Lumare 1979, Yano 1985, Primavera 1985, Crocoss and Keller 1986, Mohammed and Diwan 1991). Although many observations on endocrine systems have been conducted on the inhibition/suppression of reproductive maturation by eyestalk hormone(s) since the pioneering work of the 1940s (Panouse, 1943) however recent researches have focused mostly on organs like brain, thoracic ganglion, ovary etc functioning closely related with gonad stimulating factors or hormone(s) in crustacea (Eastman-Recks and Fingerman, 1984; Takayanagi et al., 1986; Yano, 1988; Mohammed and Diwan, 1991; Yano, 1992; Yano and Wyban, 1992). Hormones are chemical messengers synthesized and secreted by endocrine glands and released to the circulatory system. Blood carries the hormones to a (usually) distant target cell(s) where they alter the cell's physiology. Usually, these hormones mediate long term, pervasive physiological alteration, as opposed to the short term, localized events that are usually mediated by the nervous system (Chang, 1992). Hormones that regulate reproduction in crustaceans include, the gonad inhibiting hormone (GIH), which may be the same as that of the vitellogenin inhibiting hormone (VIH) and the gonad stimulating hormone (GSH) both of which have been described mainly in conjunction with female reproduction (Laufer et al., 1992). It has been reported that GIH/VIH is released from the sinus gland in the eyestalk and inhibits ovarian synthesis of yolk protein vetellogenin in vitro (Eastman-Recks and Fingerman, 1984; Quackenbush and Keeley, 1987). In contrast it is reported that a hormone (GSH) released from the thoracic ganglion (Eastman-Recks and Fingerman, 1984) and the brain (Gomes, 1965; Hinch and Bennet, 1979; Anilkumar and Adiyodi, 1980; Takayanagi,

1986; Yano and Wyban, 1992) stimulates ovarian growth and maturation. A gonad stimulating hormone releasing hormone has been reported from the brain of P. vannamei by Yano and Wyban (1992). Knowledge of the hormonal control of maturation in crustaceans however, is not well documented.

Among decapod crustaceans the control of diverse and numerous physiological processes, such as reproduction, moulting, haemolymph glucose content, rate of heart beat and chromatic adaptation, etc. is mediated by neuroendocrine factors synthesized within the specialized neurosecretory cells (NSCs) located within or associated with the ganglia of the central nervous system (CNS) (See reviews of Kleinholz, 1976, Cooke and Sullivan, 1982; Sandeman, 1982; Fingerman, 1987). Some of these peptide neurofactors, for example, pigment aggregating (Fernlund and Jossefson, 1972) and dispersing hormone (Fernlund, 1976) have been identified, purified and synthesized. Considerable information is available concerning specific processes, such as the regulatory phenomena involved in the control of chromatic adaptation (Rao and Fingerman, 1983; Fingerman, 1985,1987). In contrast, neuroendocrine control of other phenomena such as reproduction, osmoregulation etc. has received the attention of many investigators during the last two decades, however the neurofactors involved have been only partially characterized and purified. A clear picture of the control of reproduction has not been unraveled till recently. While there is substantial data available concerning the physiological effects of the homogenates of the optic, supra-oesophageal and thoracic ganglia on reproduction informations regarding the specific sites of the synthesis of neurofactors involved in the reproduction is meagre (Mc Namara 1993). The approach of cytophysiology of the endocrine systems has a great deal to offer when it comes to understanding the hormonal regulation of various life processes mainly reproduction and its control.

The gross morphology of the central nervous system of Penaeus was described by Young (1959) and its structure and function in Penaeoidea, including Penaeus by Bullock and Horridge (1965). Optic ganglia of penaeids has been described by Dall (1965) and Elofsson (1965). The layout of the central nervous system of penaeids has been described by Dall et al. (1990). The established endocrine elements in the decapod neurosecretory system are the X-organ sinus gland complex of the eyestalks, neurosecretory cells of the brain and central nervous system, post commissural organs, pericardial organs, the epithelial endocrine systems, the Y-organ and the androgenic gland ( Gabe 1953, 1956; Carlisle and Knowles, 1959; Bullock and Horridge, 1965; Cooke and Sullivan, 1982; Skinner, 1985; Charniaux-Cotton and Payen, 1985; Fingerman, 1985).

The morphology as well as the histology of the crustacean endocrine organs in the central nervous system have been described by Carlisle and Knowles, (1959); Nagabhushanam et al., (1992) and Mohammed et al., (1993). Similarly the X-organ sinus gland complex has been described by Dall (1965) in Metapenaeus bennettee and Nakamura (1974) in P. japonicus, Nanda and Ghosh (1985) in P. monodon and Mohammed et al. (1993) in P. indicus. Histological investigations of the neurosecretory cell types in the Penaeus species has been described by Ramadan and Matta (1967), Nanda and Ghosh (1985, 1992), Mohammed et al. (1993) and that of Metapenaeus by Madhysta and Rangnekar (1976) and Nagabhushanam et al. (1992).

It is now well known that physiologically active substances are produced in the neurosecretory cells located in the ganglionic centers of crustaceans (Bliss et al., 1954, Durand, 1956; Dall, 1965; Fingerman and Guru, 1968; Adiyodi and Adiyodi, 1970; Nakamura, 1974; Chandy and Kolwalker, 1985; Gyananath and Sarojini, 1985; Nanda and Ghosh, 1985 & 1992; Nagabhushanam et al., 1986 & 1992; Quackenbush, 1989; Mohammed et al., 1993).

Nevertheless investigations on penaeid neurosecretion are limited to Dall 1965, in the Australian school prawn Metapenaeus species, Nakamura (1974) in P. japonicus; Nagabhushanam et al. (1986) in P. stylifera; Nanda and Ghosh (1985 & 1992) in P. monodon; Nagabhushanam et al. (1992) in M. ensis; Mohammed et al. (1993) in P. indicus. Gyananath and Sarojini (1985) delineated the activity of neurosecretory cells in the fresh water prawn Macrobrachium rosenbergii.

Even though these studies are describing different types of neurosecretory cells in different ganglionic centers of crustaceans, only few studies have been made to correlate the changes in the neurosecretory activity with physiological events like reproduction (Aoto and Nishida, 1956; Durand, 1956; Hanoaka and Otsu, 1957; Kulkarni and Nagabhushanam, 1980; Gyananath and Sarojini, 1986; Mohammed et al., 1993). Similarly these investigators have described the secretory activity of these NSCs but not the mechanism and/or the various stages in the secretory cycle in relation to any other physiological processes. Neurosecretory cells in different stages are mentioned in the reports of Durand (1956) in crayfishes, Peryman (1969) in Procambarus clarkii; Diwan and Nagabhushanam (1974) in Barytelphusa cunicularis; Rao et al. (1981) in M. lanchesteri; Decaraman and Subramoniam (1983) in Squilla holochista; and Joshi (1989) in Potamon koolooense. However, these investigators did not explain the actual mechanism and/or cyclic stages in neurosecretion. But Damassieux and Batesdent (1977) have reported the cyclic variation in certain NSCs in Ascellus aquaticus. A detailed description of the various phases in the secretory cycle of different neurosecretory cells has been explained in P. indicus by Mohammed et al. (1993) through light microscopy. Such attempts through electron-microscopy has been restricted to the ultrastructural studies in the neurosecretory cells of shrimp Macrobrachium olfersii maintained in different salinities by Mc Namara (1993) among crustaceans.

Neurosecretory cells are located in different ganglionic centers of the central nervous system and also in the optic ganglion of decapod crustaceans (Dall, 1965). Furthermore in crustaceans, neurosecretory cells are distributed as distinct groups (Bliss, 1951; Enami, 1951). Various investigators as mentioned above, studied different ganglionic centers of various decapods through light microscopy. NSCs of the brain and their various functions have been studied by Nakamura (1974), Ramadan and Matta (1976), Fahrenbach (1976), Nanda and Ghosh (1985) and Mohammed et al. (1993). Various investigators like Enami (1951) in the crab Sesarma; Matsumoto (1954) in Eriocheir japonicus; Maynard (1985) in nine species of brachyuran crabs and Mohammed et al. (1993) in P. indicus studied the NSCs in the thoracic ganglia. Abdominal ganglia of crustaceans were not investigated as thoroughly as other ganglia in the central nervous system. Available information in this respect is restricted to reports of Hearnye (1976) in hermit crab, Skinner (1968) and Kendoh and Hsada, (1986) in crayfish and Mohammed et al. (1993) in P. indicus. In contrast, the NSC types in the optic ganglia of various crustacean species have been studied in detail by many investigators like Carlisle (1953), Smith and Naylor (1972), Hisano (1974), Smith (1975), Jaros (1978), Nakamura (1980), Nanda and Ghosh (1985) and Mohammed et al. (1993).

The X-organ sinus gland complex is the most thoroughly investigated part in crustacean endocrinology, as it is the center of many of the inhibitory hormonal factors, especially the gonad inhibitory factors (Fingerman, 1987). Similarly sinus gland is the principal neurohaemal organ involved in the storage and release of neurosecretory materials serving several endocrine functions (Gabe, 1956; Kleinholz, 1976; Highnam and Hill, 1977; Fingerman, 1987). The ultrastructure of the neurosecretory granules stored in the sinus gland of various crustaceans have been studied by many

(Fingerman and Aoto, 1959; Knowles, 1959; Meusy, 1968; Andrew et al., 1971; Smith, 1974; Brodie and Halerow, 1977; Martin et al., 1983). However, such studies in penaeids are scanty. Mohammed et al. (1993) described the light microscopy of the sinus gland but there is no such studies in penaeids through electron-microscopy. Similarly many of the above mentioned investigators have studied the various types of neurosecretory granules in the sinus gland but there are no report showing the types of neurosecretory granules during any of the particular physiological activities.

Similarly, endocrine control of reproduction has been investigated in a wide variety of crustaceans. In this respect amphipods and isopods have received considerable attention. However, the actual mechanism and hormones working behind reproduction have not been revealed in any other crustaceans (Fingerman, 1987). It has been attributed to the eyestalk factors by many (Aoto and Nishida, 1956; Demeusy, 1967; Rangnekar and Deshmukh, 1968; Diwan and Nagabhushanam, 1974; Laubier, 1978; Arnstein and Beard, 1975; Webb, 1977; Quackenbush and Herrkind, 1981; Eastman-Recks and Fingerman, 1984; Radhakrishnan and Vijayakumaran, (1984); Kulkarni et al., 1984; Anilkumar and Adiyodi, 1985; Fingerman, 1987; Quackenbush and Keeley, 1987; Mohammed and Diwan, 1991; Van Herp, 1992) after Panouse (1943) in Leander serratus.

A second decapod reproductive hormone found in the brain and thoracic ganglia has been attributed the role of gonad stimulation. 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) has reported that the thoracic ganglia secretes the GSH in decapods. However, Gomez (1965); Gomez and Nayar (1965), Diwan and Nagabhushanam (1974); Nagabhushanam et al. (1982), and Yano and Hyban (1992) reported that besides the thoracic ganglia, the brain also secretes an ovarian growth accelerating hormone in crustaceans. In this

respect cytological and ultrastructural studies of various endocrine organs during the process of gonadal maturation can give some concrete evidences about which organ is more active and what type of factors are produced etc. However, unfortunately such studies are over looked by various investigators and most of them have concentrated in the isolation of neurosecretory factors. From the foregoing literature it is obvious that very fragmentary information is available in this regard for various crustaceans, especially in marine penaeid prawns. Mohammed and Diwan (1991) figured out the light microscopical features of the various ganglionic centers of a penaeid prawn P. indicus during the process of gonadal maturation. Still there is no published information on such studies describing the characters of various NSCs engaged in the reproduction through electronmicroscopy. Hence, the present study is designed to investigate the cytological aspects of various neuroendocrine centers during the process of gonadal maturation. Different types of NSCs in various ganglionic centers of P. monodon were identified through light microscopy and its functional details were studed through electron-microscopy during the process of gonadal maturation. Ultrastructural features of the different phases of secretory cycle of these NSCs have also been studied to get a clear picture of the process of neurosecretion. The mapping of neurosecretory cells in different ganglionic masses of P. monodon has been also carried out. A thorough investigation of the ultrastructural features of the eyestalk neurosecretory system including the sinus gland, has been made in relation to reproduction during the present investigation.

## MATERIALS AND METHODS

### I. Collection of animals and preparation of tissues for hislological studies

Wild caught prawns P. monodon, ranging in size from 190 to 230 mm in total length, in various maturity stages were used in the present study. Prawns were captured from deeper offshore areas of Cochin and Madras. The prawns collected, and kept in 1 tonne capacity fibre glass tanks, containing seawater provided with a sub-gravel biological filter working on an air lift mechanism. All prawns were fed daily with fresh clam meat, and maintained carefully till their sacrifice for further studies.

Prawns of different maturity stages were selected. To study the morphology, histoloy, and electron-microscopical details of the neuroendocrine system during the process of gonadal maturation, the different neuroendocrine organs (various ganglionic centers) in the central nervous system and the eyestalks as well as the ovary, were excised from live prawns. Tissues like optic, cerebral, tritocerebral, thoracic and abdominal ganglia and the corresponding ovary were excised separately and fixed in appropriate fixatives for light and electron-microscopic studies. Dissections were carried out in crustacean saline for keeping the tissues in live conditions. Gross morphological studies were made using a sterioscopic dissection microscope. Different maturity stages were identified based on the morphological features of the gonads, like gonadal development and colour of the gonads.

### I. Light microscopical studies

Freshly excised tissues were immediately transferred to Bouin's fixative for a period of 24 to 48 hrs before further processing. Properly fixed tissues were washed over night in running tap water to remove the excess

picric acid. Washed tissues were later dehydrated through an ascending grades of ethanol series (30 to 100%) and cleared in chloroform. Dehydrated tissues were put for the cold impregnation in a wax chloroform mixture (1:1 ratio) for one day. Subsequently the tissues were transferred to molten wax (Parrafin wax with cerresin, Merck, MP. 58 to 60°C) for hot impregnation. After 2 more changes of duration, 15 minutes in fresh frozen wax, tissue blocks were prepared using L-blocks made of Brass.

Serial sections of the tissue blocks were taken at an approximate thickness of 5 to 7 u using a rotary microtome. Sections were affixed on clean glass slides using fresh Meyer's egg albumen and flattened by placing on slide warmer with few drops of distilled water. Subsequently the water was drained off and the slides were allowed to dry. These slides were then used for further histological investigations.

Routine staining was carried out for the gross morphological studies, using Mallory's triple. Mallory's triple staining of the neurosecretory material is based on the affinity of these stains for the acidic group. Sections to be stained were first deparaffinized in two changes of xylene and then dehydrated through a down series of ethanol grades. For Mallory's triple, a primary mordant (Hgcl2- acetic acid) was used prior to the fuchsin staining, and then sections were stained with Mallory's Triple stain (Mallory, 1944). Stained slides were again dehydrated in two changes of absolute alcohol and cleared in xylene before mounting in DPX mountant. DPX mounted slides were allowed to dry and after drying observations were made under a monocular microscope. Structural details of the neurosecretory cells and their changes during different phases of the neurosecretory cycle as well as during the process of gonadal maturation, were studied. Micrometric measurements of the various types of neurosecretory

ells at different phases of neurosecretion were taken using an occular icrometer calibrated with a stage micrometer. As the neurosecretory cells rongly deviate from a spherical shape, the largest and the smallest axis f the cell was taken and the average was used as the actual cell diameter.

Photomicrographs of histological investigations of the neuroendocrine ells were taken using an Olympus Universal Research Microscope (Vannox odel PM 10 AD) equipped with an automatic exposure system. Black and white 5 mm film (Ilford, 125 ISO) was used for photography.

## II. Ultrastructural studies

Various ganglionic centers and gonadal tissues excised at different aturity stages were used for ultrastructural studies. Initially small ieces of ganglionic centers and the corresponding gonadal tissues were bathed n cold fixative (4°C) by pipetting fixative, directly on to the tissue n situ. The tissues were then removed from the abdomen and placed in resh primary fixative which is 4% gluteraldehyde, buffered with 0.2M acodylate buffer containing 2% tannic acid and 6% glucose at pH 7.2 for 1.5 hrs at 4°C. After primary fixation tissues were washed 3 times in acodylate buffer, each about 15 min. duration and post fixed in 1% buffered mium tetroxide for about 1 hr. at 4°C. Properly fixed tissues again washed cacodylate buffer and then dehydrated in a graded acetone series. The ssues were infiltrated and embedded in low viscosity resin embedding medium purr, 1969) and kept at 60°C for 36 hrs.

After proper trimming semithin sections (0.5 to 1.0 u) were cut from e polymerized blocks and stained with toludine blue and observed under ght microscope in order to identify the interested area. These blocks re again trimmed for ultramicrotomy, and ultrathin sections were cut with

a diamond knife on an ultramicrotome "Ultracut E". Ultra-thin sections of 300 - 600 A° collected on uncoated copper grids (300 mesh), were post stained in 10% uranyl acetate in methanol (Watson, 1968) and lead citrate (Reynold, 1963). After drying, the grids were examined with a "Phillips CM 10" transmission electron-microscope at 60 KV. Ultrastructural details of the neurosecretory cells as well as the corresponding gonadal tissues were examined critically and interested areas were photographed using Kodak-100 ASA film.

## RESULTS

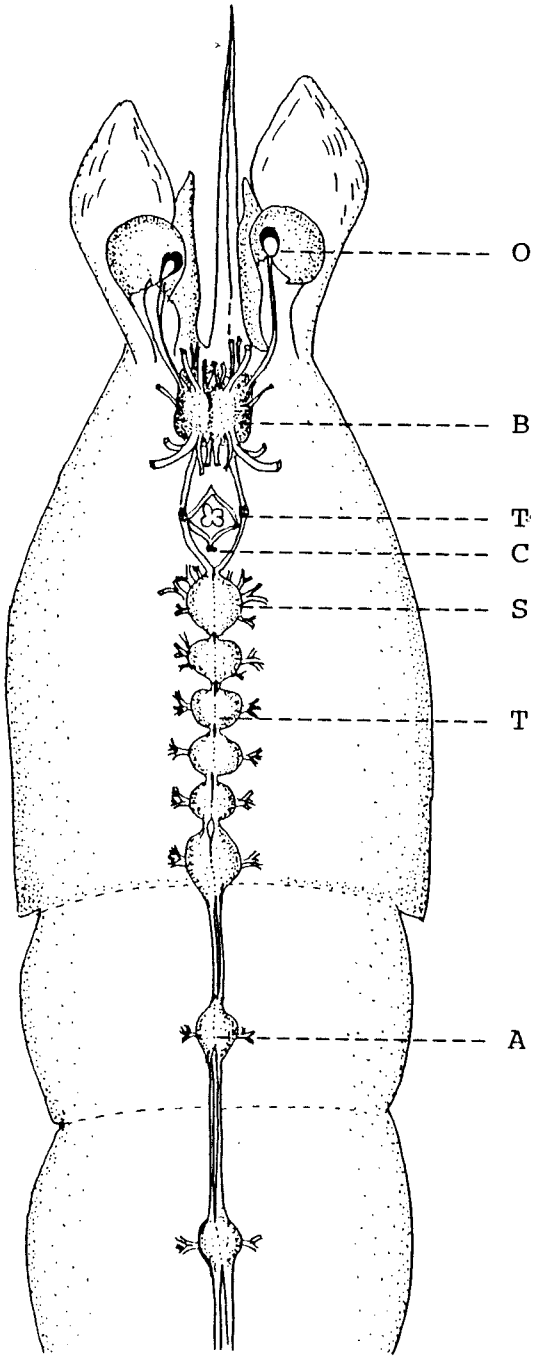
The long double ventral nerve cord and the paired ganglia corresponding to each segment are the characteristic features of the crustacean dendrobranchiate central nervous system. Typical of annulata, the central nervous system of Penaeus monodon comprises of a dorsal brain connected to the ganglionated ventral nerve cord below the gut by two large tracts so that the gut passes between these tracts. The dorsal brain or supraoesophageal ganglion, is found to be located within the head lobe in the dorsal part of the protocephalon, protected dorsally by the broadening base of the rostrum. It receives nerves from the sense organs of the head and supplies nerves to the adjacent muscles. The supraoesophageal ganglion consisted of three primary lobes; the protocerebrum, deuterocerebrum and tritocerebrum. From the dorsal surface of the brain ie from the protocerebrum arises a pair of stout optic nerves, which ends in the optic ganglia. The medulla interna and medulla terminalis of the optic ganglia are derived from the protocerebral part of the brain. Deuterocerebrum was the middle part of brain and this supplied nerves to the antennular, antennal appendages and statocyst. While the third part, the tritocerebrum was the postero-ventral part of the brain and supplies nerves to the labrum, the preoral stomato-gastric system and the post oral tritocerebral commisure.

It was found that the brain or supra oesophageal ganglion was linked to the ventral nerve cord by two connectives, the tritocerebral connectives that passes around the oesophagus. Midway through the tritocerebral connectives are the pair of tritocerebral ganglia. The tritocerebral connectives arising from the brain meet ventral to the oesophagus, in a large suboesophageal ganglion on the second thoracic segment. The first ganglion of the ventral nerve cord, the sub-oesophageal ganglion, together with the

following metameric ventral ganglia, receive impulses from sensory end organs of the body and appendages and sends motor impulses to the muscles moving these structures. Present investigation revealed that like the brain, the sub-oesophageal ganglion of P. monodon is a compound of several major ganglia supplying nerves to mouth parts like mandibles, maxillules, and maxillae.

The ventral nerve cord of P. monodon was the fusion product of a "ladder" nervous system where the paired ganglia of each segment have come together at the midline. It contained a longitudinal series of ganglia, inter connected by fused pairs of intersegmental nerve tracts. It was found that this ventral nerve cord continued posteriorly from the sub-oesophageal ganglion to the five thoracic ganglia each corresponding to the five thoracic segments. Even though these thoracic ganglia are found to be fused across the mid-line, they were longitudinally separated from one another by short paired connectives. The thoracic ganglia are found from the 3rd segment onwards, and the 1st thoracic ganglion in the ventral nerve cord supply nerves to the three maxillipedes. The next three thoracic ganglions supplies nerves to each of the 3 pereiopods. The 5th thoracic ganglia in the seventh and eighth segments were found fused together and it supplied nerves to the 4th and 5th pereiopods.

The ventral nerve cord narrowed as it entered the abdomen, where there were six abdominal ganglia. Anterior five abdominal ganglia were found to be identical but the last one was some what enlarged. These abdominal ganglia are also found fused across the midline like thoracic ganglia. The connectives that joins the abdominal ganglia are found to be comparatively longer than that of the thoracic ganglia. The different ganglionic centers identified in P. monodon on the basis of the present investigation, and their relative position in the nervous system are depicted in fig (97).




97 Diagramatic representation of the neuroendocrine system of P. monodon

O - optic ganglia, B -brain (supra oesophageal ganglion) S - suboesophageal ganglion, Tr - tritocerebral ganglion, T - thoracic ganglion, A - abdominal ganglion, C - circumoesophgeal commissure

## A. Neurosecretory cells (NSCs) and their secretory cycle

### A.1 Neurosecretory cell types - Ultrastructure

Light microscopical investigations in the ventral nerve cord of P. monodon revealed different types of neurosecretory cells. From the cross sections of the ganglia it was found that neurosecretory cells are aggregated only in the ganglionic masses and not in the nerve cord. Nerve cord contained only axons of nerve fibers. In order to classify the neurosecretory cells in P. monodon, serial sections of all the ganglionic masses were taken and stained with Mallory's triple stain to distinguish the cells clearly. NSCs were found in groups towards the periphery of each ganglionic masses. NSCSs were characterized by the presence of large, centrally located, weakly stained, spherical nucleus, abundant cytoplasm and conspicuously staining granules in their perikarya. Distinct cyclic changes were noticed in their granular cytoplasm during different phases of maturation of the animal. Along with these NSCs in each ganglia some other non-neurosecretory cells were also detected in abundance. They were structurally different from the neurosecretory cells. They were tiny, deep stained cells where as, neurosecretory cells were larger and not so deeply stained.

Histological investigation revealed that among the NSCs there were differences in size, general shape of the cell body, presence or absence of vacuoles in cytoplasm and in the appearance of their secretory product. Based on these characters the NSCs found in different ganglions of P. monodon were classified arbitrarily in to 4 morphological types viz Giant neurons (GN) (fig. 98), A cells (fig. 100), B cells (102), and C cells (fig. 104).

Ultrastructural examinations revealed that all the ganglionic masses were richly endowed with neurons and NSC groups comprised of heterogenous cells. Ultrastructure of these cells showed a comparatively large electron

dense nucleus and a less electron-dense but active cytoplasm. The cytoplasm contained abundant supplies of cell orgenelles like stacks of RER, large accumulations of ovoid granules and electron lucent vesicles.

**a. Cell type I Giant neurons (GN- cells)**

Giant neurons as indicated by its name are the largest of the NSC observed in P. monodon. Generally cell bodies of these GN cells were large and their shape varied from oval to spherical (fig. 98). It was found that most of the GN cells were unipolar with a short axon (fig. 98). Pericellular capillary plexus were also noticed around each GN cells. Glial cell nuclei were found distributed in limited numbers in all the ganglia except the optic ganglia. Size of these large cell bodies varied between 110 to 154 in diameter and possessed much cytoplasm and a large nucleus of 28 to 44 in diameter. From the present investigation it was found that the large nuclei of the GN cells sometimes differed considerably from the round nuclei of other cells (fig. 99). The modified large nuclei appeared as flat, oval crescent shaped in different cells (fig. 99). The nucleus often contained more number of nucleoli, the number varied between 8 to 14. These nucleoli were found dispersed in the chromatin material usually towards the peripheral region lying against the nuclear membrane (fig. 98). Even though there were minor differences in the size and shape among GN cells, all of them were rich in cytoplasm with granular materials around the nucleus. Further, in all of the GN cells showed the presence of large amount of secretory materials at all times. In certain stages it was found that a very thin rim of cytoplasm encompasses the large nucleus in these cells. Occurrence of small vacuoles in the cytoplasm was a noticeable feature in these cells on certain occasions. Round, oval or horse-shoe shaped vacuoles of varying

sizes occurred in the perikarya of GN cells with reduced amount of cytoplasm. In these cells, cytoplasm was not granular in nature due to the absence of secretory granules.

Ultrastructurally it was observed that the GN cells were richly endowed with abundance of highly active cell orgenelles and secretory granules in their perikaryon (fig. 99). Variably shaped large central nucleus were provided with numerous gaps or nuclear pores (fig 99). The electron dense cytoplasm around the nucleus contained stacks of rough endoplasmic reticulum (RER), Golgi complexus, electron-dense Golgi derived granules and glycogen particles associated with electron-lucent vesicles made of RER (fig. 99,115). These large and highly electron-dense secretory granules appeared as round or spherical, homogenous and bound by a thin limiting membrane. Most of the granules were found dispersed throughout the cytoplasm. Free and poly ribosomes and abundant oval to spherical mitochondria were noticed in the cytoplasm (fig. 117). Multivesicular bodies and inclusion bodies containing flocculent materials were also encountered.

The RER consisted of flattened cisternae running parallel to each other as well as oval to polygonal granule filled electron lucent vesicles (fig. 99,115). The intra-cisternal space of the parallel RER was slightly electron dense owing to the presence of some flocculent material. Highly active large Golgi complex were found in the cytoplasm in association with these RER (fig. 117). In the well developed Golgi complex the cisternae were occasionally enlarged at the ends and contained a moderately dense material, apparently giving rise to the elementary granule (fig. 116). Highly electron-dense large round or spherical secretory granules as well as variably shaped vesicular bodies were found in the perikayon of these cells. The perikarya of these GN cells were delimited by a capsular glial like system of 5-6 paired membranes. These cells possessed glial cell nuclei in between these layers.

**b. Cell type II A- cells**

A cells were much smaller than GN cells (fig. 100) and were found in all the ganglia examined. Even though these neurosecretory cells were observed in all ganglia examined, their occurrence was limited especially in optic and abdominal ganglia. All the other ganglia contained comparatively more number of A cells. Light microscopical examinations revealed that these cells possessed an average cell diameter of 79.5 ± 7.36 μ. The centrally placed nucleus was spherical or oval with a nuclear diameter ranging between 18.5 to 27.8 μ. The vesicular nucleus contained a small nucleolus. The cytoplasm was more or less homogeneously distributed with secretory inclusions. Some of these cells occurred with axonal process but most of the A cells were without long axons, like GN cells.

The large central nucleus and the characteristic, clumped distribution of the nuclear pores within the nuclear envelope are evident in these cells. The perikarya of most of the A cells were highly active with abundant cell organelles. RER in these cells were observed as narrow elongated cisternae aligned parallely in stacks of up to 8 cisternae or as randomly oriented lamified cisternae (fig. 121). Large Golgi complex also occurred in association with those RER elements (fig.124). Active mitochondria were found to occur in groups in the perikarya of the A cells. Their profiles were rounded or ovoid and the cristae were distinct and rather straight (fig. 121). Free and poly-ribosomes were apparent in association with these mitochondrial bodies. Multivesicular bodies and inclusion bodies containing flocculent to dense material were also occasionally found in the cytoplasm (fig. 125). The perikarya of these cells were delimited by the capsular system found in the GN cells. Here the limiting membrane is double layered with glial cell nuclei located in between these layers.

**c. Cell Type III B- cells**

These medium sized cells were apparent in large numbers in all the ganglia examined. Their shape varied from oval to polygonal with an average cell diameter of 39.69 ± 6.12 μ. From the light microscopical investigations it was noted that these medium sized cells were always found in groups of 10 to 25 cells (fig. 102). The nuclear diameter varied between 10 to 20 μ. Nucleolus was found near the nuclear membranes. Axonal processes were not distinct in all cells but during certain stages, small axonal processes were feebly visible. Generally B cells showed only little sign of secretory activity differing from the other GN and A cells. Their cytoplasm is fairly homogenous and of compact appearance. The fine cytoplasmic inclusions are sparcely distributed within the perikarya. Occurrence of peripheral vacuoles lacking secretory inclusions were also encountered. Capillary plexus and glial cells were apparent in their pericellular regions during certain times.

Ultrastructural studies revealed that these oval to polygonal cells possessed a large nucleus (fig. 101). It was observed that perikarya of most of the B cells was electron lucent and devoid of many cell orgenelles (fig. 127). The fine structure of these perikarya was appreciably different from that of the larger GN and A cells. Large concentrically arranged membraneous system was encountered in the perikarya B cells, apart from the usual RER system on certain occasions. Numerous round to oval motochondria occurred in the perikaryon but their cristae were not distinct (fig. 135).

**d. Cell type IV C- cells**

C type cells were the smallest NSCs observed, and were found in very limited numbers in all the ganglia examined. These cells were usually found in groups in association with the B cells. These small cells were

characterized by their conical or elliptical shape tapering in narrow axons. The size range of these cells vary from 12 to 24 $\mu$ with an average diameter of 18.6 ± 3.5 $\mu$. The centrally placed round nuclei contained conspicuous intra-nucleolar materials. The nucleus cytoplasm ratio was high due to the lesser amount of cytoplasm. Occurence of peripheral vacuoles were noticed in these C cells. Here also, it was found that the cells were limited by the neuroglia and the capillary net works.

Electron-microscopical studies revealed that the fine structure of the perikarya of these C cells were appreciably different from that of the other larger cell bodies. The nucleus of these neurosecretory cells were characterized by a conical to polygonal shape fig. 105). This centrally placed nucleus contained highly electron-dense chromatin materials. Nuclear pores were not so evident as in the other cells. The electron-lucent perikaryon of these cells were devoid of secretory granules or cell orgenelle (fig. 105). The relatively abundant RER had an electron-lucent narrow lumen but the slender parallel cisternae was slightly more electron-dense than the remaining cytoplasm due to the presence of a flocculent material (fig. 130). Vacuoles of varying size and shape were also found here and there in the cytoplasm. Highly electron-dense small granular materials were observed in certain maturing C cells indicating its secretory activity (fig. 155).

**. Non-neurosecretory cells**

Existence of a few clusters of unique tiny, deep stained easily dentifiable neurons were found freely and in association with the afore aid cell types in all the ganglia examined. Most of these non-neurosecretory ells were round in shape but varied occasionally (fig. 106). The size range these cells varied between 8.4 to 14.5 $\mu$. The disproportionately large

nucleus were round and had an average diameter of 10.5 μ. This nuclei did not take up any stains but contained a large deeply stained nucleoli. Ultrastructural observations revealed that these cells contained a large nucleus and a thin rim of cytoplasm. Neurosecretory materials were not found in the cytoplasm of these cells. The limiting membrane of these cells were not distinguishable. Details of the neurosecretory and the non-neurosecretory cells were presented in the table 2.

| Cell type | Cell-diameter (μ) | Nucleus-diameter (μ) | No. of nucleoli |
|---|---|---|---|
| GN cell | 130.12 ± 12.55 | 35.65 ± 3.95 | 8-14 |
| A cell | 79.50 ± 7.36 | 22.20 ± 5.17 | 1 |
| B cell | 39.69 ± 6.12 | 16.90 ± 2.90 | 1 |
| C cell | 18.60 ± 3.50 | 10.10 ± 2.20 | 1 |

Table 2. Details of different nerusecretory cell types

## 4.2 Neurosecretory cells and their secretory cycle

Light microscopical as well as ultrastructural studies revealed, that there are cyclic changes in the perikarya of the NSCs in concurrence with the synthesis of the neurosecretory materials. Different phases were identified during the secretory cycle, on the basis of the cytoplasmic changes and appearance of the secretory products in their perikaryon. The identifying characters of the NSCs are the presence of large amounts of cytoplasm and the abundant supply of secretory granules in their perikaryon. However, it was found that these characters were not fixed, but changing

cyclically during the secretory cycle. Light microscopical studies showed distinguishable differences in the amount of secretory materials present in different groups of NSCs. Further it was observed that some of these cells were at the peak of their secretory activity while some others were still at their resting stage.

Based on all these observations, the secretory cycle of these NSCs were classified into 3 different phases viz. quiescent, vacuolar and secretory. These three phases of the secretory cycle were found to be common for all the four types of NSCs. Generally the appearance of the cell body, presence or absence of vacuoles in the cytoplasm and the appearance of secretory products in the cytoplasm etc were used as the main criteria in separating different stages of the neurosecretory cycle. From the present investigation it was found that even though there were distinguishable differences in the morphological nature of the secretory granules, there was a basic pattern in their mode of secretion and discharge pattern, in different NSCs.

**a. Quiescent phase (Q- phase)**

This formed the inactive or resting phase of NSCs. The NSCs in their quiescent phase contained only a thin rim of homogenous lightly staining cytoplasm. But these cells were characterized by a large round, easily distinguishable nucleus filling almost the entire cell volume. The limiting membrane of the nucleus as well as the cytoplasm of these resting cells were very prominent compared to that of the other phases (figs. 108-113) The nucleus of the GN and A cells contained highly distinguishable scattered nucleoli (fig. 108,110). While B and C cells possessed deeply stained clumped nucleolus in the centre of the karyoplasm (fig. 112). A few glial cells

were also found surrounding the outer margin of these resting cells (fig. 110,112). Capillary plexes were also encountered infrequently (fig. 110). Axonal filaments of these quiescent cells were found devoid of any flocculent cytoplasmic materials (fig. 108).

Ultrastructurally, the perikarya of the resting NSCs were found smooth and electron-lucent with minimal cytoplasmic activity. It was found that the large electron-dense nucleus occupied almost the entire cellular volume (fig. 109,111,113). The characteristic clumped distribution of the nuclear pores within the nuclear envelop was not evident in these resting cells. Here the nuclear envelop was found continuous and smooth without any interruptions (fig. 111,113). The electron-lucent perikaryon of these NSCs contained only a minimum number of cell orgenelles. The most dominant type of cell organelle found in the perikaryon of these quiescent phase NSCs was the rough and smooth endoplasmic reticular elements. Smooth ER was found either as randomly oriented ramified cisternae or as narrow elongated cisternae aligned parallely in stacks of up to 15 cisternae. These narrow elongated parallel cisternae were found dominated. Small parallel stacks of Golgi complex were also found in association with these endoplasmic reticular elements. Some free and poly-ribosomes and oval to spherical mitochondria were also encountered in the electron-lucent perikarya. A few numbers of electron-lucent multivesicular bodies and inclusion bodies were found along the outer perikaryon. Electron-lucent and moderately electron-dense vacuoles and/or vesicles were less abundant. Ultimately it was found that the electron-lucent perikaryon of these quiescent phase neurosecretory cells were not yet triggered for any type of secretory activity as indicated by its less abundant supply of cell orgenelles.

**b. Vacuolar phase (V- phase).**

As its name indicates, NSCs of this particular phase were characterized by the presence of numerous vacuoles (fig. 114,120). Irrespective of its size and shape all the NSC types possessed vacuoles of different size and shape during its V-phase of the secretory cycle. Even though there were minor differences, the vacuoles of all the NSC types possessed certain basic characters. These vacuoles were usually originated as small round membrane bound bodies in the cytoplasm and later towards the final stages these vacuoles undergo some structural changes and get transformed into larger vacuoles of various size and shape in different cell types.

Histologically, it was observed that GN and A cells possessed variably shaped larger vacuoles (fig. 114,121). Most of the times, these expanded vacuoles were observed to be almost free of inclusions except for a few fuchisinophilic granules. These vacuoles were rather larger and measured up to 42 $\mu$ in size and most of the time found around the periphery of the cell, although some times it was located more centrally in the cytoplasm. These largely expanded oval to horse-shoe shaped vacuoles were usually found empty but occasionally a lightly staining material was encountered in some of these vacuoles. The cytoplasm around these vacuoles was flaky in appearance due to the presence of small secretory granules.

The nucleus of these vacuolar phase NSCs contained rather diffused chromatin materials. The limiting membranes of the nucleus as well the cytoplasm was not so clearly detectable as in the case of resting cells (fig. 114,120). The glial cell nuclei around these neurosecretory cells exhibited hyper activity during this particular stage (fig. 120). The entire cell volume was found to be encompassed by these active glial cells.

Where as B and C cells possessed rather small, uniform round vacuoles (fig. 126). These vacuoles were not sharply delimited from the cytoplasm. The diameter of these smaller vacuoles varied between 8 to 12μ. These numerous small vacuoles gave a coarse appearance to the cytoplasm. Vacuoles in these cells were not restricted to a particular position but observed throughout the entire cytoplasm. Along with these lightly stained vacuoles the cytoplasm contained some darkly stained secretory granules (fig. 126). These granules were observed apparently at random, throughout the cytoplasm. There also the limiting membranes of the nucleus as well as the cytoplasm was not distinct as in the case of resting cells. Clumped nucleolar materials were found at the central part of the nucleus. The hypertrophied glial nuclei along with some capillary plexes were observed around the pericellular margin of these cells.

In sharp contrast to the resting condition, ultrastructural features of the NSCs at the vacuolar phase exhibited conspicuous structural changes that are indicative of highly active condition of these cells during this phase. These V-phase NSCs possessed a highly active nucleus and cytoplasm. The large nucleus with its electron-dense nucleolar materials were located at the centre of these NSCs. The characteristic distribution of the nuclear pores within the nuclear envelope were particularly evident in these cells (fig. 121). Streaming of small granular materials from the nucleus to the perinucleolar cytoplasm was apparent through these nuclear pores. Numerous vesicles packed with granules of dense nature were also apparent in the cytoplasm around the nucleus (fig. 115,121,127). The perikarya around this active nucleus contained numerous highly active organelles (fig. 116,125). This perikarya possessed active subcellular organelles like rough endoplasmic reticulum (fig. 115), large Golgi complex (fig. 117), round to oval

itochondria with distorted cristae (fig. 119,124) large numbers of moderately ectron-dense multivesicular bodies with developing secretory granules (fig. 8) and various types of lysosomal structures (fig. 122).

Numerous rough and smooth endoplasmic reticular elements were apparent ar the nuclear as well as plasma membrane of these vacuolar phase NSCs. ese ER were observed in different morphological forms like short cisternae less dilations, randomly oriented ramified cisternae or parallely arranged acks of cisternae (fig. 121). Parallely arranged stacks of cisternae re found usually around the nuclear membrane, where as ramified cisternae re more apparent in close apposition to the outer plasma membrane. Active R in the form of short cisternae were also found through out the erikaryon. Small electron-lucent vesicles were found budded off from these R elements (fig. 125).

Large Golgi complex were found in association with these stacked R elements and also distributed randomly throughout the cytoplasm (fig. 6,117). These Golgi complex were found either in the form of 'C-shaped' ofiles or in the form of ramified cisternae comprised of 4-5 saccules (fig. 7). It was found that the Golgi complex produced small vesicles of 2 pes, a frequently encountered one, which is often irregular and electron ucent located around the lateral margins of the Golgi saccules and casionally encountered vesicles exhibiting an electron dense matrix with diffuse coating, associated with the trans or mature face of Golgi complex. ese vacuoles were observed mainly in association with the Golgi complex ith 'C-shaped' profiles. Where as the saccules of Golgi complex associated ith the ramified cisternae were found filled with highly elecrron-dense terials (fig. 116). The central Golgi region contained various electron ucent vesicular and tubular structures.

Large numbers of active mitochondia were apparent throughout the cytoplasm. These mitochondrial bodies were observed in groups and scattered in the dense cytoplasm of the vacuolar phase NSCs (Fig. 118,124). Their profiles vary from round or ovoid to irregular and their cristae were distinct and rather straight. Matrix of the mitochondria possessed some small irregularly shaped electron dense deposits. Rough endoplasmic reticulum bound vacuoles of variable sizes were found in abundance in the perikaryon of these neurosecretory cells (fig. 125). Their profiles were usually rounded or ovoid and these electron lucent bodies were found empty during most of the times. Apart from these empty vacuoles, single vesicular structures and multivesicular bodies were found in moderate numbers in the perikaryon of these vacuolar phase NSCs (fig. 128). Different types of vesicular bodies encountered in the cytoplasm, round to oval membrane bound vesicles packed with moderately electron-dense granules occurred in close proximity to the nucleus and a moderate number of clear vesicles occurred rather dispersed through out the cytoplasm.

Another important cell organelle encountered in these NSCs were the variably shaped lysosomal bodies. Lysosomes containing secretory granules were also present in very few numbers (fig. 128). Free and poly-ribosomes were also abound through out the active dense cytoplasm. The glial cells encompassing these NSCs were also very active during this vacuolar phase (fig. 123) and these cells contained active nuclei and highly active cell organelles. Abundant supply of small cisterns of RER was found in their cytoplasm. Other cell organelles found in the cytoplasm are the vesicular bodies and inclusion bodies with flocculent to dense materials. As a whole, remarkable increase in the synthetic activity was found during this vacuolar phase of the NSCs compared to the previous resting phase. However, there

was a paucity of neurosecretory material or membrane limited neurosecretory granules within the cytoplasm, apparently indicating that the sub-cellular processes have not yet been completed.

**c. Secretory phase**

Irrespective of the cellular dimensions or cell types all the NSCs in this particular phase were rich in darkly stained granular cytoplasm (fig. 132,146). NSCs in the secretory phases were found apparently ready for the release of its secretory products (fig. 132,138,144). In contrast to its previous stages these cells stained darkly due to the presence of secretory products. All the cell types showed an increase in size due to the accumulation of secretory granules in their cytoplasm.

It was found that the large vacuoles found in the GN and A cells underwent some morphological changes, when the cell enters the secretory phase. Instead of variably shaped vacuoles of the preceding stages, small round vacuoles were found in this stage (fig. 132,138). Moreover most of these vacuoles contained small secretory granules. These small sized vacuoles were gradually shifted to the outer cytoplasm from their previous perinuclear position (fig. 132,138). Due to the extremely granular nature in the outer cytoplasm, the cell membrane had a broken appearance. The cytoplasm in the perinuclear area became highly granular and deeply stained.

During the secretory cycle, in addition to the changes apparent in the cytoplasm, the nucleus also exhibited some concomitant changes in its size and morphological appearance (fig. 132,138). The clearly round profile of the nucleus became irregular and broken and small granules made its appearance in the vicinity of the modified nuclei. Streaming of granules from the nucleus was apparent during this S-phase (fig. 132). At the later stages

of the secretory cycle the round nucleus became oval to crescent shaped and then expanded, and rapidly regained its round shape during its next resting or quisecent phase. The axonal processes of these cells also became more prominent during this phase. Granules of similar nature, observed in the cytoplasm were found to some extent in the axons of these cells (fig. 132). Streaming of secretory granules towards the axonal process was apparent in these cell bodies. Randomly distributed hypertrophied glial cell nuclei (2-3 u) were particularly evident in these secretory cells. Blood capillaries were found at the outer margin of these cells (fig. 132).

Cytoplasm of B and C cells exhibited similar pattern of secretory activity as observed in the GN and A cells, but in a lesser extent. Peak secretory activity in these cells were evident from the deeply stained secretory granules observed throughout the cytoplasm (fig. 144). These B & C cells at their secretory phase were found thickly packed with their granular secretory materials.

Light microscopical observations revealed that the scattered vacuoles apparent throughout the cytoplasm during the preceding vacuolar phase showed considerable reduction in their size during this stage (fig. 144). These smaller vacuoles were found shifted towards the outer cytoplasm and the entire cell volume got filled with these deeply stained secretory granules (fig. 144). Small deeply stained secretory granules were encountered in most of these round to oval vacuoles. Aggregations of small secretory granules were noticed on the nuclear membranes of the centrally located nucleus and perinucleolar area (fig. 144). Where as the deeply stained secretory granules inter spaced by small vacuoles containing granular materials gave a coarse appearance to the cytoplasm (fig. 146). The hypertrophied glial cells and capillary net works were not prominent around the B & C type cells as in the case of GN and A cells.

Ultrastructural features of these secretory phase NSCs were conspicuously different from that observed in the perikarya of the previous stages. From the observations it was found that the distribution of cellular organelles were limited in the S- phase cell's than the vacuolar phase cells however, greater quantity of various secretory granules were apparent in the perikarya of these secretory phase NSCs (fig. 133-143). A marked increase in the number of highly electron dense secretory granules were noted in all the NSC types. The most noticeable increase in the activity of subcellular synthetic processes was registered in the perikarya of GN & A cells (fig. 133-143). However, the abundant supply of various kinds of secretory granules in the NSC types during their secretory phase apparently indicated the heightened subcellular synthetic activity of these cells. Irrespective of the cell types, all the NSCs during their secretory phase possessed RER (fig. 137-142), highly active Golgi complex (fig. 136), mitochondria (fig. 137-141), lysosomes (fig. 134-135) etc in addition to their electron dense secretory granules and developing vesicles (fig. 137-143).

The rough endoplasmic reticulum in the form of short dilated cisternae dominated over other morphological forms which include flattened, parallely arranged elongated cisternae (fig. 136) and concentrically arranged membraneous whorl of elements (fig. 133). The process of granule synthesis appeared to commence in the cisternae of rough endoplasmic reticulum, which contained a flocculent material. Numerous, small subspherical to oval vesicular bodies were found in the cytoplasm in association with the RER (fig. 142). Similar type of small secretory granules observed in the inner side of the cisternae of RER were also found in these vesicles as it matures (fig. 142). These moderately electron dense vesicles, packed with

secretory granules and derived from RER elements, were abundant in the perikaryon especially around the RER and active Golgi elements of this neurosecretory cells. During the next stage these vesicular bodies with moderate electron density observed near the Golgi complex were found fused with the outer most Golgi saccule. It was observed that the outer Golgi saccules were generally dilated and contained granules of low and moderate electron density (fig. 136). Size of these granules found decreased towards the maturing or trans-face, however, the electron density of their contents were found to be increased. Accumulations of finely granular, electron-dense materials were apparent within the saccules especially at the cis and trans saccules of these Golgi complex (fig. 136). Closer examinations revealed that, after the passage of these precursor granules through the Golgi complex, the mature neurosecretory granules with their electron dense contents were budded off from the margins of the trans-most saccules (fig. 136). It was again noted that on occasions, the entire trans-most saccule appeared to separate from the Golgi complex and subsequently get divided into spherical membrane bounded, electron dense neurosecretory granules in the cytoplasm. In addition to these highly electron dense granules, the Golgi complex in these activated perikarya produced large numbers of coated vesicles (fig. 136). These coated vesicles contained an electron-lucent materials and were most frequently but not exclusively associated with the trans-most saccule of the Golgi complex which were not at all incorporated in the production of electron dense neurosecretory granules.

In the process of secretory granule formation three consecutive morphological stages were identified on the basis of their electron density, shape and their relative position. The stages include trans, ventral and peripheral granules. Trans-granules were the newly formed granules and they

ere found still connected to the Golgi apparatus (fig. 136). These trans cretory granules showed the same high electron density as the dense material the Golgi apparatus. Whereas the second stage or central secretory anules had been already budded off from the Golgi apparatus and occurred some distance from the trans-face of the Golgi apparatus (fig. 136). ese central granules were slightly more electron-dense and smaller than e trans-secretory granules. Thirdly, the peripheral secretory granules d left the Golgi apparatus completely and found some where in the perikarya. ese secretory granules exhibited the same electron density but the were re regular and spherical in morphological appearance.

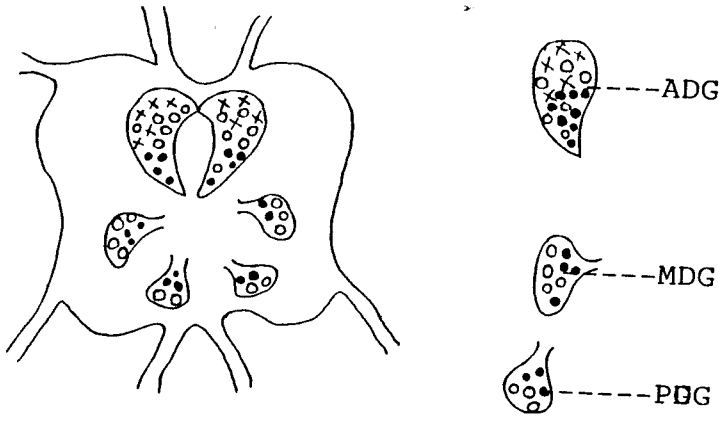
Moderate numbers of mitochondria were found particularly close to e plasma membrane (fig. 135) their profiles were irregular and cristae ten indistinct and distorted. Small granular accumulations visible during e vacuolar phase were not visible in these cells of secretory phase. rious types of lysosomal structures containing secretory granules were countered in these secretory cells (fig. 135,140). The most dominant bcellular structures encountered in these NSCs are the various types of nse cored secretory granules and electron lucent vacuoles of variable size d shape (fig. 137,139,143). Detailed investigations revealed slight but stinguishable differences in the morphology and electron-density of these cretory granules in the same and different types of NSCs. These urosecretory granules include highly electron-dense, homogenous and rather rge round granules bounded by a thin limiting membrane (fig. 137,139). st of this type of granules were found dispersed throughout the cytoplasm. ese moderately electron dense round granules formed the 2nd dominant type granules observed (fig. 139,141). Comparatively smaller & highly electron nse round to oval granules not bounded by any type of limiting membranes

composed the third type of secretory granules (fig. 139,143). The number of these smaller granules were very less in most of the NSC types. Another morphological type observed were round to oval, highly electron-dense granules with an electron lucent area in the centre (fig. 143). Apart from these scattered granules some grouped elementary granules were also encountered in some of these NSCs. Lysosome like dense bodies of various sizes and shapes often packed with small elementary granules were present along with these various types of neurosecretory granules (fig. 140,146). These electron dense granular elements were found in association with the RER elements. In addition to these variably shaped electron dense neurosecretory granules numerous electron-lucent vesicles and vacuolar bodies of different size and shape were also apparent in these NSCs (fig. 140,143).

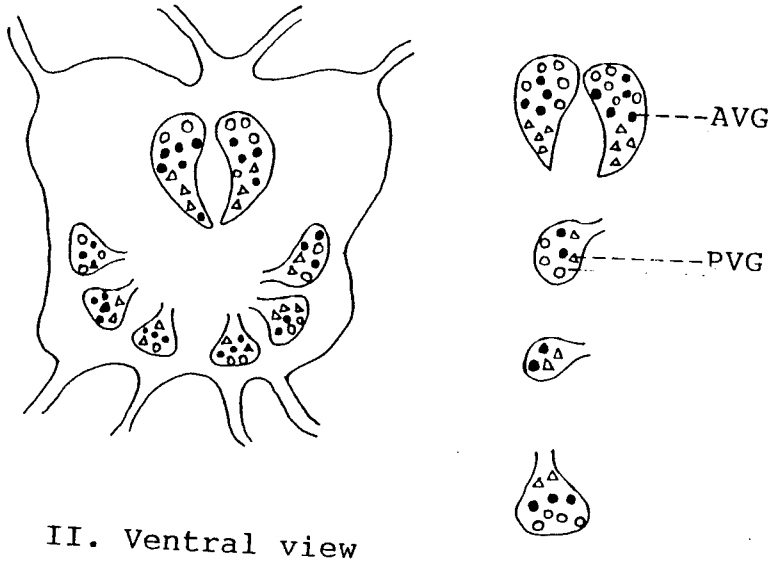
## 3. Distribution and mapping of NSCs in different ganglia of central nervous system

### a. Supra-oesophageal ganglion

Supra-oesophageal ganglion or cerebral ganglion is richly endowed with an abundant supply of NSC groups, formed of different NSCs. These NSC groups were found in the peripheral regions immediately underneath the connective tissue sheath. Totally 14 NSC groups are observed in this ganglion. Light microscopical studies revealed that these NSC groups were not identical. Some of them were larger and contained greater number of secretory cells. NSC types were also found to be different in these cell groups. From the present study, it was found that dorsal side of the cerebral ganglion contained 6 NSC groups (fig. 151), and these groups were larger with greater number of NSCs. These paired NSC groups were found to be located around the junctions of nerve branches with its main body. The anterior




I. Dorsal view




II. Ventral view

150 Diagramatic representation of the distribution and mapping of NSCs in the supra oesophageal ganglion

I Dorsal view II Ventral view

ADG - anterior dorsal group, MDG - median dorsal group, PDG - posterior dorsal group, AVG - anterior ventral group, MVG - median ventral group, PVG - posterior ventral group.

x - GN cells, o - Acells, o - B cells, - C cells

rsal groups (ADG) are the first and the largest NSC group found in the rebral ganglion. This pear shaped NSC group consisted of a few GN cells large number of A cells and a very few number of B cells (fig. 153). This oup was found at the centre of the anterior most region, in between the nction of the optic nerves and anternnary nerves. GN cells were found cated at the anterior most region of these pear shaped cell group. Most minated A cells were found in the entire cell group. A pair of median rsal groups (MDG) formed the second pair of NSC groups, was observed in e middle part of the ganglion in front of the junction of the maxillary rve. This NSC group contained majority of B cells and a few A cells at e outer margin of the cell group. The third or posterior dorsal group PDG) found at the point of origin of the circum oesophageal connectives t the posterior most region of the main body. These groups were composed f 4-5 A cells and a few B cells. This was the smallest NSC group found n the dorsal side of the cerebral ganglion.

Whereas the ventral side of this ganglion had 8 NSC groups, and ll these cell groups were smaller with fewer number of NSCs. Distribution f the cell groups in ventral is plane found almost similar to that in the rsal side. However, the ventral side differing from dorsal side, 2 pairs f median cell groups were encountered. The first pair of anterior ventral roup (AVG) was the largest one observed in the ventral plane and found in ront of the optic nerve. GN cells were totally absent in this cell group ut 3-4 A cells were found at the anterior most margin. B cells were the minant cell type and a few numbers of C cells were also encountered (fig. 154). In the middle part there were two NSC groups. The 1st median ventral roups (I MVG) contained A, B and C cell types. B cells were found xclusively dominating in this group over 2-3 A cells, and a few C cells

observed in the upper and lower margins respectively. Whereas the second median ventral group (II MVG) was formed exclusively of B and C cells. These two median ventral groups were found in front of the maxillary nerve. Apart from all these, a pair of posterior ventral group (PVG) was observed in front of the circum-oesophageal connective. This NSC group contained A, B and C cells in almost equal proportions. From the light microscopical studies, it was found that GN cells were totally absent in the ventral part of the cerebral ganglion in contrast to the dorsal part where it is found in abundance. Similarly C cells were found in almost all the NSC groups in the ventral side but they were completely absent in the dorsal side. Whereas A & B cells were found in dorsal and ventral sides of the cerebral ganglion.

**b. Tritocerebral ganglion**

Tritocerebral ganglion was the smallest ganglion observed in the CNS of P. monodon (fig. 150). This paired ganglion was found in the midway of each tritocerebral connectives, connecting the cerebral ganglion to the suboesophageal ganglion. Light microscopical studies during the present investigation revealed that this ganglion possessed a single NSC group with one or two GN and A cells (fig. 155). A cells were always found in association with those GN cells. GN cells of this ganglion were found conspicuously larger.

**c. Suboesophageal ganglion**

Suboesophageal ganglion is a compound structure supplying nerves to different mouth parts. Because of the same reason it was observed that this ganglion possessed the highest number of NSC groups (fig. 151). A maximum number of 16 NSC groups were identified in this ganglion on its

dorsal, median and ventral planes. Unlike the other ganglions NSC groups with all the cell types were present in median plane of this ganglia and the total number of the secretory cells were also higher.

Light microscopical investigations on the dorsal side of this ganglion revealed 3 NSC groups. The first one, the anterior dorsal group (ADG) was not paired but united as single large compound NSC group. This comprised mainly of GN and A cells in equal proportions and a few B cells (fig. 156). Scatterly distributed GN and A cells were found almost in the entire area of the cell group. Whereas limited numbers of B cells were found at the interior side of the cell group. The posterior side of the dorsal plane was found with a paired neurosecretory cell group, the posterior dorsal group (PDG). These PDG were endowed exclusively with B cells and a few C cells. In the median plane of this ganglion, a total of 9 NSC groups with varying sizes were observed (fig. 150). A single NSC group observed in the central region of the median plane, 'the central median' (CM) comprised of large numbers of GN, A and B cells (fig. 157). The remaining 4 pairs of NSC groups, the median lateral group (MLG) were found at the peripheral regions of the ganglia and contained only A and B cells. It was noted that these medio-laterals were smaller compared to the central median group. Only 4 NSC groups encountered, in the ventral side of the suboesophageal ganglion (fig. 151). Even though there were only 4 NSC groups in the ventral plane of the suboesophageal ganglion, they contained the highest number of NSCs. The anterior ventral group (AVG) was a single large NSC group observed at the anterior most region of the ventral plane which contained a few GN and A cells, but majority of cell type observed were B cells (fig. 158). The large ventro-median group (VMG) observed at the centre was the largest NSC group in the ventral plane. It was composed mainly of GN and A cells

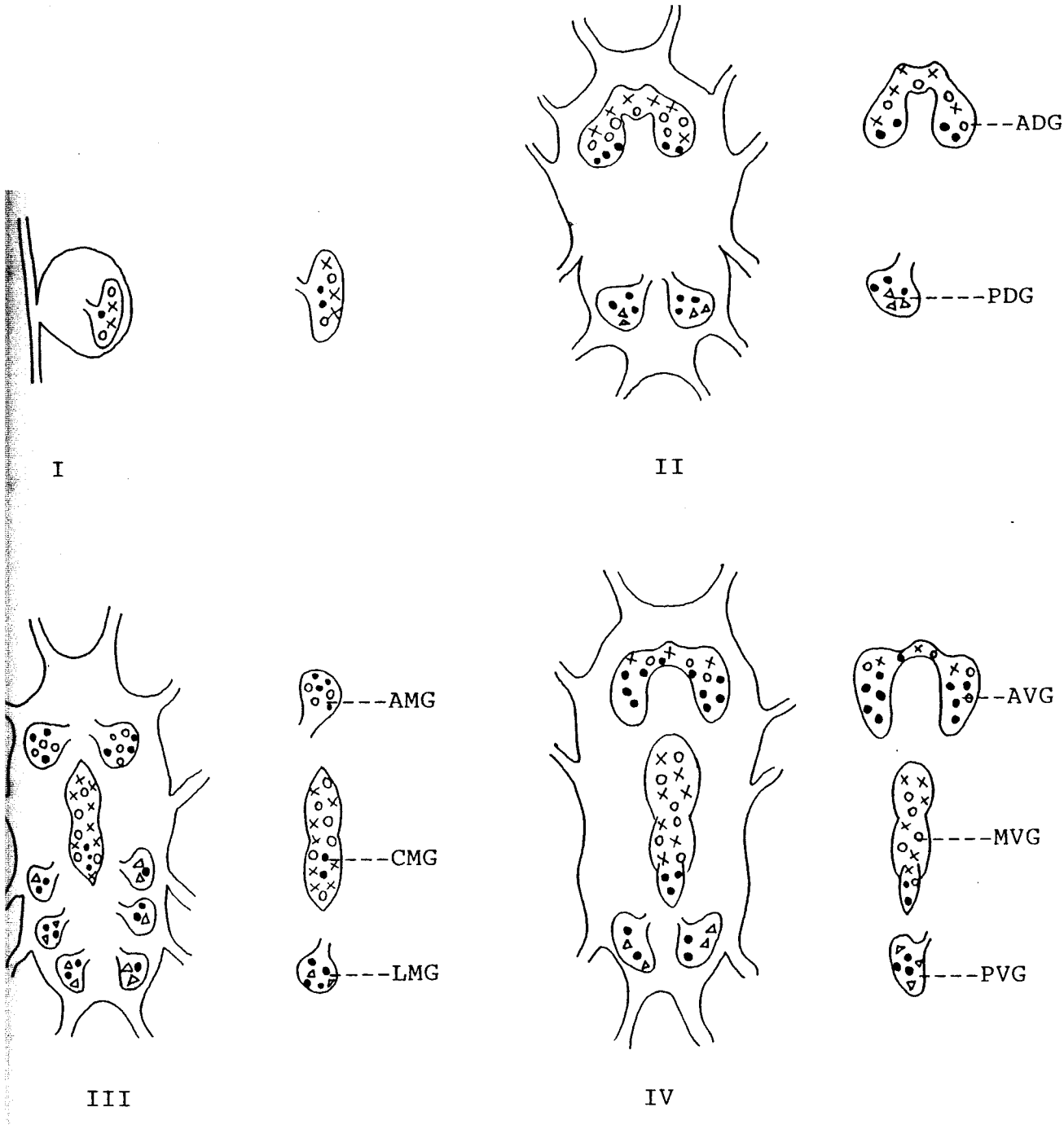



**Fig 151** Diagramatic representation of the distribution and mapping of NSCs in the sub-oesophageal and tritocerebral ganglion.

I - Tritocerebral ganglion II -Dorsal, III - median
IV - Ventral views

ADG - anterior dorsal group, PDG - posterior dorsal group, AMG - antero median grops, CMG - central median group, LMG - latero median group, AVG - anterior ventral group, MVG - median ventral group, PVG - posterior ventral group.
x - GN cells, o - Acells, o - B cells, - C cells

equal proportions. A few B cells were also encountered in the lower most rt of this cell group. At the posterior end of this ganglion a pair of all NSC groups viz. the posterior ventral group (PVG) were noted with a w NSCs which was exclusively formed of oval to polygonal B cells and small cells. From the investigations it was found that, suboesophageal ganglion ntained the highest number of NSC groups and cell types. There were a t of GN and A cells in the dorsal and ventral planes. While the median lane contained more B and C cells. The larger GN and A type cells were und to be completely absent in the posterior end of the ganglion.

**Thoracic ganglion**

There were five thoracic ganglia innervating the five thoracic gments. These ganglia were connected intheir longitudinal axis by short ired connectives. Histological studies revealed that these five ganglia d the similar type of distribution of NSC group. In all these ganglions was found that there were 3 NSC groups with few GN and A cells, and higher and C cells in their dorsal side (fig. 152). The anterior most region d a large single NSC group the anterior dorsal group (ADG). This ADG ntained only few GN & A cells at their outer margin and here B and C cells minated in this cell group (fig. 159). Posteriorly there was a pair of all NSC group named posterior dorsal group (PDG). The PDG was found in nt of the junction of the ventral nerve cord. This cell group contained 3 A cells and comparatively more number of B and C cells.

A single median NSC group encountered in the ventral side was termed verntro-median group (VMG) (fig. 152). Light microscopical studies showed at the VMG was oblong with flattened anterior and posterior ends. Like

the VMG group of the suboesophageal ganglion this was composed of mainly GN and A cells (Fig. 160). Very few B cells were also encountered at the posterior end of the cell group however C cells were found absent.

**e. Abdominal ganglion**

There were five abdominal ganglia corresponding to each abdominal appendages. It was found that these are usually smaller compared to other ganglia. Histological studies revealed that this ganglia contained only few NSCs. Even though there were 8 NSC groups together in both dorsal and ventral planes, the total number of neurosecretory cells were less compared to other ganglia (fig. 152). Both the dorsal and ventral sides contained two pairs of NSC groups, one at the anterior most region just in front of the junction where the ventral nerve cord joins the ganglia, the 'anterior dorsals' (fig, 161) and the other at the posterior end, the 'posterior dorsals' just in front of the junction from where the VNC leaves the ganglion in the dorsal side and the 'anterior ventrals' and 'posterior ventrals' in the ventral plane of this ganglia. Both of these NSC groups contained the three types of NSCs A, B, and C cells, however their number seems to be very less compared to other ganglia.

**C. Eyestalk neurosecretory system**

**C.1 Gross morphology**

Present study on the endocrine system in the eyestalk of P. monodon revealed that, the large prominent, stalked compound eyes of this animal are uniformly rounded and slightly larger than a hemisphere. The eye is found attached to the head region by the proximal end of the optic stalk

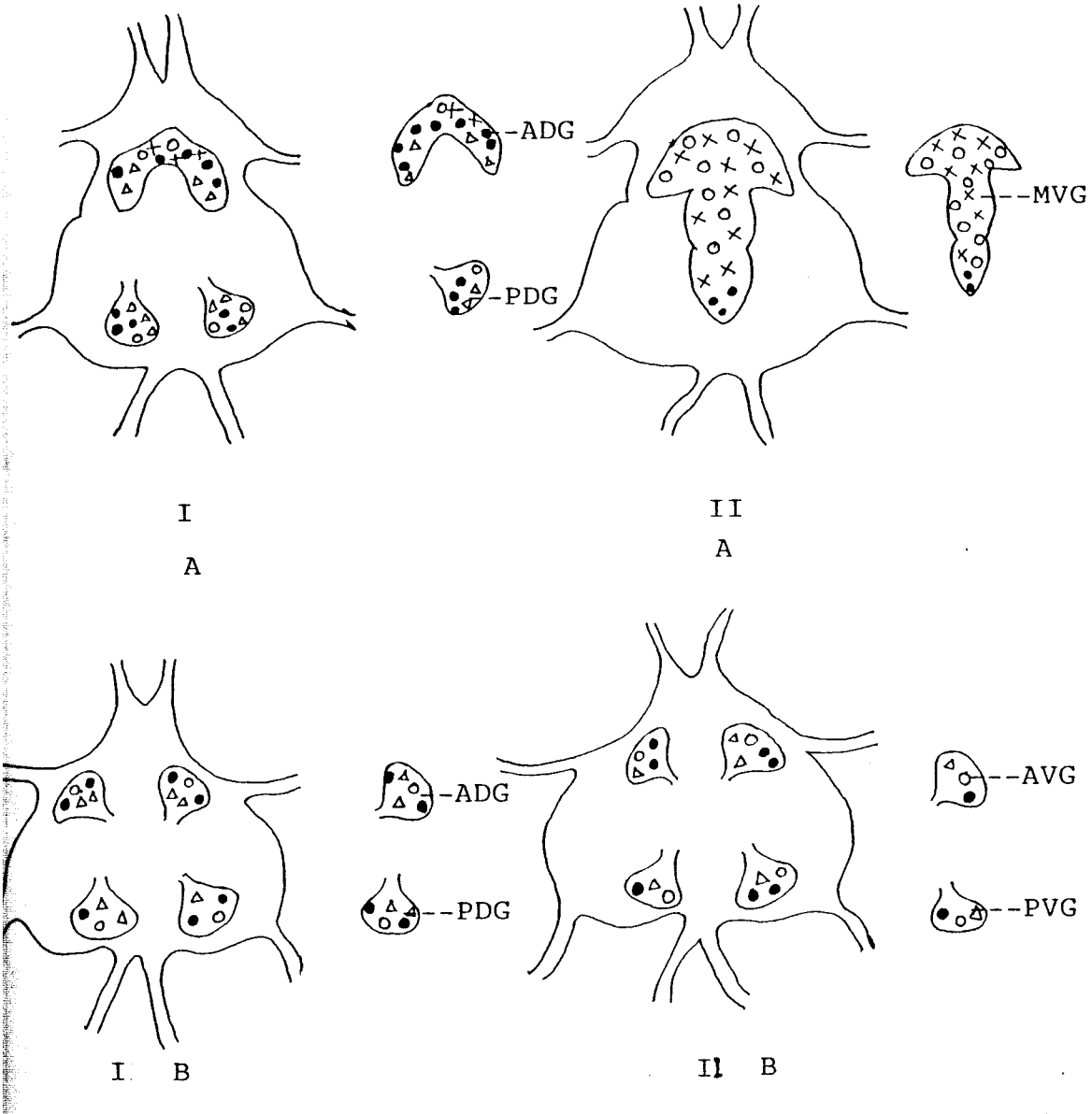



Fig 152 Diagramatic representation of the distribution and mapping of NSCs in the thoracic and abdominal ganglia

A Thoracic and B abdominal ganglia
I Dorsal view II Ventral view

ADG - anterior dorsal group, PDG - posterior dorsal group, MVG - median ventral group, PVG - posterior ventral group.

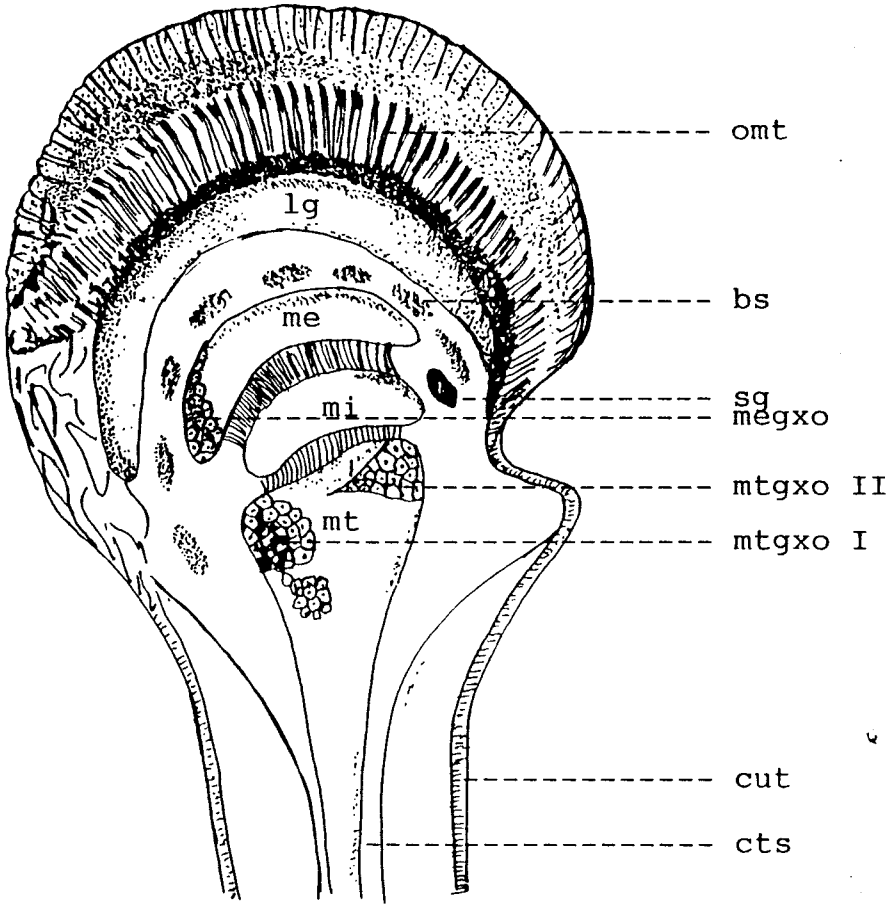
x - GN cells, o - Acells, o - B cells, - C cells

or peduncle. Light microscopical investigations revealed that the eyestalk is a complex structure composed of radial units the ommatidia. A schematic representation of the eyestalk is given in (fig. 163). Standard eyestalk nomenclature described by Bell et al., 1988 has been used throughout the description.

From the light microscopical investigations it was found that the eyestalks are covered externally by a thin layer of transparent cuticle (fig. 163,165) it was demarcated into facets. The clear 'V' shaped notch on the dorsal side of the eye-stalk is prominent. The outer lying cuticle of the eye is found subtended basally by a layer of epicorneagenous cells and crystalline cone cells (fig. 163,165). Below the crystalline cone cells are the retinular cells. These together form the dioptric portion (fig. 163). It is found that, a basement membrane divides the distal dioptric portion of the ommatidia from the proximal ganglionic portion. Proximal ganglionic portion of the eye contained 3 ganglia, the distal one the medulla externa (ME), followed by the medial one the medulla interna (MI) and the most proximal one the medulla terminalis (MT). Between the ommatidia and the medulla externa was the lamina ganglionaris (fig. 163).

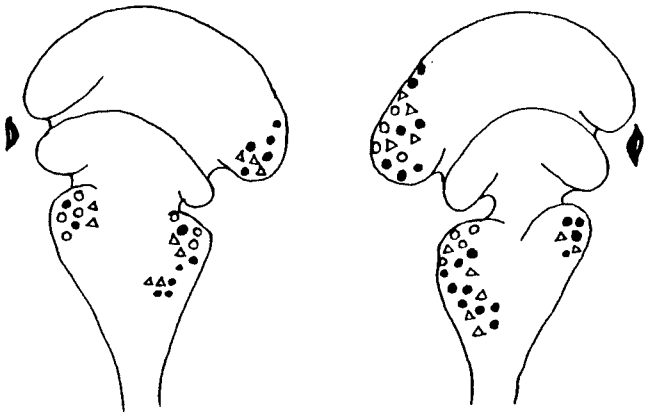
Lamina ganglionaris was located exactly between the distal fasciculated zone of the dioptric portion and the proximal medulla externa (fig. 163). LG is found to be devoid of any NSCs. Between the LG and the ME, a vascular layer was observed. Ten to fifteen haemolymph lacunae (blood sinuses) were visible at the distal end of the LG. The neurohaemal organ, the sinus gland, is invariably found associated with one such vascular process.

The ME is an inverted cup shaped lobe immediately beneath the LG. The distal surface of the lobe is undulating and it was found that the NSCs are present in the dorsal extremities of ME (fig 166,167). Fibers of the neuropile and blood capillaries are found in this region. MI is found to

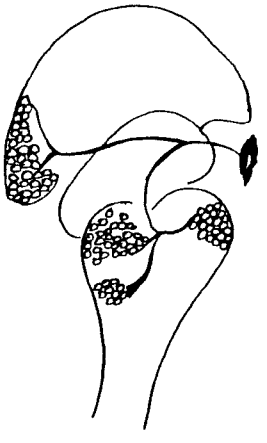



g. 163 Schematic representation of the important structures in the right eyestalk as seen from the dorsal side.

omt - ommatidia, lg - lamina ganglionaris, bs - blood sinus, me - medulla externa, megxo - medulla externa ganglionic X-organ, sg - sinus gland, mi - medulla interna, mt - medulla terminalis, mtgxo I - medulla terminalis ganglionic X- organ one, mtgxo II - medulla terminals ganglionic X- organ two, cut - cuticle, and cts - connective tissue sheath.

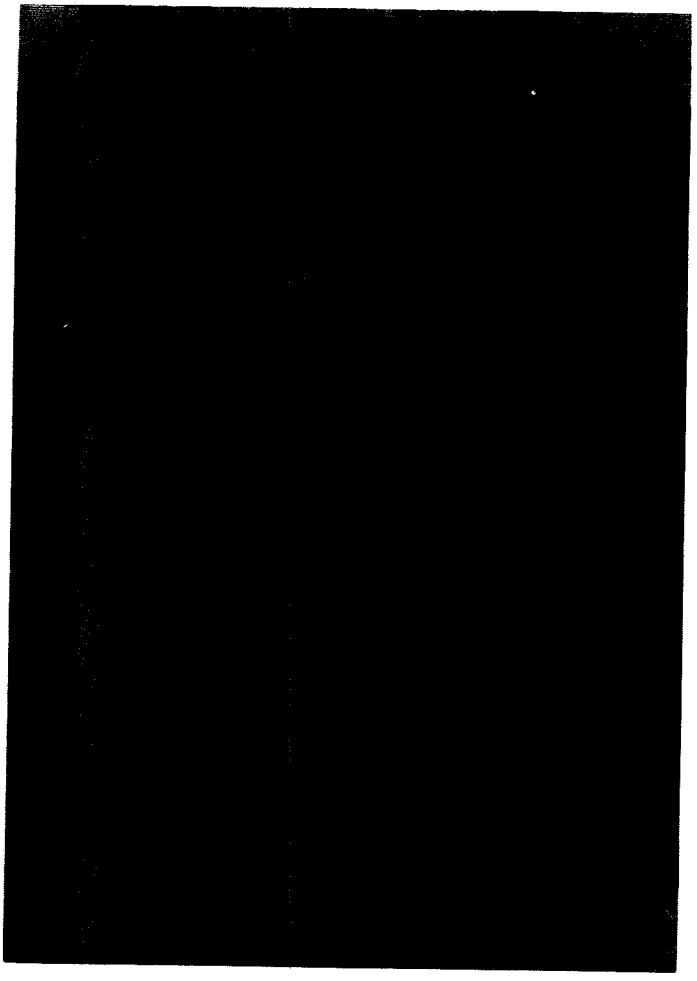


I. Mapping of NSCs in the eyestalk



II. X-Organ sinus gland tract in the eyestalk

Fig. 164 Diagramatic representation of the distribution and mapping of NSCs in the eyestalk (I) and the X- organ sinus gland tract (II), me - medulla externa, mi - medulla interna, mt - medulla terminalis sg - sinus gland o - A cell, o - B cell, - C cell



. 165 Light micrograph of the right eye stalk of P. monodon showing important structures (omt - ommatidia, lg - lamina ganglionaris, bs - blood sinus, me - medulla externa, megxo - medulla externa, ganglionic X-organ, sg - sinus gland, mi - medulla interna, mt - medulla terminalis, mtgxoI - medulla terminalis ganglionic X- organ one, mtgxo II - medulla terminals ganglionic X- organ two, cut - cuticle and cts - connective tissue sheath.

be similar in structure to the ME including the inter-spaced capillaries, though slightly smaller in size and devoid of NSC (fig. 166). The sinus gland is found between this ME and MI (fig. 166). The most proximal ganglia is the knob like medulla terminalis (fig. 166,169) on the surface of which, were the principal NSC groups are found to be located. Fibers from these cells directly formed the optic nerve (fig. 170). Immediately surrounding the medulla terminalis and the other ganglia is the broad cortical glia, followed by a thin, dense neurilemma. MT, like other ganglia contained matrix of capillaries and lacunae.

**C.2 Distribution of NSC in the eyestalk**

Histological investigations revealed that there are mainly 3 NSC groups in the eye stalks of P. monodon. These cell groups were named on the basis of their location. Among the medullary lobes, neurosecretory cells were found only in the MT and ME (fig. 166). NSCs groups were absent in the lamina ganglionaris and MI of the eyestalk. The principal NSC groups are found to be located at the apical portion of the MT (fig. 163). Serial sections revealed 2 NSC groups together in the dorsal and ventral planes of the MT. Dorsally there were two distinct NSC groups, were found at the topmost region of the lateral sides of MT (fig. 163). However, there were only one NSC group at the ventral side, of the MT. Among the two NSC groups encountered in the dorsal side one found at the right side of the right eye is termed the medulla terminalis X organ-I, (MTGXO-I) and the NSC group on the left side was termed the medulla terminalis X organ-II (MTGXO-II) (fig. 170). The MTGXO-I is found to be small and contained comparatively lesser number of NSCs. The MTGXO I is pyriform in shape with a tapering end from where the axon terminal leaves the ganglion (fig. 170). The axon terminal is found to join the main axon coming from MTGXO II before it leaves

MT. The sinus gland is positioned immediately distal to this MTGXO-I group (fig. 166). Present investigation revealed that, MTGXO-I was the only NSC group found in the right side of the right eye where the sinus gland was located. All other NSC groups are found at the opposite or left side of the right eye (fig. 163,166). Light microscopical studies revealed that MTGXO-I contained a few A & C cells, and majority of B cells (fig. 171).

MTGXO-II is also found in the upper most area of the MT on the opposite side of MTGXO I, which is a comparatively larger NSC group containing more secretory cells. Eventhough a few secretory cells are found dispersed in the lower part of the MT, majority of the cells are found located in the apical part itself (fig. 168). Further a small group of NSCs was observed in the close proximity to the MTGXO II. The MEGXO was observed on the lateral surface of the ME, on its rostral side (fig. 167). This group was observed to be diffuse, with a number of B & C and 2-3 A cells found on dorsal and ventral surfaces (fig. 166). It was noted that, a neurosecretory axon was leaving from this ellipsoid cell group (MTGXO II) at its tapering lower end which is found to join the axon coming from the MTGXO-I before leaving the MT. The neurosecretory cell distribution of this group has been shown in the (fig. 168). Type B & C cells were the most abundant cell types on both dorsal and ventral aspects of this cell group with few larger A cells. Details of these cells are shown in the figure [illegible].

**The sinus gland and the X-organ sinus gland tract.**

The sinus gland is the principal neurohaemal organ in the eyestalk. It is found to be located between the medulla externa and medulla interna in the dorso-lateral surface of the eyestalk, connected with some axon bundle coming from the ganglionic part of the optic ganglia (fig. 164,171). In

P. monodon, the sinus gland is always found in association with the finger like blood sinuses around the medullary lobes. It is observed that, the sinus gland itself is a blood sinus surrounded by numerous heavily stained axon endings on the dorso-lateral side of the optic ganglia (fig. 173). In the right eyestalk, the sinus gland is located in the right side between the ME and MI. Light microscopically this sinus gland is roughly elliptical and at its lateral plane is more flattened. The central part of the gland is occupied by the internal blood sinus (fig. 173). The heavily stained swollen axonal endings with its neurosecretory materials was found to be separated from the blood sinus by a thin membrane. In adult animals (above 50 mm TL) the sinus gland measured about 250-275 $\mu$ long and 100-120 $\mu$ wide.

Ultrastructural studies revealed, that the sinus gland is composed of axons which terminate in the bulb shaped enlargements just beneath the basement membrane lining the blood sinus (fig. 174-179). Present investigations on the biomolecular aspects of the sinus gland of P. monodon revealed that, it is found to be built up of 4 main components, the axons, axon terminals, glial cells and blood sinus. A thin membrane separated the sinus gland with its heavily stained axonal endings and neurosecretory materials from the blood sinus. As a neurohaemal organ, the sinus gland received the axons from the perikarya of the neurosecretory cells which are localized in other ganglionic parts of the neuroendocrine systems. It is observed that profiles of these unmyelinated axons were filled to a greater or lesser extent by bound granules. Transversely cut ends of these axons in their pre-terminal part showed the presence of micro-tubules at their central part. Pre-terminally, these narrow axons are fit closely together and contained only neuro-tubules, running along its entire length. However, the terminal region is characterized by the presence of a greater number of membrane bound granules (fig. 174). This terminal area of the axon is

found to be swollen, for increasing its storage volume, and also abuts on to a basement membrane that separates the axon from the blood sinus. These axon terminals abutting on the blood sinus of the gland, contained the neurosecretory granules, electron lucent vesicles and small mitochondria (fig. 174,179). Golgi complex was also found in certain times (fig. 176). The granules were of varying electron density and generally rounded to oval in shape. A few granules were dumbbell shaped or had an electron dense head and a tail of decreasing electron-density. Besides these electron-dense granules, electron-lucent vesicles with a diameter of 20-50 nm, the so called synaptic vesicles were also apparent in these axon terminals (fig. 176). Two types of electron-lucent vesicles with a diameter of 500-600 A° were found usually scattered among neurosecretory granules in certain axon terminals (fig. 177). The other larger electron-lucent vesicles were irregular in shape and had a diameter of 2 μ. Those terminals which possessed these vesicles contained only few neurosecretory granules. This suggested, the presence of vesicles as an indication of the active neuron (fig. 177).

Passing between many of the neurosecretory axons were the glial cells (fig. 177) that form the nuclear component of the sinus gland. The greater part of the glial cell was occupied by the nucleus, which was irregular in shape and measured up to 3μ (fig. 177). The cytoplasm contained microtubules, vesicular structures, mitochondria, endoplasmic reticulum and free ribosomes. Rough endoplasmic reticulum was often situated around the mitochondria. Golgi complex were encountered rarely within the glial cells (fig. 176).

The blood sinus forms the remaining major component of the sinus gland. It was found that the structure less basement membrane forms an intercellular boundary between the axons and internal blood sinus, and it varies from 150-450 nm in thickness. Some times the basement membrane was found convoluted in certain areas, and extensions of which was found in between

axon terminals (fig. 175). Axon terminals in the neighbourhood of the blood lacunae were characterized by the storage and release of neurosecretory granules. These granules vary in shape, size and electron-density.

In the present investigation, five different types of axon terminals have been tentatively identified in the sinus gland of P. monodon, on the basis of characters of its granules. It was noted that these neurosecretory granules were not found mixed within the individual axons. The five different types of neurosecretory granules found in the axon terminals are described here as type I to V.

The type I terminals (fig. 174,176) contained considerably large neurosecretory granules averaging (1700 A°) in diameter. These granules were round, elliptical or oval in shape and had very electron dense, homogenous contents, which completely filled the enclosing membranes. It was found that these less frequent axon terminals were compactly packed with highly electron-dense granules of almost same size and shape. Nothing other than the granules was apparent in these terminals.

The type II axons were the most common type found in the sinus gland of P. monodon. These axon terminals contained granules range from (1200-1800 A°)in diameter and were round to oval in shape (fig. 174,176,177). Although very similar to the type I granules in shape and dimensions, the type II axons contained granules of varying diameters and electron-ensity. A few numbers of larger granules with high electron density were observed, along with the other comparatively larger, moderately electron-dense granules. These axon terminals contained loosely packed granules, in contrast with the compactly packed granules of type I terminals. Electron-lucent vesicles, microtubules and mitochondria were found in these axon terminals. Golgi complexes were also apparent in certain axons (fig. 176).

Type III terminals contained the smallest neurosecretory granules served in the sinus gland (fig. 177). The diameter of these small granules ried between (700-2300 A°). These round granules were identical in size d shape and contained homogenous, moderately dense cores. Nothing other an the loosely packed small granules were apparent in these axon terminals. ese small axon terminals were not very common in the sinus gland.

The type IV terminals contained the largest neurosecretory granules g. 175), averaging (700-1200 A°) in diameter. These granules were round oval in shape and possessed highly electron-dense homogenous contents ich completely filled the enclosing membranes. These axon terminals ntained only thickly packed large granules. Other electron lucent vesicles cell organelle like mitochondria, Golgi complex etc were not at all parent.

The type V axon terminals contained granules of varying diameters d shape (fig. 178,179). The diameters of these round to irregular rosecretory granules varied between (1800-2600 A°). Granules were found her dispersed than aggregated. Mainly 2 types of granules were found these axon terminals. Very few numbers of larger highly electron-dense nules with a diameter of (1900 A°) and large number of smaller granules h less electron density. Along with these granules some other microtubules electron-lucent vesicles were also encountered. During the present estigation it was noted that occurrence of type V axon terminals was not y common in the sinus gland.

Light microscopic investigations on the serial sections of the optic glion showed a prominent axon bundle in the ganglionic part of the optic glion (fig. 170). It was also noticed that, this nerve bundle originated m the NSC groups and terminated in the sinus gland (fig. 170,171). This ous tract from the X organ to the sinus gland termed as X-organ sinus

land tract, was observed rather infrequently (fig. 165). The entire route f this tract was never traced completely in any of the sections. Eventhough lear picturisation of this tract was not possible through light microscopy, rial sections of a number of eyestalks has given a skeletal picture of he X-organ sinus gland tract. The predominant nerve bundle found at the nglionic part of optic ganglion was clearly visible in the MI (fig. 165) pecially at its terminating end at the sinus gland, and also in the middle rt of the medulla interna & medulla terminalis. Further tracing of this ndle at its proximal region showed that, it was connected with NSC group cated at the dorso-lateral surface of the medulla terminalis (fig. 170) he so called MTGXO-I. From the semithin sections it was found that, small on profiles of the NSCs in this group united and formed the main bulk of he axon bundle. Additional axons, originating from the small NSC group n the immediate neighbourhood and small axon profiles from the NSCs located the other side of the MT, joined together and formed a small axon bundle. is bundle was also found joining the main tract as it passes forwards. on bundles from the MEGXO was also found joining this tract during its rse through the MI to the sinus gland. The exact points where these small on bundles joining the main tract was not clearly visible in any of the rial sections. The schematic representation of the X organ sinus gland ct on the basis of the histological observation is given in (fig. 164).

The X organ sinus gland tract near the sinus gland is found roximately 18-20 $\mu$ in thickness (fig. 171) however, it showed only 10-12 thickness when it leaves MTGXO-I (fig. 170). When the axon from the XO-II joined with the axons of MTGXO-I above the MGXOII, its thickness creased from 12 to 15 $\mu$. These two together entered the MI where the axon m MEGXO also joined them and these three axons together proceeded so-laterally towards the sinus gland.

## D. Neuroendocrine control of reproduction

Light microscopical as well as electron-microscopical studies on the NSCs in different ganglionic masses during different maturity stages revealed concomittant changes in the secretory phases of these cells during the process of maturation (fig. 186-197). These specific and dominant changes indicated their significant roles in the control of reproduction. The mean percentage occurrence of these NSCs at different secretory phases are found varying during the process of maturation. Present investigation revealed dramatic changes between the secretory figures of the eyestalk X-organ complex and different ganglionic centres of the CNS. The mean percentage occurrence of NSCs in the eyestalk and central nervous system at different stageas of their secretory cycle during the various maturity stages of P. monodon are presented in the table 3.

| | Eyestalk | | Central nervous system | |
|---|---|---|---|---|
| Secretory phases | Immature (%) | Maturing (%) | Immture (%) | Maturing (%) |
| Q | 20-35 | 45-60 | 45-50 | 50-60 |
| V | 15-25 | 20-27 | 20-35 | 25-30 |
| S | 50-55 | 15-25 | 15-25 | 10-15 |

Table 3. Percentage of total neurosecretory cells in eyestalk and central nervous system of immature and maturing P. monodon

### 1 Eye stalk neurosecretory cells during maturation.

Light microscopical investigations in the eyestalk X-organ complex immature animals showed a higher number of active cells (fig. 180,181). was found that more than 70% of the NSCs in both the MTGXO and MEGXO were

their physiologically active vacuolar as well as secretory phases. Due the presence of large numbers of active NSCs, both the cell groups were rticularly prominent and clear in the eyestalks of immature animals than other maturing stages. NSCs at the S-phase contained highly granular toplasm and V-phase were characterized by the presence of vacuoles. reaming of cytoplasmic granules were apparent in many of the S-phase NSCs. rastructural investigations revealed that, the neurosecretory granules different size, shape and electron-density were apparent in the perikarya the active NSCs of eyestalk X-organ complex from the immature animals. reased secretory activity was evidenced from its abundant supply of eloping vacuoles and vesicular bodies. Perikaryon of most of these cells essed numerous highly active cell organelles, especially the very active gi complex with moderately electron-dense flocculent materials. Free polyribosomes were apparent throughout the cytoplasm of these active ls. Multivesicular bodies were also encountered in association with somal structures. Exocytosis of fully developed granules were apparent the outer membranes of these NSCs. Present investigation revealed that g the different types of NSCs, A type cells exhibited more secretory vity followed by B cells in the eyestalk X-organ complex of immature ns (fig. 180,181). It was noted that more than 60% of the A cells were the S-phase with highly granulated cytoplasm. Vacuolar phase A cells also apparent in equal numbers in these NSC groups. A cells at their ing or quiescent phase were not completely absent. Remaining 10-12% hese A cells were at the resting or Q-phase in all the cell groups of eyestalk. Such a heightened neurosecretory activity was not observed & C cell types. However 50% of the B cells exhibited secretory activity dicated by its active coarse cytoplasm due to the presence of secretory les inter spaced with small active cytoplasmic vacuoles. Among the

cells 20-35% of them exhibited coarse cytoplasm which was an indication f the secretory activity. However, most of C cells, were at the Q-phase f its secretory cycle.

In the early maturing stage of the animal it was found that the mber of the S-phase cells decreased considerably. Concomitant decrease n the percentage of the V-phase cells were observed in the eyestalk X-organ mplex of these animals. Consequently the percentage of Q-phase cells ncreased drastically after the initiation of the vitellogenesis. However, e streaming of the granular cytoplasm and hypertrophied glial cell nuclei mpassing these cells (fig. 182,189) evidences the secretory activity the existing S-phase. Where as the eyestalk X-organ complex of the mature imals showed a higher percentage of Quiescent phase NSCs. It was noted at about 60% of the NSCs were at the resting or Q-phase of the secretory cle. Remaining NSCs were still at their vacuolar as well as secretory ses. Even though the cytoplasm of these active cells appeared coarse granular, the cytoplasmic streaming observed in the previous stages was apparent in any of these active cells. Different cell types showed stic changes from the preceding stage. Surprising increase in the number Q-phase cells were observed in case of A cells (fig. 184,185). A four d increase was found in the percentage of Q-phase A cells ie. from 12 46%. Other two types of NSCs also exhibited concomitant increase in the centage of their Q-phase cells.

**Neurosecretory cells in different ganglionic centers and their correlation with ovarian maturation**

Light microscopically it was found that, in sharp contrast to the ts observed in the eyestalk X-organ complex, most of the NSCs in the lions of CNS from the immature animals were at the Q-phase of their

d in the ganglia of the late vitellogenic animal. Light and lectronmicroscopical investigations in the ganglionic centers of fully atured animals just before ovulation revealed large numbers of almost pty NSCs (fig. 196,197). Ultrastructurally it was observed that, these pty cells possessed only their centrally placed nucleus and the outer lasmamembrane with a few membrane systems. However, the large numbers f resting NSCs observed among these empty cells indicated that, they gained their resting phase within short fractions of time. All the cell pes in a ganglia did not react in the same manner. Hyper activity was und in GN and A cells. However, B cells exhibited only moderate activity hereas comparatively less activity was encountered in the smallest C type lls.

Highest secretory activity was encountered in the GN cells followed A cells. Almost 90% of the GN cells were either at S or V phase of cretion in these ganglions. Q-phase GN cells were found only in limited mbers. Similarly more than 70% of the A cells showed granular as well vacuolar cytoplasm. Streaming of cytoplasmic granules were observed some extent in their axonal processes. Whereas the B & C types of cells not exhibit such a heightened neurosecretory activity, as found in and A cells. The cytoplasm of these cells, at their active S & V phases, ntained small round vacuoles inter spaced by granular cytoplasm. The ertrophied glial cell nuclei were not prominent in these cells, like other cells. Streaming of the cytoplasm was not apparent in these ll cells.

Even though the general pattern of NSC phases were found similar all the ganglionic centres of the CNS, slight differences were noticed ween different ganglionic centers. Highest percentage of active rosecretory cells were found in the suboesophageal ganglion, followed

thoracic ganglions. Cerebral ganglion contained comparatively lesser mber of active cells in the mature animals. However, higher number of phase NSCs were observed in the cerebral ganglion during the early turing state of ovary. Biomolecular studies strongly support these servations. Neurosecretory cells packed with granules of variable size, ape and electron-density were apparent in various ganglionic centers mature animals.

From the light and electron-microscopical investigations of the ferent NSCs located in various ganglionic centers during the process gonadal maturation, it can be concluded that these cells are playing ive roles in the regulation of reproduction in P. monodon. Significant nges were noticed in the secretory activity of these cells during ferent stages of gonadal maturation. In the immature animals the NSCs eyestalk, especially A & B type of cells were found highly active icating that, these cells are engaged in the secretion of the d-inhibiting factors. At the same time most of the NSCs in various lionic centers of CNS were at their resting Q-phase. This shows they not actively participating in the secretion of GIH. In contrast during early stages of gonadal maturation, the NSCs of eyestalks did not show ightened secretory activity as that of immature animals. During the stages of maturation, GN and A cells of brain are found to secrete factors which in tern activate the thoracic and sub-oesophageal ganglia ecrete the gonad stimulating factors. Concomitantly, an increase in umber of S-phase GN and A type NSCs were noticed in sub-oesophageal horacic ganglia. Heightened secretory activity was noticed in these and the other B & C cells of these ganglia, during the subsequent of oogenesis, indicating its active role in the synthesis and ion of gonad-stimulating factors.

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## DISCUSSION

Crustaceans utilise neuropeptide hormones for regulating diverse biological processes including reproduction. The endocrine control of production has been investigated with a wide variety of crustaceans. this respect, the amphipods and isopods have received considerable ntion (Fingerman, 1987). Investigations in decapods are, mainly in crabs and crayfishes and the Penaeid group has not received that much ntion in this respect. However, the neuroendocrine control of oduction has not been elucidated clearly in any group of crustaceans , 1992). Therefore, during the present study an attempt was made through t and electronmicroscopy to study the cytological features of the various oendocrine organs during the process of gonadal maturation in the penaeid n P. monodon.

The central nervous system of P. monodon comprises of a dorsal brain, a ventral nerve cord with a pair of ganglia in each segment. According ac Laughlin (1983), the central nervous system (CNS) of crustaceans ally consists of a large supra-oesophageal ganglion, the brain, and ntral nerve cord with a pair of ganglia corresponding to each embryonic te. In spite of the considerable variation in the number of segmental lia and structure of the ventral nerve cord a more or less similar type ntral nervous system (CNS) was described in many invertebrates by various stigators (Enami, 1951; Matsumoto, 1958; Fingerman and Aoto, 1959; Lake, ; Dall et al., 1990; Mohammed et al., 1993). Among the penaeids, the endocrine system of P. indicus described by Mohammed et al. (1993) ly resembles the pattern observed during the present investigation.

Four different types of neurosecretory and a non-neurosecretory cell were identified in the ganglionic centers of P. monodon during the nt investigation. Neurosecretory cells (NSC) observed in the ganglionic

ters of P. monodon during the present investigation, were found to be tinct from the small non-neurosecretory cells. NSCs described here, were acteristically similar to the NSCs described in other crustaceans by ous investigators (Enami, 1951; Durand, 1956; Matsumoto, 1958; Fingerman Aoto, 1959; Lake, 1970; Nakamura, 1974; Diwan and Nagabhushanam, 1974; Herp, et al., 1977; Chandy and Kolwalker, 1985; Nanda and Ghosh, 1985, ; Mohammed et al., 1993). All the four NSC types described in P. don are found to be comparatively larger than those described in other taceans. Similarly, 4 different types of NSCs have been described in brain or supra-oesophageal ganglion of the same species by Nanda and h (1992). In P. indicus, Mohammed et al. (1993) also reported four erent types of NSCs while in P. japonicus, Nakamura (1974) described fferent types of NSCs. However, only three different types of NSCs were tified in the ganglionic centers of P. japonicus and P. kerathurus by an and Matta (1976). In the ganglionic centers of P. stylifera, hushanam, et al. (1976) identified 8 different types of NSCs. In the water prawn Palaemon paucidens, Hisano (1974) and Macrobrachium bergii, Deitz (1982) had identified six different types of NSCs. iptions of the NSC types, found in the optic and other ganglia of ent species are varied, probably because of the differences in logical staining procedures, cyclic secretory activity, species rences and human subjectivity. In spite of this, various investigators Durand (1956), Passano (1960), Adiyodi and Adiyodi (1970), Quackenbush ), Nagabhushanam et al. (1992) and Mohammed et al. (1993) have made pts to relate the variously distributed NSCs in crustaceans.

Classification of the NSCs in P. monodon during the present study was based on their shape, size, (descending order) and morphological and lecular characteristics of these cells and their secretory products.

GN cells were the largest NSCs observed in P. monodon. Similar type of cells has been described only in P. indicus in the same terminology by ammed et al. (1993). Similarly, the GN cells described in the present dy are comparable to cell type I and II of P. japonicus (Nakamura, 1974) to the giant A cells described by Matsumoto, (1954) in the thoracic glia of the Japanese freshwater crab Eriocheir japonicus in its shape, e and histological features. All the above mentioned investigators have cribed only the light microscopical features. A perusal of the available erature showed a paucity of information in the ultrastructural features the NSCs in Crustacea. Only few published reports are available with rd to electron-microscopic studies of the NSCs in crustaceans (Fingerman Aoto, 1959; Hisano, 1976; Andrew et al., 1987; Chataigner et al., 1978; mura, 1980; Mc Namara, 1993). Among the various investigators, astructural descriptions of the various NSC types have been reported by Hisano (1976) in fresh water prawn P. paucidens; and Chataigner l., (1978) in the isopod Spheroma serratum. In penaeids, Nakamura (1980) ied the PAS positive cells of supra-oesophageal ganglion of P. japonicus, Mohammed et al. (1993) have showed the neurosecretory granules of P. cus. Ultrastructural studies conducted in the various NSC types of P. don showed the ultrastructural features of the highly active perikarya hese cells as well as the mode of synthesis of neurosecretory materials n their cytoplasm. The perikarya of the GN cells of P. monodon during rocess of gonadal maturation possessed an active perikarya with numerous organelles and secretory granules. More or less similar ultrastructure een described for the A type NSCs of freshwater prawn P. paucidens sano, (1976) and the A type cells of the isopod S. serratum by Chataigner (1978). It was found that the highly active perikarya of these cells ed by the above investigators as well as the GN cells of P. monodon

ring the present study, contained active cell organelles like Golgi plex, RER, cisternal types of reticular elements and mitochondria and e secretory granules of varying size and shape.

In its shape, size and histological features A cells of P. monodon e comparable with the 'V' cells of Sesarma dehaani (Enami, 1951); Type and III cells of P. paucidens (Hisano, 1974); type III cells P. japonicus kamura, 1974); B cells of P. serratus (Van Herp et al., 1977); and A cells Charybdis lucifera (Chandy and Kolwalkar, 1985). The size and tomorphological features of A cells observed in the present investigation wed close resemblance to the A cells described by Nanda and Ghosh (1985, 2) in the same species. Apart from those the A cells observed here were ilar to the cell types I, II, III and IV of P. stylifera (Nagabhushanam al., 1986) and the A cells of P. indicus (Mohammed et al., 1993). astructural studies in the A cells of P. monodon during the present stigation showed a highly active perikarya with abundant active cell nelles and numerous secretory granules. Ultrastructurally this showed e resemblance to the B & C type cells in P. paucidens (Hisano, 1976) to the 'B type cells in the isopod S. serratum (Chataigner et al., 1978).

B cells observed during the present investigation in P. monodon were ler than GN and A cells. The B cells were found similar to the B cells ribed by Nanda and Ghosh in the same species. These cells exhibited or less similar characters of the type IV cells of P. paucidens ribed by Hisano (1974) and of the cell types V, VI & VII of P. indicus mmed et al., 1993). Ultrastructurally perikarya of these cells did xhibit a high secretory activity. Active cell organelles found in the arya of certain B cells of P. monodon were similar to the D and E type of S. serratum (Chataigner et al., 1978). However, secretory granules less in the perikarya of B cells of P. monodon. The C cells noticed

the present study were found to be the smallest NSC type and showed similar phological characters to the C cells described by Nanda and Ghosh (1985) the same species and the C cells of P. indicus (Mohammed et al., 1993). cells described by Van Herp (1977) in P. serratus were also comparable the pyriform C cells of P. monodon. The electron lucent cytoplasm was oid of much cell organelles. Neurosecretory granules were also absent most of the C cells. Ultrastructurally the C cells of P. monodon were ilar to the F cells of P. paucidens (Hisano, 1978).

Apart from the NSC types, a single type of non-neurosecretory cells also observed in the ganglionic centers of P. monodon through light roscopy. Almost all the investigators referred this as the small NSCs sumably based on their mere occurrence amidst the NSC group. However, the present observation it was found that these small cells are neurosecretory. In P. indicus Mohammed et al. (1993) described similar es of non-neurosecretory cells. The non-neurosecretory cells of P. odon did not show any signs of secretory activity similar to such cells cribed in P. indicus by Mohammed et al. (1993). Matsumoto (1954) sified such cells as D-cells and emphatically stated the absence of etory activity in these cells. These cells are usually found in groups P. monodon and they contain a single conspicuous nucleoli as described Matsumoto, (1954) in E. japonicus and Mohammed et al. (1993) in P. icus. The non-neurosecretory cells of the present study contain a thin of cytoplasm which was not identifiable through light microscopy. umoto (1954) and Mohammed et al. (1993) also have reported that the cell daries of such cells are difficult to discern.

The non-neurosecretory cells of P. monodon during the period of al maturation revealed a cyclic secretory activity. In crustaceans, few workers like Enami (1951) in Sesarma dehaani and Matsumoto (1954

1958) in E. japonicus have described the cyclic activity of the NSCs in ail. These authors have noted the formation of vacuoles as a significant t of the secretory cycle. In P. monodon also the vacuole formation was first phase in the activation of the cells during its secretory cycle. uoles of various shapes and sizes were observed in the NSCs of P. monodon cially in GN and A type cells. The diameter of these NSCs was found be increasing as the vacuole formation continued. This vacuolar stage followed by a secretory stage during which the entire cytoplasm gets led with the secretory materials at the expense of the vacuoles. The e and variously sized vacuoles of GN and A cells were found towards periphery of the cells. Identical observations have been made by various ors in several other crustaceans (Matsumoto, 1954; Parameswaran, 1956; dy and Kolwalker, 1985). Peripheral vacuoles forming pericellular olar ring was observed in the B & C type NSCs of P. monodon. Similar of observations has been reported in P. indicus were also observed hammed et al. (1993).

Electron-microscopical observations in the NSCs of P. monodon g the gonadal maturation showed, the ultrastructural features of these ells during the secretory cycle. The activation of the sub-cellular etic mechanism occurred in the NSCs during the process of gonadal ation. Unfortunately there is no published information available about ecretory activity of the NSCs during the process of gonadal maturation ustaceans. However, Mc Namara, (1993) described the activation of the llular machinery in the NSCs of M. olfersii exposed to a high salinity.

The ultrastructure of the perikarya of the NSCs particularly GN type cells in the present observation, was found to be similar to observed in P. paucidens by Hisano (1976) in the isopod S. serratum nataigner et al. (1978) and in M. olfersii by Mc Namara (1993). It

s observed that perikarya of the NSCs at their quiescent phase contained ly electron lucent cytoplasm with few numbers of cell organelles. Even ough ultrastructural studies are not available on these aspects, light croscopical studies of Gyananath and Sarojini (1985) in M. dobsonii, abhushanam et al. (1992) in M. affinis and Mohammed et al. (1993) in P. dicus reported that the NSCs are with smooth cytoplasm and less synthetic ivity during their resting phase. The ultrastructural studies of Mc Namara 93), in M. olfersii showed less cytoplasmic activity in the NSCs during resting stage of the animal. According to him (Mc Namara, 1993) the rikarya of NSCs at their resting phase did not contain much sub-cellular anelles or secretory materials.

However, drastic changes were observed in the ultrastructure of vacuolar phase NSCs, especially that of the GN and A cells of P. monodon, ing the present investigation. The characteristic features of the olar phase NSCs were the highly active cell organelles and the abundant ply of variously shaped vacuoles of different sizes. The highly active -cellular machinery of these vacuolar phase NSCs indicated its active e in the neurosecretion. Similar types of neurosecretory cells with highly ive cell organelles were described in M. olfersii after its introduction a higher salinity (Mc Namara, 1993). According to Mc Namara (1993), the arance of the highly active sub-cellular organelles and variously sized shaped vesicles are indications of the activations of sub-cellular inery for the synthesis of its secretory products. In the present rvation also it was noticed that these vesicles and vacuoles later on converted into vesicular bodies packed with neurosecretory granules. larly, variously shaped vacuoles were described in association with cisterns of RER and Golgi bodies in the NSCs of P. paucidens by Hisano 6) and in the isopod S. serratum by Chataigner, et al. (1978).

The perikaryon of the B & C type of NSCs in P. monodon also showed more or less similar characters during the vacuolar phase. However, the cuoles as well as the cell organelles were not so prominent as that observed the other two cell types. However, in M olfersii, Mc Namara (1993) ported that, the perikarya of all the NSC types showed similar trastructure during its osmotic adaptations to various external salinities. m the present investigation it was evident that the subcellular machinery the B & C type of NSCs was not activated to the same extent as that of er GN & A cells for the synthesis of secretory granules. However, a few l organelles and empty vacuoles were encountered in the perikaryon of ells, but the C cells did not show even this.

In contrast to the vacuolar phase NSCs, the ultrastructure of the retory phase NSCs of P. monodon during the present study, contained erous thick and highly electron-dense secretory granules and granule filled e vesicles along with the acitve cell organelles. In these cells no empty vesicles are encountered, however, the fully packed secretory ules gave the high electron-density to these cells. A remarkable increase the synthetic activity of the neurosecretory granules was reported in NSCs of M. olfersii when exposed to seawater (Mc Namara, 1993).

The NSCs of P. monodon during the present investigation showed increased synthetic activity during its secretory phase followed by the olar phase. Similarly a high synthetic activity was noticed in ciation with the Golgi complex as well as cisternae of RER of these NSCs e abundant vacuoles of varying sizes and shapes were apparent (Mc Namara, ), and he further observed that these vesicles act as transport vesicles g the synthetic activity of the secretory materials. These transport les with electron-dense flocculent materials, underwent some structural fications at the proximity of the Golgi complex and near the RER and

ot modified into coated vesicles and some into granule filled dense vesicles Mc Namara, 1993). Secretory activity was more pronounced in GN and A type ells of P. monodon in contrast to the Mc Namaras observations in M. olfersii here he observed an increased synthetic activity in all the cell types. owever, the subcellular machinery and its synthetic mechanism was found re or less similar in both of these crustaceans. Sub-cellular organelles ke Golgi complex as well as cisterns of RER were found actively rticipating in the synthesis of secretory materials in the NSCs of P. nodon and from the observation it is obvious that the vacuoles found in he preceding V-phase have specific and important roles in the process of urosecretion. A series of structural and functional transformations were oticed in these vesicular bodies during the synthesis of neurosecretory terials in the NSCs of P. monodon during the present investigation.

As in the NSCs of M. olfersii described by Mc Namara (1993), the lgi complex are the most actively participating sub-cellular organelle the NSCs of P. monodon during the synthesis of neurosecretory materials. milar to the observations of Hisano (1976) in P. paucidens and Chataigner t al. (1978) in S. serratum and Mc Namara (1993) in M. olfersii the cisterns RER was the most dominating type of cell organelle found in the secretory pe NSCs of P. monodon. Here the commencement of neurosecretion was found these cisternae of RER and the small vesicles associated with them. Later , some of these vesicles with moderately electron-dense flocculent materials e found to be moved to the proximity of Golgi bodies as noticed in M. fersii (Mc Namara, 1993), get modified into the secretory vacuoles with me structural modifications. The highly active sub-cellular organelles scribed in the freshwater prawn P. paucidens by Hisano (1976) and in the opod S. serratum by Chataigner et al. (1978) supports the present servation, even though these authors have not described the formation

of the secretory granules. In contrast to M. olfersii, synthesis of neurosecretory materials was found also in association with the active mitochondria in the NSCs of P. monodon during the present investigation. Abundant supply of small free and poly-ribosomes in the cytoplasm as well as attached to the ER elements was a characteristic feature noticed in the secretory phase NSCs of P. monodon during present investigation. Similar observations have been made in the NSCs of other crustaceans by Hisano (1976), Chataigner et al. (1978) and Mc Namara (1993). The most dominating feature noticed in the secretory phase of NSCs of P. monodon during this study was the five various types of neurosecretory granules. Similarly in the NSCs of the eyestalk of P. paucidens, Hisano (1976) has described 6 various types of neurosecretory granules. Synthesis of a few moderately electron -dense materials was noticed in the B & C type NSCs during secretory phase. However, the perikarya of these NSCs did not show an increased synthetic activity like that of GN and A cells in P. monodon during the process of gonadal maturation.

The role of neuroglia in the secretory cycle of P. monodon was found similar to that in P. indicus described by Mohammed et al. (1993). Some indications of glial activity has been reported by Lake (1970) in the crab Paragrapsus gaimardii. During the present study it was found that the glial cells surrounding the NSCs become hypertrophied during vacuolar phase of the NSCs and give a broken appearance to the plasma membrane of the NSCs during its secretory phase. The glio-neuronal relationship is vague in crustacean NSCs. However, Mohammed et al. (1993) reported that glial cells may serve as a source of nutritive material during the synthetic phase of the NSCs and they may also serve as a media between the NSC and the surrounding capillary network, thus giving a glio-vacuolar relationship similar to one existing in the vertebrates (Gless and Meller, 1969).

Ultrastructural investigations in these glial cells surrounding the NSCs of P. monodon showed a 5 layered membraneous system with numerous RER elements. This is indicative of their role in the transport of materials to the circulatory cells. Some sort of synthetic activity was also found in the membrane systems surrounding the active nuclei. Nuclear and nucleolar participation in the cellular synthetic activity is well established in all active cells (De Robertis et al., 1975). Whereas in the light microscopic studies of Mohammed et al. (1993) in P. indicus the nuclear involvement was found only in the B & C type cells. However, participation of nucleus has also been observed in the synthetic activity of these neurosecretory materials from nuclear pores and aggregations of nuclear materials around these pores in M olfersii (Mc Namara, 1993).

Mapping of the NSC types in different ganglionic centers of decapod crustaceans was thoroughly investigated in pleocyematans by Adiyodi and Adiyodi (1970) and in penaeids by Mohammed et al. (1993). The neurosecretory cell groups of various ganglionic centers of other penaeids are meagre. Present investigation on the cerebral ganglion of P. monodon revealed that it is richly endowed with an abundant supply of NSC groups of different types of NSCs. A total of 14 NSC groups were found in the cerebral ganglia of P. monodon during the present study, whereas Nakamura (1974) observed only 8 NSC groups in P. japonicus and Mohammed et al. (1993) observed 11 NSC groups in the cerebral ganglia of P. indicus. The location of the NSC groups in the cerebral ganglion of P. monodon was basically similar to those observed in P. japonicus, P. stylifera, P. indicus and Caridina laevis by Nakamura (1974), Nagabhushanam et al. (1986) Mohammed et al. (1993) and Pillai (1961) respectively. However, no central dorsal group, as found in P. indicus, was found in the cerebral ganglion of P. monodon. Here 6 NSC groups were located in the dorsal side and 8 NSC groups

in the ventral side. Mohammed et al. (1993) observed only 5 NSC groups in the dorsal and 6 in the ventral side of the cerebral ganglion.

With regard to the remaining ventral ganglia there is surprisingly only one study in penaeids by Mohammed et al. (1993) in P. indicus. The tritocerebral ganglion of P. monodon contained one or two large GN and one or two medium sized A cells. In contrast, in crabs Sesarma dehaani (Enami, 1951) and Paragrapsus giamardi (Lake, 1970) the tritocerebral ganglia contain only medium and small sized NSCs. However, in the tritocerebral ganglia of P. indicus, Mohammed et al. (1993) usually found one and occasionally two GN cells and a medium sized A cell. The sub-oesophageal ganglion of P. monodon contained a total of 16 NSC groups. By virtue of its size, magnitude and number of NSC groups, the suboesophageal ganglion of P. monodon is comparable to the suboesophageal ganglion of P. indicus (Mohammed et al. 1993) and fused thoracic ganglion of pleocymatans (Adiyodi and Adiyodi, 1970). All the four neurosecretory cell types were found in this ganglion with a slight dominance of GN and A cells, whereas 15 NSC groups were found in the sub-oesophageal ganglion of P. indicus and here the GN and A cells are the dominant types Mohammed et al. (1993). Thoracic ganglia of P. monodon contained 4 large NSC groups with a prominent dominance of GN and A cells. In P. indicus (Mohammed et al. 1993) also reported 4 NSC groups. Similarly 4 NSC groups with A, B and C type cells were identified in the abdominal ganglia of P. monodon during the present investigation which are found to be identical in P. indicus (Mohammed et al. 1993).

There is apparently a general agreement on the major structural components of the dendrobranchiate eyestalks, but controversy exists with regard to the NSC groups associated with the ganglia. The major controversy

ists in the presence of MIGX-organ. Some are of the opinion that it exists all, 1965; Hisano, 1976; Nanda and Ghosh, 1985; Dietz, 1987) while others not (Nakamura, 1974; Van Herp, 1977; Mohammed et al., 1993). During e present investigation such as NSC was not observed in the MI. The screpancies are perhaps due to incorrect identification. There are 3 C groups in the optic ganglia of P. monodon viz. the MTGXO I, MTGXO II, d MEGXO. This is not similar to the observations made by Nanda & Ghosh 985) in the same species. They found 4 NSC groups in the optic ganglia P. monodon. However, during the present investigation the MTGXO II was nd scattered over the dorsal side of the MT towards the MI, and sometimes ese NSC may have been identified by Nanda and Ghosh (1985) as the MIGXO. milar observations have been reported by other investigators like Van rp et al. (1977) in P. serratus, Nakamura, (1974) in P. japonicus; Dietz 982) in M. rosenbergii and Mohammed et al. (1993) in P. indicus.

The sensory and glandular organ of Bellonci (Sensory pore X-organ) the pars distalis X-organ was not observed in the eyestalk of P. monodon ring the present observation. This observation is consistent with the orts in other dendrobranchiates by several workers (Van Herp, 1977 and hammed et al., 1993). In contrast Nanda and Ghosh (1985) observed the organ Bellonci or (Sensory pore X-organ) at the adaxial part of MT of P. nodon. During the present investigation small dispersed NSC groups of GXO II with a dominance of B & C type cells were found at the adaxial part MT. Similarly Smith and Naylor (1972) stated that the organ of Bellonci distinct in dendrobranchiates but maybe absent in others. However, similar the present observations Dall (1965) has reported that in Metapenaeus , there is no characteristic onion like bodies. Paradoxically in decapod self the organ is diversly located ie. at the level of ME in Lysmata

ticaudata (Carlisle, 1953), at the ME in P. serratus (Pasteur, 1958), closed within the ME, in P. paucidens (Hisano, 1974), within the MT in ndalus borealis (Carlisle, 1959) and superficial and partially surrounded MT in P. serratus (Van Herp, 1977) and in M. rosenbergii (Dietz, 1982).

The most striking feature noticed in the NSC groups in the optic nglia of P. monodon is the total absence of GN cells. MEGXO of P. monodon a well defined NSC group with 3 different cell types viz. A, B and C cells. tive NSCs were found in the MEGX-organ of immature animals. However, l these cell types were at their quiescent stage during the process of productive maturation. Similarly NSCs in the ME form a well defined oup in Natantia such as Pandalus borealis (Carlisle, 1959), P. serratus mbert, 1965; Van Herp, et al., 1977), P. paucidens (Hisano, 1974, 1976) d P. japonicus (Nakamura, 1974). However, in other Natantia, such as phlatya garciai (Juberthie-Jupeau, 1976) and Atyaephyra desmaresti (Boissou al., 1976) the NSCs in the MEGXO are more or less scattered in the ME. ly two different cell types were reported in the MEGXO of crabs by tsumoto, (1958), in Paragrapsus giamardii by Lake, (1970) and in Palaemon ratus by Humbert et al. (1981). During the present investigation 3 ferent types of NSCs were identified in the MEGXO of P. monodon. Similarly different NSC types were identified in the penaeid prawn P. indicus by ammed et al. (1993). Whereas ultrastructural studies of Hisano (1976) P. paucidens revealed 6 different types of NSCs.

The present study showed that MTGXO I is composed of a small NSC up located at the dorsal side of the MT near the SG. It contained the ee A, B and C types of NSCs. Similar descriptions has been given for MTGXO of the same species by Nanda and Ghosh (1985) and also for P. icus by Mohammed et al., (1993). The MTGXO II of P. monodon during

present investigations is found to be composed of a cluster of NSC groups ated at the opposite side of the MTGXO I. Here a few NSC are found to e invaded the dorsal part of the MT region. This cell group may have n identified as the MIGXO by Nanda and Ghosh (1985) in P. monodon. ever, in P. indicus Mohammed et al. (1993) described this as MTGXO II ch is similar or identical with the observations made here in P. monodon.

The sinus gland (SG) of P. monodon appeared as an elongated finger e body, associated with one of the vascular process in the optic ganglia ing the present investigation. Bulbous axonal endings of various shapes size and the internal blood sinus are the major components of the SG P. monodon. Similar findings have been made by Nanda and Ghosh (1985) le describing the SG in P. monodon. Mohammed et al. (1993) also cribed a similar type of SG in P. indicus. The structure of the SG P. monodon as well as its dorso-lateral position in between the MI and closely resembles the findings of earlier workers in other crustaceans ll, 1965; Nakamura, 1974 and Van Herp, 1977). The 'S' shaped X-organ s gland tract in P. monodon investigated here, has been found to be tical to that of other decapod crustaceans (Adiyodi and Adiyodi, 1970). ng the present investigation it was found that the axon bundles from O I and MTGXO II unite at the level of MT and then moves dorsally through space in between the MI and ME and on the way it joins with the axon le from the MEGXO before entry into the SG. The pathway observed in present study clearly indicated that the mode of discharge of osecretory material in the eyestalk is via the axonal transport. Based the electro-physiological and cobalt ion ionophoretic studies of Andrew Saleddin (1978) in Orconectes virilis and Jarose (1978) in O. limosus d a more or less similar pathway for the X-organ sinus gland tract.

Ultrastructural studies carried out here revealed that the SG P. monodon is composed of axons which terminate in the bulb like largements just beneath the basement membrane lining the blood sinus. ed on the ultrastructural studies the earlier workers described a similar ucture for the SG of various crustaceans like Procambarus clarkii by Bunt Ashby (1967), Uca pugilator by Silverthorn (1975), Carcinus maenas by dmann (1977) and Ligia oceanica by Martin et al. (1983). Ultrastructural minations of the sinus gland also showed five different types of axon lings in P. monodon based on morphology of the granules occupying the axon. s observation is comparable to the sinus gland of other decapods. However, y three types in Porcellio dilatatus (Martin, 1972) and two types in arcinus lateralis (Hodge and Chapman, 1958), Camberallus shufeldti ngerman and Aoto, 1959) and O. nais (Shivers, 1969).

In crustacea unlike the vertebrates and other invertebrates like ects, very few studies have been made to correlate changes in the rosecretory system with physiological events particularly reproduction, though neurosecretory elements are known to control this phenomenon yodi and Adiyodi, 1970). Similarly information regarding the sub-cellular inery responsible for the synthesis of neurosecretory materials in tion to reproduction are lacking in penaeids. Light and tronmicroscopical studies carried out on the NSCs of different ganglionic ers during the process of maturation revealed concomitant changes in activity of NSCs. Demassieux and Balesdent, (1977) have reported a ic variation in function of certain NSCs in Ascellus aquaticus in tion to reproduction. Gyananath and Sarojini (1985) delineated the cyclic vity of NSCs in relation to reproduction in a freshwater prawn, M. rii. In P. indicus, Mohammed et al. (1993) described similar types of

Light microscopical investigations revealed that the neurosecretory cells of the supra-oesophageal, sub-oesophageal and thoracic ganglion were highly active in the early maturing and late-maturing animals of P. monodon in the present study. However ultrstructural investigations showed a slight discrepancy in their activity during the gonadal maturation. It was found that the NSCs of brain showed its maximum secretory activity during early-vitellogenic phases. However, NSCs of suboesophageal and thoracic ganglion were maximally active in the late maturing P. monodon. In the thoracic ganglia of crabs, Matsumoto (1958) reported that the NSCs were maximally active in its early stages of maturation. Similarly in the crayfish Procambarus simulors, Perryman (1969) correlated the stages of ovarian development with varying amount of neurosecretory material in cell type III of the cerebral ganglion. Diwan and Nagabhushanam (1974) demonstrated a seasonal activity in the thoracic ganglia of the freshwater crab Barytelphusa cunicularis. Such a high secretory activity during ovarian maturation has also been observed in specific NSCs of the thoracic ganglion of Macrobrachium lanchestrii (Rao et al., 1981), M. kistensis (Mirajkar et al., 1983) and Potamon koolooense (Joshi, 1989).

CHAPTER IV

# NEUROENDOCRINE MANIPULATIONS AND THEIR IMPACT ON GONADAL MATURATION

A *Influence of eyestalk ablation on ovarian maturation*

B *Effect of ganglionic extract injections on ovarian maturation*

C *Effect of synthetic hormone injections on ovarian maturation*

# CHAPTER IV

## NEUROENDOCRINE MANIPULATION AND THEIR IMPACT ON GONADAL MATURATION

### ntroduction

Successful domestication of the candidate species is the foot step any economically viable culture programmes. The key to domestication es in controlled and enhanced reprodution of the broodstock animals. w, the aquaculture technology of penaeid prawns has developed remarkably d at present the prawns are cultured on a large scale in captivity. The olution of modern shrimp culture demands captive reproduction and seed oduction through larviculture in hatcheries at required time. However, e fundamental problem in shrimp mariculture industry is the lack of edictable abundant supplies of offsprings of known heritage. The growing mand for quality prawn seed from the farmers and entrepreneurs, coupled th uncertainty of their availability from nature at the appropriate time required quantities has prompted research on problems connected with awn seed production.

In many decapod crustaceans, control of gonadal maturation is a major oblem in developing commercial aquaculture programmes, (Yano, 1992). The ant tiger prawn Penaeus monodon is one of the largest and hardiest penaeid ecies in the world, and it has been identified as the most favourable ndidate species for intensive aquaculture by many of the countries. The st successful technique for inducing maturation in captivity, has been e removal of the eyestalk which is known to be the site of production and orage of gonad inhibiting hormones (Fingerman, 1987).

The classical experiments of Panous, (1943) on the female shrimp der serratus demonstrated for the first time that, removal of eyestalk ng sexual inactivity led to the rapid increase in ovarian size and cious egg deposition. His (Panouse, 1943), studies have been elaborated last 40 years in various crustaceans, such as Pandalus kessleri (Aoto Nishida, 1956), Carcinus meanas (Demeusy, 1967), Scylla serrata (Rangnekar Deshmukh, 1968) Cragon cragon (Bomirski and Klek, 1974), Barytelphusa cularis (Nagabhushanam and Diwan, 1974), Panulirus argus, (Quackenbush Harrnkind, 1981), Uca pugilator, (Quackenbush and Keeley, 1988). However importance of this finding to penaeid shrimp culture lay dormant for ears until the eyestalk removal/gonadal development principle was applied naeus duorarum by Caillouet, (1972). Later a flurry of research activity similar lines ie. by applying unilateral eyestalk ablation to various eid species, under various conditions continued until recent times and of the worth mentioning contributions made on these lines are by tein and Beard (1975); Alikunhi et al. (1975); Aquacope (1975); Muthu Laxminarayana (1979); Lumare (1979); Emmerson (1980); Kulkarni and hushanam (1980); Primavera (1982); Chamberlain and Gervais (1984); Choy 7); Wyban et al. (1987) and Mohammed and Diwan (1991).

The successful application of the eyestalk ablation technique to ce maturation and spawning of P. monodon under controlled conditions been a commercial practice in many of the Indo-Pacefic countries where s present in large quantities in natural seas. However, the percentage nimals getting fully matured and spawned in case of P. monodon is still in our country, and problems are more acute especially in areas where natural population of this species is comparatively less. Even though are reports about the achievements of maturation from pond reared

P. monodon, the farmers still depended on the capture of wild ones, Liao, (1992). So there is still a need to refine eyestalk ablation conceptual chnique particularly for this species. Therefore eyestalk ablation periments were carried out in different environments to find out its effect maturation in this species.

In decapod crustaceans gonad maturation and function are profoundly fluenced by two antagonistic hormones one stimulating, presumed to be from ain & thoracic ganglion and the other inhibiting, from the eyestalk. rious investigations are under way to determine the roles of hormones on arian maturation which may lead to practices of induced maturation in cordance with the need for increased aquaculture practices. Many workers ggested that ovarian maturation is regulated by a gonad-inhibiting hormone om the X-organ sinus gland complex of the eyestalk, (Bomirski et al. 1981; ackenbush and Herrkind, 1983; Meusy et al. 1987, Mohammed, et al., 1991).

In crustaceans, vitellogenin (Vg) is the precursor of egg protein d is a necessary prerequisite for the ovarian oocytes to reach full turation (Chang et al., 1993). Hormonal regulation of synthesis of Vg is ll documented in oviparous vertebrates, (Tata, 1978). Knowledge of hormonal duction of gonadal development in crustaceans, however is fragmentary. su, (1960) reported that accumulation of yolk granules in the oocytes s stimulated by repeated implantation of pieces of the thoracic ganglion immature female crab Potamon dehaani. Oyama (1968), Hinch and Bennett 79), Nagabhushanam Kulkarni (1982), Eastman-Reck and Fingerman (1984) Takayanagi et al. (1986) reported similar effects of the thoracic glion on ovarian maturation in in vivo and in vitro experiments in various staceans. Yano et al. (1988) demonstrated that yolk accumulation of P. namei can be induced and accelerated by implantation of thoracic ganglion

rom maturing female lobster Homarus americanus. Similarly advancement of arian stages were demonstrated in P. vannamei by injection of brain extract ano and Wyban, 1992) and in P. japonicus by injection of thoracic ganglion tract (Yano, 1992) from maturing females. Paradoxically, several of the ustacean reproductive hormones are only postulated, but direct chemical idences and its regulatory activities are lacking (Aiken and Waddy, 1980). in the present study investigations were made to find out the effect of ese ganglionic extracts on gonadal maturation in the tiger prawn P. monodon.

An area of research where enticing prospects lie both from the int of view of basic and applied research, is the study of role of the eroids in the reprodution of invertebrates. Involvement of ovarian steroids reproduction in higher vertebrates is well understood but the role of mones in invertebrates especially in crustacean reproductive biology still unknown. In crustaceans the steroids are apparently necessary r moulting (Stevenson et al. 1979) and reproduction (Adiyodi, 1978). Little k has been done with regard to the effect of exogenous administration steroids on gametogenesis in crustaceans (Kulkarni et al. 1979, 1992; abhushanam et al. 1982 and 1987; and Koskela et al. 1992).

Various steroid hormones have been identified in the ovaries of apod crustaceans including estrogen in marine invertebrates (Hagerman al. 1957), estrone in P. monodon (Fairs et al. 1990), the estrogenic pound 17B-estradiol in H. americanus (Couch et al. 1987) and in Nephrops vegicus (Fairs et al. 1989) and progesterone and its related compounds, Panulirus japonicus (Kanazawa and Teshima, 1971); in Astacus leptodactylus leveir et al. 1986); in H. americanus (Couch et al. 1987); and in Nephrops vegicus (Fairs et al. 1989). Occurrence of precursors of steroid hormones enzyme systems involved in the steroid metabolism has been reported in

ustaceans (Kanazawa and Teshima, 1971 and Teshima and Kanazawa, 1971). nera et al. (1977) first proposed that a vitellogenin stimulating ovarian mone was present in the ovary of the amphipod, Orchestia gammarella. nce then progesterone (Kulkarni et al., 1979; Yano, 1985), and 17a- hydroxy gesterone (Nagabhushanam et al., 1980; Yano, 1987; Tsukimura and Kamemoto, 88 and Koskela et al., 1992) have all be implicated in this role. More ently, D'croz et al. (1988) isolated 5 prostaglandins and three related pounds from the polycheate Americonuphis reesei, which is used as a dietary plement to accelerate ovarian maturation of penaeid prawns in Central erica. However, the detailed mechanism of ovarian induction by steroids crustaceans is still unknown. Involvement of estrogenic compounds in tellogenesis and development of egg has been suggested by Couch, (1987) d Ghosh and Ray, (1992). To see the effects of exogenous hormones, Viz. lpha - progesteron and estradiol on gonadal development of penaeid prawn monodon, a detailed investigation was made.

## MATERIALS AND METHODS

### Collection and acclimatization of animals

Live adult male and female tiger prawns, Penaeus monodon ranging from 180-270 mm in total length with immature ovaries were used for the present investigations. Animals were collected from two sources, culture ponds and the sea. Five to six month old cultured prawns were collected from filtration ponds around the Cochin back waters. Wild animals both male and female were collected from a number of trawling operations in the Gulf of Mannar, off Mandapam. Healthy animals were selected and carefully transported to the laboratory in well aerated containers. Later they were transferred to 1 tonne capacity tanks provided with a sand bottom gravel filter where water is injected through perforated pvc pipes which allows a self cleaning action and avoid reduction of substrate. Water level in the tanks were maintained at 2 m depth. Hydrological parameters such as temperature, pH, salinity and dissolved oxygen were monitored everyday. The stock was fed twice in a day with frozen squid and fresh clam meat ad libitum. At least one week acclimatized prawns were used for the experimental purposes. After the acclimatization period, animals were subjected to different endocrine manipulations such as eyestalk ablation, injection of different ganglionic extract and synthetic hormones.

### Influence of unilateral eyestalk ablation on gonadal maturation

Several important species of penaeid prawns including P. monodon have been induced to mature and spawn in captivity by the well known eyestalk ablation technique. However, the percentage of animals getting matured varied greatly due to the lack of understanding of the actual mechanism involved. Therefore, in the present study three series of eyestalk ablation experiments

were conducted (fig. 198). The first series of experiments were conducted in full strength seawater (S 34%.) using wild stock animals. The second series of experiments also were conducted in full strength seawater (S 34%.) using animals collected from the filtration ponds. The third sereis of experiments were carried out at a low salinity (S 20% ) using animals collected from the filtration ponds.

**1. Eyestalk ablation in wild stock P. monodon at full strength seawater (S 34% )**

These experiments were done in the out door prawn hatchery of CMFRI, Mandapam camp. Adult P. monodon collected from the offshore waters of Gulf of Mannar coast near Mandapam camp were used for eyestalk ablation experiments. As soon as shrimps were brought to the laboratory, a quarantine treatment of 20 ppm formalin for half an hour was given to them and they were later kept in well aerated seawater tanks for acclimatization for a period of 20 min. Ten numbers of treated prawns ranging from 210-270 mm were subjected to unilateral eyestalk ablation using an electro-cautery apparatus at 12 volts. Advantage of this method over other crude eyestalk ablation techniques is the simultaneously sealing ofthe wound, resulting in cent percentage survival (Muthu and Laxminarayana, 1982). Two eyestalk ablated females and one unablated male were housed together in each one tonne capacity tanks fitted with sub-gravel biological filter. Like wise, five sets each containing two female prawns were arranged. A water depth of 2 m was maintained in the tanks throughout the experiment. Water temperature in the experimental tanks varied between 25-29°C but pH of the water was regulated between 8-8.2 Compressed air was supplied continuously to the experimental units through air lift pumps and free diffuser stones. Prawns were fed ad libitum with frozen squid meat and fresh clam meat. Tanks were

hon-cleaned every morning by replacing with fresh seawater (salinity 34%). ht intensity was reduced by covering the tanks with black cloth. To uce stress, tanks were previously painted in black. Simultaneously one containing two unablated females and one male were housed in another k as control under similar experimental conditions. All the females were erved once in three days for assessing ovarian development for a period 30 days.

**Eyestalk ablation in P. monodon at full strength seawater (S 34% ) using animals from filtration ponds**

Using the animals collected from the filtration ponds of Vypeen land near Cochin, eyestalk ablation experiments were done in the in door wn hatchery of CIBA Narackal. Properly acclimatized twenty healthy adult ale prawns were selected and the above described quarantine treatment 20 ppm formalin was given to them. Ten numbers of selected animals nging between 190-240 mm in total length were subjected to unilateral estalk ablation and divided into five groups, each containing two eyestalk lated females. Two eyestalk ablated females and one unablated male housed gether in each one tonne capacity tanks fitted with sub gravel biological lter. A water depth of 2 m was maintained in each tank. Water temperature the experimental tanks varied from 28 to 30 °C and pH between 8.00-8.2. linity in the tanks was 34%.. Every morning about 60% of the water in e tanks along with the sediments and faecal strands were siphoned-out d replaced with fresh filtered seawater. Experimental parameters were nitored as described in the earlier experiment. Two unablated females th one male housed in another tank under similar experimental conditions re kept as control group. The assessment of the ovarian development was ne periodically. These experiments were conducted for a period of one month.

**Eyestalk ablation in P. monodon at low saline (S 20‰.) conditions using animals from filtration ponds**

In order to study whether eyestalk ablation can induce gonadal turation at lower salinities, experiments were conducted at a salinity 20 ‰ . Adult prawns acclimatized to laboratory conditions were used for is experimental purpose. Among the 20 animals selected half of them re used for eyestalk ablation. The remaining animals reared under the milar experimental conditions without eyestalk ablation formed the control. imals ranging from 180 to 210 mm size were used for eyestalk ablation. lated female prawns were housed with unablated males in the ratio of 2:1 1 tonne capacity tanks provided with a biological filter. The experimental ocedure was similar to that described earlier in section A.1 of this apter with exception to the salinity. Throughout the experimental period e salinity was maintained at 20 ppt in both groups of experiments. Every rning about 60 % of the water was siphoned out and replaced with fresh ‰. seawater. Water temperature in the tanks varied between 28-30°C and between 8 - 8.2.

The total experimental duration was 30 days. The impact of unilateral estalk ablation on gonadal development in all the three groups of animals z. 1, 2 and 3 were assessed. The development of gonad was determined ternally by holding each female prawn against a powerful light and observing e state of the ovaries through the cephalothorax and abdomen. Fully matured imals were transferred to conical spawning tanks of 100 l capacity during e night hours. Egg counts of spawned ones were taken in the morning hours the following day. Animals which did not spawn, were sacrificed for the sessment of gonadal development after 30 days of the experiment. rphological features like colour, nature, and development of ovarian lobes

re noted and small pieces of ovarian tissue were fixed in Bouins fixative r histological evaluation. Staging of gonadal development was done based the descriptions given in chapter

## EFFECT OF GANGLIONIC EXTRACT INJECTIONS ON OVARIAN MATURATION

In order to assess the effect of ganglionic factors on gonadal turation the following ganglionic extracts were prepared and injected into lt immature female P. monodon.

### Preparation and storage of different ganglionic extracts using- cerebral, thoracic and optic ganglion

Twenty female green tiger prawns, Penaeus semisulcatus with vitellogenic aries were collected from trawlers operating in the Gulf of Mannar. Size the prawns collected were ranged between 80g - 90g. Cerebral, thoracic d optic ganglia of these prawns were excised separately and rinsed in stacean saline solutions. These ganglionic masses were homogenized parately in 5ml of saline solution and centrifuged at 10,000 rpm for 15 nutes. After centrifugation the supernatant was removed and stored at 6 °C until use, as the crude tissue extract. To evaluate the effects these ganglionic extracts on maturation, different experiments were planned follows.

### Injection of various crude ganglionic extracts

Twenty five numbers of adult immature females collected from offshore ters of Gulf of Mannar were grouped into 4 groups of 5 animals each. Females e housed with males in the ratio of 2:1 and a quarantine treatment of pm formalin was given before starting the experiments. Different intra scular injections were given to each group as follows.

## II.1 Cerebral ganglion

Each prawn in group I was injected with one ml of crude extract of cerebral ganglion. Three injections were given in the ascending concentrations of 100 µl, 300 µl and 600 µl to each animal respectively, on alternate days. Injections were given through the lateral somite of the abdomen with the help of a 27 guage needle with a hypodermic syringe. Each ml of the stock solution contained the extract from the 4 cerebral ganglions. Two injected females with one male were housed in each one tonne tank.

## II.2 Thoracic ganglion

Each prawn in group II was injected with 1 ml of crude thoracic ganglion extract containing materials from 4 thoracic ganglia. Three successive injections were given in an ascending concentration of 100 µl, 300 µl and 600 µl. respectively, on alternate days to each animal through the lateral sides of the 1st abdominal somite with the help of a 27 guage needle with a hypodermic syringe. Two injected females with one male were housed in each one tonne capacity tank.

## II.2 Optic ganglion

1 ml of optic ganglion extract containing materials from 4 pairs of optic ganglia were injected to the third group of animals by three successive injections as described above. Here also injections were given in ascending concentrations of 100 µl, 300 µl and 600 µl respectively on alternate days using 27 guage needle with a hypodermic syringe. Two injected females with one male were housed in each one tonne capacity tanks.

The fourth group of animals were injected with 1 ml of saline solution alone and kept as control. Here also 1 ml of saline was injected in three

ccessive injections of 100 ul, 300 ul and 600 ul respectively, on alternate ys. The same type of 27 guage needle with a hypodermic syringe was used r the injection which was administered through the lateral side of the dominal somite. Two injected females and one male were housed together a ratio of 2:1 in each one tonne tanks.

Experimental procedures described in section A.1 of this chapter were llowed in all these four experimental trials. Experiments were carried t for a period of 30 days. Eyestalks of the prawns injected with crude tracts of ganglia were not ablated in these experiments. Animals were served once in 3 days under a powerful light source and the fully matured imals were transferred to conical spawning tanks of 100 l capacity during ght hrs. Egg counts were done on the following day in spawned animals. imals which did not spawn were sacrificed for the assessment of gonadal velopment after 30 days of experiment. Morphological features like colour, ture and development of ovarian lobes were noted and small pieces of ovarian ssue fixed in Bouin's fixative for histological evaluation.

## EFFECT OF SYNTHETIC HORMONE INJECTION ON OVARIAN MATURATION

Twenty numbers of adult female P. monodon ranging from 200-260 mm in tal length with immature ovary collected from the offshore waters of Gulf Mannar were used for these experiments. After the acclimatization period imals were divided into 4 groups each containing 5 animals. Two females re housed with one male after a quarantine treatment of 20ppm formalin s given before starting the experiments. Different intramuscular injections re given to each group as follows.

**1. 17a- Progesterone**

The group I animals were injected with a total of 1 ml of progesteron luted in 10% ethanol. 1 ml containing 250 µg was given through 3 ccessive injections in increasing concentrations of 150 µl, 300 µl and 0 µl respectively on alternate days.

**2. Estradiol**

Each prawn in group II was injected with a total of 1 ml of estradiol luted with 10% ethanol containing 800 µg of estradiol. A total of three jections were given in increasing concentrations of 150 µl, 300 µl and 0 µl in the above experiment.

Animals in group III were injected with 10% ethanol as described ove and kept as control I. While the animals in group IV were kept as ch without any injections or eyestalk ablation as control II. In the rst three sets, injections were given through the lateral side of the dominal somite using 27 guage needle with a hypodermic syringe. drological parameters were the same as those described in section A.1 of is chapter. Temperature in the experimental tanks varied between 28-30°C t pH was regulated between 8-8.2. Salinity of the water was 34% . periments were carried out for 30 days. Animals were examined once in ree days for ovarian development under a powerful light source till the rmination of the experiment. At the termination of the experiments animals re sacrificed and dissected for morphological studies and small pieces ovarian tissues were excised and fixed in Bouin's fixation for istological examination.

All results were analysed for significante using nalysis of variance (ANOVA) and least significant difference.

## RESULTS

In decapod crustaceans most of the physiological processes, luding reproductive maturation, are controlled by the neuroendocrine tem. The present cytological studies conducted in the neuroendocrine ans in relation to gonadal maturation, revealed that different NSCs play ignificant role in controlling gonadal maturation. Therefore, experiments e carried out by administering the crude extracts of various ganglionic ters like cerebral, thoracic and optic ganglion to see the effects of crine factors originating centers on gonadal maturation in P. monodon. ects of exogenous synthetic hormones were also assessed experimentally administering them intra-muscularly.

### Influence of unilateral eyestalk ablation on gonadal maturation

The relationship between the removal of a one eyestalk of female apod crustacean and ensuing gonadal development was a well known fact. most commonly accepted theory is that, a gonad inhibitory hormone is duced in the X-organ/sinus gland complex and in order to check the effect the eyestalk factors on maturation, eyestalks of adult immature females lected from different sources were ablated using electro-cautery apparatus. first series of experiments were carried-out in full strength seawater 34%.) using wild animals collected from open sea. The second series of eriments were also conducted in full strength seawater (S 34 %.) using mals collected from filtration ponds. The third series of experiments e carried out in low saline conditions (S 20 %.) using animals collected m filtration ponds.

## 4.1 Eyestalk ablation in wild stock *P. monodon* in full strength seawater (S 34 ‰.)

These experiments were carried out using wild animals collected from offshore areas of Mandapam. Adult female prawns with undeveloped ovaries were subjected to unilateral eyestalk ablation. Animals collected from the same area, with similar biological characters and reared under the similar experimental conditions but without eyestalk ablation served the control. Results of the experiment are presented in the table (4). At the termination of the experiment, no apparent ovarian development or spawning was observed in the control animals. The ovaries of these animals appeared translucent and/or opaque with a white to cream colour and smooth texture. The under-developed ovaries were evidenced by the low mean GSI value of 1.81 to 3.08. Light microscopic observations revealed, that the ovarian lobes were packed with developing oogonial cells and primary oocytes, with a prominent nucleus and chromatin materials. The oocyte diameter varied between 42 to 180 µ in control animals. The haematoxyline stained cytoplasm did not indicate any signs of yolk formation. There was no mortality in the control animals, but 50% of them moulted during the experimental period. Their feeding and swimming behaviours were normal.

However, all the unilaterally eyestalk ablated females exhibited significant increase in their GSI and oocyte diameter. Among the eyestalk ablated animals, 50% of them demonstrated well defined ovarian maturation. 30% of the females subsequently spawned within the 30 days experiment, with an average fecundity of 8,00,000/spawn. Half of the eyestalk ablated animals spawned twice after a period of 5-6 days from the Ist spawning in the same intermoult period with an average fecundity of 4,00,000/ spawn. The shortest period observed from ablation to the onset of maturation was 22 days and from ablation to first spawning was 25 days. Full mature ovaries in the

**(at full strength seawater using wild stock animals)**

| S.No. | Total length | Body wieght (g) | Gonadal | GSI | colour and nature of the ovary | Oocyte diameter (u) | Maturity stages | | | | | Spawning | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 0 | I | II | III | IV | 1 | 2 |
| 1 | 246 | 148 | 7.3 | 4.97 | Greenish yellow firm and granular | 265 | - | - | II | - | - | - | - |
| 2 | 265 | 172 | - | - | - | - | - | - | - | - | - | 25 | 30 |
| 3 | 253 | 156 | 8.4 | 5.38 | Green firm and granular | 365 | - | - | - | III | - | - | - |
| 4 | 249 | 152 | 8.7 | 5.72 | Green firm and granular | 386 | - | - | - | - | IV | - | - |
| 5 | 271 | 169 | 8.9 | 5.67 | Green granular and firm | 395 | - | - | - | - | IV | - | - |
| 6 | 267 | 174 | - | - | - | - | - | - | - | - | - | 26 | 33 |
| 7 | 258 | 153 | - | - | - | - | - | - | - | - | - | 28 | 34 |
| 8 | 251 | 146 | 7.4 | 5.07 | Green firm and granular | 330 | - | - | - | III | - | - | - |
| 9 | 269 | 152 | 7.9 | 5.12 | Green firm and granular | 305 | - | - | - | III | - | - | - |
| 10 | 260 | 151 | 106.9 | 4.57 | Green firm and firm | 292 | - | - | - | III | - | - | - |
| Control | | | | | | | | | | | | | |
| 1 | 197 | 124.5 | 2.02 | 1.62 | White opaque and smooth | 104.5 | - | I | - | - | - | - | - |
| 2 | 246 | 143.5 | 3.03 | 2.10 | Cream opaque and smooth | 116.14 | - | - | II | - | - | - | - |
| 3 | 190 | 124.5 | 1.96 | 1.57 | Flesh coloured and smooth | 81.7 | - | I | - | - | - | - | - |
| 4 | 212 | 130.5 | 2.14 | 1.6 | White opaque and smooth | 96.8 | - | I | - | - | - | - | - |
| 5 | 221 | 142.7 | 1.29 | 0.90 | Transluscent thin and smooth | 32.5 | 0 | - | - | - | - | - | - |
| 6 | 212 | 124.5 | 2.35 | 1.93 | Opaque white firm and granular | 94.5 | - | I | - | - | - | - | - |
| 7 | 189 | 131.6 | 5.34 | 4.05 | Yellow firm and granular | 198.5 | - | - | II | - | - | - | - |
| 8 | 199 | 124.5 | 2.97 | 2.38 | White granular and firm | 49.6 | - | I | - | - | - | - | - |
| 9 | 231 | 132.5 | 2.12 | 1.6 | Transluscent and smooth | 48.5 | 0 | - | - | - | - | - | - |
| 10 | 226 | 134.5 | 3.05 | 2.26 | Cream opaque granular and firm | 89.5 | - | I | - | - | - | - | - |

itellogenic phase, was observed in 20% of the eyestalk ablated animals at e termination of the experiment. In these animals fully developed, ominent dark band of ovary with diamond shaped expansion at the first dominal segment was clearly visible through the exoskeleton. Upon ssection, the ovary appeared dark olive green in colour with firm granular xture and clumps of discernible eggs. The high average GSI value of 8.9 1.2 indicated the progressive development of the gonads. Histological udies revealed that the oocytes were at their cortical rod phase and fully tured stages. The oocyte diameter varied from between to 410 µ, with an erage diameter of 395 µ. Atretic oocytes were also noted in some cases. wever, another 40% of the animals were found to be reach only the III stage itellogenic stage) with a solid and linear band of ovary visible through e exoskeleton from the cephalothoracic region to the base of the telson. e ovaries in these animals appeared deep yellow to greenish yellow in colour th firm and granular texture. The GSI value varied from 5.1 to 5.67. ght microscopical studies revealed that in addition to early vitellogenic cytes, some vitellogenic oocytes were also found in these ovaries. The ameters of such oocytes were found to vary between 305 to 365 µ. Atretic cytes were abundant in these ovaries. The remaining 10% of the animals re still at their early vitellogenic stage (stage II) of development. re ovaries were only faintly visible through the exoskeleton as a thick lid band along the dorsal side. The colour of the dissected ovaries varied om creamy to light yellow and GSI value between 3.83 to 4.97. stologically it was observed that, pre-vitellogenic oocytes at perinucleolar age and early vitellogenic oocytes at cisternal phase dominated. The erage oocyte diameter was 265 µ.

**A.2. Eyestalk ablation in P. monodon at full strength seawater (S 34‰) using animals from filtration ponds**

Adult female prawns ranging in size from 150 to 210 mm with immature ovary collected from filtration ponds were used for unilateral eyestalk ablation. Animals collected from the same area with similar biological characters formed the control animals. At the end of the experiment both the ablated and control animals were examined critically for the gonadal conditions. Results of the experiments are presented in the table (5). It was found that, in the control animals there were no apparent gonadal development or spawning till the termination of the experiment. The feeding and swimming activity of the animals were found to be normal except for their moulting period. However, all the control animals moulted once during the experiment and 20% of them moulted twice. Morphological examinations revealed that ovary was not visible externally through the dorsal exoskeleton. Upon dissection it appeared as colourless white or flesh coloured, flaccid body devoid of visible eggs. Histologically it was found that these ovaries were characterized by the predominance of developing oogonia and pre-vitellogenic oocytes with oocyte diameter varying between 01 to 125 $\mu$.

Spawning could not be achieved even in the eyestalk ablated animals, however 60% of the animals demonstrated well defined ovarian maturation. In this group all the experimental animals moulted before the commencement of the gonadal development and 30% of them moulted 2-3 times before the gonadal development began. Mortality rate was negligible in both the eyestalk ablated and control group of animals. Morphological examinations revealed that, among the 10 eyestalk ablated animals 4 of them reached the late vitellogenic stage of ovarian development at the termination of the experiment. In these animals ovary was visible through the dorsal exoskeleton

| S.No. | Total length | Body wieght (g) | Gonadal | GSI | colour and nature of the ovary | Oocyte diameter (u) | Maturity stages | | | | | Spawning | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 0 | I | II | III | IV | 1 | 2 |
| 1 | 210 | 152 | 7.9 | 5.2 | Greenish yellow firm and granular | 310 | - | - | - | III | - | - | - |
| 2 | 185 | 120 | 4.08 | 3.4 | Yellow firm and granular | 135 | - | - | II | - | - | - | - |
| 3 | 205 | 149 | 7.7 | 5.2 | Green firm and granular | 296 | - | - | - | III | - | - | - |
| 4 | 190 | 136 | 4.78 | 3.8 | Greenish yellow frim and granular | 142 | - | - | II | - | - | - | - |
| 5 | 187 | 101 | 3.1 | 3 | White firm | 97 | - | I | | - | - | - | - |
| 6 | 175 | 149 | 6.4 | 4.3 | Light green frim and granular | 284 | - | - | - | III | - | - | - |
| 7 | 186 | 143 | 6.7 | 4.7 | Greenish yellow and thick | 279 | - | - | - | III | - | - | - |
| 8 | 190 | 104 | 2.8 | 2.7 | Yellow | 92 | - | I | - | - | - | - | - |
| 9 | 168 | 145 | 5.9 | 4.1 | Green firm and granular | 296 | - | - | II | - | - | - | - |
| 10 | 155 | 118 | 4.3 | 3.7 | Greenish yellow | 175 | - | - | II | - | - | - | - |
| **Control** | | | | | | | | | | | | | |
| 1 | 197 | 124.5 | 2.02 | 1.62 | White opaque and smooth | 104.5 | - | | I | - | - | - | - |
| 2 | 246 | 143.5 | 3.03 | 2.10 | Cream opaque and smooth | 116.14 | - | | - | II | - | - | - |
| 3 | 190 | 124.5 | 1.96 | 1.57 | Flesh coloured and smooth | 81.7 | - | | I | - | - | - | - |
| 4 | 212 | 130.5 | 2.14 | 1.6 | White opaque and smooth | 96.8 | - | | I | - | - | - | - |
| 5 | 221 | 142.7 | 1.29 | 0.90 | Transluscent thin and smooth | 32.5 | 0 | | - | - | - | - | - |
| 6 | 189 | 156.2 | 7.76 | 4.96 | Greenish yellow and firm | 295 | - | | I | - | - | - | - |
| 7 | 215 | 143.0 | 2.95 | 2.06 | Cream opaque and smooth | 108.3 | - | | - | II | - | - | - |
| 8 | 210 | 142.7 | 1.29 | 0.90 | Transluscent thin and smooth | 32.4 | 0 | | - | - | - | - | - |
| 9 | 197 | 143.5 | 3.23 | 2.25 | Cream opaque and smooth | 108.3 | - | | I | - | - | - | - |
| 10 | 192 | 148.5 | 6.9 | 4.64 | Greenish yeloow firm and granular | 265 | - | | - | II | - | - | - |

as a thick, solid, linear band as it expanded at the posterior thoracic and anterior abdominal segments. The development of the gonad is evidenced by the higher GSI value of 8.1 to 9.2. The ovaries here appeared greenish yellow to olive green in colour. Light microscopic studies revealed that, these yolky oocytes in the late vitellogenic ovary are equipped with the characteristic cortical rods. The oocyte diameter varied from 265- 310 $\mu$. Atretic oocytes are found to be abundant in these ovaries.

Among the remaining experimental animals 40% of them were at their early vitellogenic stage of ovarian development. Ovary was feebly visible through the exoskeleton, and appeared as a relatively thick streak extending from the cephalothoracic region to the anterior side of the telson. Its GSI value varied from 3.4 to 3.9. The colour of the ovary varied between light yellow to greenish yellow. Histological observations revealed that these oocytes were characterized by the presence of highly eosinophilic granular cytoplasm due to the accumulating yolk granules. These granular cisternal and/or platelet phase oocytes with an oocyte diameter of 135 to 175 $\mu$ indicated the early vitellogenic stage of development. Among the 10 experimental animals remaining two prawns did not show any visible signs of ovarian development. Ovary was not visible externally from the dorsal exoskeleton. It appeared as a colourless, thin streak on the dorsal side of the digestive tract. Histologically it was observed that this stage was characterized by the abundance of developing oogonia and primary oocytes, in which the oocyte diameter varied from 45 to 95 $\mu$m. The follicle cells were not developed around each oocyte. The cytoplasm was found to be stained with hematoxylin. The cytoplasm to nucleus ratio was also found to be low.

## A.3. Eyestalk ablation in P. monodon at low saline (S 20‰.) conditions using animals from filtration ponds

This study was carried out using cultured prawns collected from filtration ponds. Adult female prawns with immature ovaries ranging from 170 to 250 mm in total length were selected for the experiment. Results of the experiment are presented in the table (6). After 30 days at the termination of the experiment, control animals did not show any signs of ovarian development. Ovary was not visible through the dorsal exoskeleton. Ovarian lobes were translucent and smaller in diameter. Low GSI indicated the underdeveloped condition of the ovary. It was found that the ovarian lobes were packed with developing oogonia and pre-vitellogenic oocytes, which are characteristic features of immature ovaries. Among the control group 20% of the prawns moulted during the experimental period. Their feeding and swimming activities were normal.

Even the eyestalk ablated animals did not show any progressive stages of ovarian maturation during the experiment. The major difference noted in this experimental animals compared to the control animals was, the repeated shedding of the exoskeleton after the ablation of eyestalks. All the eyestalk ablated animals moulted more than once. Among the experimental animals 30% of them moulted three times and rest of them moulted twice during the experimental tenure of 30 days. Consequently there was no ovarian development in any of the eyestalk ablated animals. Externally, ovary was not visible through the exoskeleton, and at the termination of the experiment, it was noted that among the 10 eyestalk ablated animals 3 of them were at the 2nd stage (early vitellogenic stage) only. Colour of the ovary varied between white to yellow. Texture of the ovary was smooth and visible eggs were not at the developed condition. The GSI value varied between 2.9 to 3.7. Histologically it was observed that, the cisternal phase oocytes with an

**TABLE 3 EFFECT OF EYESTALK ABLATION ON GONADAL MATURAL IN FEMALE PENAEUS MONODON (at low saline (S 20%.) conditions using animals from filtration ponds**

| S.No. | Total length | Body wieght (g) | Gonadal | GSI | colour and nature of the ovary | Oocyte diameter (u) | Maturity stages | | | | | Spawning | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 0 | I | II | III | IV | 1 | 2 |
| 1 | 245 | 140 | 2.7 | 1.81 | Transparent and smooth | 142 | - | I | - | - | - | - | - |
| 2 | 250 | 147 | 3.0 | 2.0 | Opaque cream and granular | 136 | - | I | - | - | - | - | - |
| 3 | 248 | 149 | 4.6 | 3.8 | Opaque yellow and granular | 180 | - | - | II | - | - | - | - |
| 4 | 170 | 124 | 4.8 | 3.9 | Greenish yellow and firm | 184 | - | - | II | - | - | - | - |
| 5 | 183 | 109 | 2.6 | 2.4 | White firm and smooth | 84 | - | I | - | - | - | - | - |
| 6 | 205 | 140 | 6.8 | 4.8 | Green firm and granular | 265 | - | - | II | - | - | - | - |
| 7 | 181 | 120 | 2.1 | 1.75 | Transparent | 61 | 0 | - | - | - | - | - | - |
| 8 | 196 | 132 | 4.2 | 3.06 | Opaque yellow and granular | 193 | - | - | II | - | - | - | - |
| 9 | 176 | 114 | 1.8 | 1.58 | Tranparent | 67 | 0 | - | - | - | - | - | - |
| 10 | 202 | 107 | 2.8 | 2.6 | Opaque firm and granular | 97 | - | I | - | - | - | - | - |
| **Control** | | | | | | | | | | | | | |
| 1 | 196 | 132.2 | 2.18 | 1.57 | Thin transparent and smooth | 43.4 | 0 | - | - | - | - | - | - |
| 2 | 234 | 145.6 | 3.18 | 2.32 | Cream granular and opaque | 103.4 | - | I | - | - | - | - | - |
| 3 | 186 | 136.7 | 1.98 | 1.44 | Thin transparent and smooth | 57.4 | 0 | - | - | - | - | - | - |
| 4 | 218 | 146 | 2.91 | 1.99 | White firm and opaque | 87.5 | - | I | - | - | - | - | - |
| 5 | 224 | 149 | 3.48 | 3.1 | Cream firm and opaque | 105.4 | - | I | - | - | - | - | - |
| 6 | 212 | 124.5 | 2.35 | 1.93 | Opaque white firm and granular | 94.5 | - | I | - | - | - | - | - |
| 7 | 221 | 143 | 2.95 | 2.06 | Cream opaque and granular | 80.5 | - | I | - | - | - | - | - |
| 8 | 199 | 124.5 | 2.97 | 2.38 | White granular and firm | 89.6 | - | I | - | - | - | - | - |
| 4 | 231 | 132.5 | 2.12 | 1.6 | Transluscent and smooth | 48.5 | 0 | - | - | - | - | - | - |
| 5 | 226 | 134.5 | 3.05 | 2.26 | Cream opaque granular and firm | 89.5 | - | I | - | - | - | - | - |

verage diameter of 182.7 μ were predominating. Eosinophilic cytoplasm was ot much granular. Many of the oocytes were individually encompassed by he cuboidal follicle cells. Along with the developing oocytes an abundant mber of atretic oocytes were also observed in these ovaries.

40% of the eyestalk ablated animals have been observed in the 1st r pre-vitellogenic stage of gonadal development at the time of termination f the experiment. In these animals gonads were not visible externally hrough the dorsal exoskeleton. The ovaries appeared white to flesh coloured, laccid and devoid of visible eggs. The GSI value varied between 2.1 to .7. Anterior lobes of the ovary were not fully developed. Diameter of he ovarian lobes were slightly more than that of the gut. Light microscopic tudies showed the presence of large numbers of the developing oogonia and few centrally shifted primary oocytes at the chromatin nucleolus stage. ocytes with an average diameter of 75 μ were found to be individually rrounded by follicle cells. Remaining 30% of the animals were still at he immature stage itself. It was observed that thin transparent ovarian bes were not even fully developed and the diameter of these ovarian lobes s smaller than that of the underlying gut. Low GSI value of 1.00 to 1.2 idenced under-developed ovarian condition. Light microscopical examinations vealed that, the germinal zone of the ovary was active with developing gonia and few primary oocytes at the central region of the ovarian tubule.

**Effect of ganglionic extract injections on ovarian maturation**

**1. Cerebral ganglion**

Adult female prawns, with immature ovaries ranging in size from 180 254 mm in total length collected from wild, were used for this experiment. e results of these experiments are given in the table (7). Females injected

with cerebral ganglion extract exhibited more advanced stages of ovarian development than those recieved ethanol injection or those did not recieve any injections. Prominent ovarian development towards maturation was found in 80% of the experimental animals while the remaining 20% animals did not show any signs of ovarian maturation. Among 80% of the developing animals half of them were at the late vitellogenic (stage III) stage of ovarian maturation at the termination of the experiment on the 30th day. Developing ovary was visible externally through the dorsal exoskeleton as a thick solid band extending the entire length of the body. A slight diamond shaped expansion was visible at the 1st abdominal segment. The ovaries appeared olive green in colour, and firm and granular in texture with clumps of discernible eggs. The GSI values were also high indicating the advanced stages of the ovary, ie. between 4.96 to 5.01. Histological studies revealed that, most of the oocytes were at the platelet phase or early cortical rod phase. Highly eosinophilic yolky substances were observed in these oocytes with a varying diameter of 295-315 u. Spherical or slightly elongated cortical bodies were observed in some of these oocytes at the periphery of the ooplasm. Thin highly stretched follicle cells were not easily distinguishable from the oocytes. Remaining 40% of the animals were at the early vitellogenic phase (stage II) of their gonadal development. In these animals, ovary was not clearly visible externally through the dorsal exoskeleton. The ovaries are found to be relatively thicker in the anterior and middle regions. Colour varied from light yellow to greenish yellow. Small individual eggs were quite discernible in the firm granular ovarian nodules. The GSI value varied between 3.45 to 3.88 indicating the developing stages of the gonads. Histologically, round to oval oocytes at cisternal and platelet phase oocytes with an average oocyte diameter of 305.65 $\mu$ dominated.

Remaining 20% of the animals were at the pre vitellogenic stage of ian development. Ovary was not visible externally through the dorsal keleton and it appeared colourless, white or flesh coloured and devoid visible eggs. The average GSI value was only 1.8. Histological rvations revealed that, these ovaries contained developing oogonia and ture oocytes, with an average oocyte diameter of 74.5 $\mu$. The feeding swimming activities of these experimental animals were normal. Among e 60% of the animals moulted once during the experimental tenure. There no mortality during the entire period of observation.

**Thoracic ganglion**

Adult female prawns ranging in size from 198 to 247 mm were cted for the injections. Results of the injections are presented in table (7). Females injected with thoracic ganglion extract exhibited advanced ovarian stages than those received saline injection or those without any injections. 20% of the experimental animals spawned with average fecundity of 6,00,000/spawn after 24 days of the injections. her 20% of the animals reached at full maturity at the termination of experiment. The thick dark band of ovary with diamond shaped expansion the first abdominal segment was clearly visible through the dorsal keleton in these animals. Ovary in this stage appeared dark green in ur, and firm and granular in texture with clumps of discernible eggs. ovary filled up all the available space of the body cavity and its average value was 9.03. Histological observation showed fully matured and ical rod phase oocytes with an average oocyte diameter of 391 $\mu$.

Early vitellogenic (stage II) ovaries were found in 40% of the cted animals. In such animals ovary was not clearly visible through dorsal exoskeleton. The ovary was yellow to greenish yellow in colour.

| S.No. | Total length | Body wieght (g) | Gonadal | GSI | colour and nature of the ovary | Oocyte diameter (u) | Maturity stages | | | | | Spawning |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 0 | I | II | III | IV | |
| **1. Effect of injections of crude extracts of cerebral ganglion** | | | | | | | | | | | | |
| 1. | 215 .3 | 148.7 | 5.6 | 3.45 | Yellow firm and granular | 178.7 | - | - | II | - | - | - |
| 2 | 230.4 | 157.5 | 7.9 | 5.01 | Greenish yellow Granular | 315.6 | - | - | - | III | - | - |
| 3 | 205.5 | 146.5 | 2.94 | 1.80 | White opaque and Smooth | 74.5 | - | I | - | - | - | - |
| 4 | 180.00 | 123.5 | 4.8 | 3.88 | Yeloow for and granular | 295.6 | - | - | II | - | - | - |
| 5 | 254.3 | 156.2 | 7.76 | 4.96 | Green granular firm | 295.7 | - | - | - | III | - | - |
| **II Effect of injections of crude extracts of thoracic ganglion** | | | | | | | | | | | | |
| 1 | 240 | 1485 | 6.9 | 4.5 | Greenish yellow firm | 265 | - | - | II | - | - | - |
| 2 | 236 | 143.5 | - | - | - | - | - | - | - | - | - | 24 |
| 3 | 247.5 | 150.5 | 13.6 | 9.03 | Green granular and thick | 391 | - | - | - | - | IV | - |
| 4 | 212.7 | 134.5 | 6.8 | 4.05 | Yellow firm granular | 234 | - | - | II | - | - | - |
| 5 | 198.5 | 123.6 | 3.24 | 2.17 | White opaque and firm | 92 | - | I | - | - | - | - |
| **III Effect of injections of crude extracts of eye stalk** | | | | | | | | | | | | |
| 1 | 245 | 143.5 | 3.23 | 2.25 | Cream opaque smooth | 108.3 | - | I | - | - | - | - |
| 2 | 236 | 135.6 | 2.34 | 1.72 | Transluscent and smooth | 74.5 | 0 | - | - | - | - | - |
| 3 | 212 | 126.5 | 1.74 | 1.37 | Transluscent and smooth | 54.03 | 0 | - | - | - | - | - |
| 4 | 197 | 124.5 | 2.02 | 1.62 | White opaque and smooth | 98.4 | 0 | - | - | - | - | - |
| 5 | 221 | 143.0 | 2.95 | 2.06 | Cream opaque and smooth | 80.5 | - | I | - | - | - | - |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **IV Control I injected with saline** | | | | | | | | | | | | |
| 1 | 197 | 124.5 | 2.02 | 1.62 | White opaque and smooth | 104.5 | - | I | - | - | - | - |
| 2 | 246 | 143.5 | 3.03 | 2.10 | Cream opaque and smooth | 116.14 | - | - | II | - | - | - |
| 3 | 190 | 124.5 | 1.96 | 1.57 | Flesh coloured and smooth | 81.7 | - | I | - | - | - | - |
| 4 | 212 | 130.5 | 2.14 | 1.6 | White opaque and smooth | 96.8 | - | I | - | - | - | - |
| 5 | 221 | 142.7 | 1.29 | 0.90 | Transluscent thin and smooth | 32.5 | 0 | - | - | - | - | - |
| **V Control II with out any injections** | | | | | | | | | | | | |
| 1 | 189 | 156.2 | 7.76 | 4.96 | Greenish yellow and firm | 295 | - | I | - | - | - | - |
| 2 | 215 | 143.0 | 2.95 | 2.06 | Cream opaque and smooth | 108.3 | - | - | II | - | - | - |
| 3 | 210 | 142.7 | 1.29 | 0.90 | Transluscent thin and smooth | 32.4 | 0 | - | - | - | - | - |
| 4 | 197 | 143.5 | 3.23 | 2.25 | Cream opaque and smooth | 108.3 | - | I | - | - | - | - |
| 5 | 192 | 148.5 | 6.9 | 4.64 | Greenish yeloow firm and granular | 265 | - | - | II | - | - | - |

owever, it was not smooth and discernible individual eggs. The GSI value aried between 4.05 to 4.64. Oocytes were at the cisternal and platelet hase of yolk formation and its average oocyte diameter was 249 μ. These ocytes contained large accumulations of yolk granules dispersed through t the cytoplasm. While the rest of the experimental animals were at the re -vitellogenic stage of ovarian development. In these animals ovary was t visible externally through the dorsal exoskeleton. The ovaries were lourless, white or flesh coloured, translucent or opaque and devoid of sible eggs. Histological studies revealed that, the ovary is composed developing oogonia, developing oocytes and oocytes at chromatin nucleolus age. Large oocytes at perinucleolus stages were found to be encompassed individual follicle cells. The feeding and swimming behaviour of these perimental animals were normal. No mortality occurred during the perimental period.

### 3. Effect of eyestalk extract injection on maturation

Adult female prawns ranging in size from 197 to 245 mm collected m wild were selected for this experiment. Prawns with immature ovaries re injected with crude extracts of eyestalks. Results of the experiments owing the ovarian developmental stages are presented in the table (7). mals injected crude eyestalks extracts did not show any signs of ovarian elopment till the termination of the experiment. Dissected ovaries were urless, white or flesh coloured, thin and translucent. Anterior lobes e ovary were not fully developed. The under developed ovarian condition videnced by the low GSI value of 1.3 to 2.3. Histological investigations ed that the ovary was composed of developing oogonia and tellogenic oocytes at chromatin nucleolus and peri nucleolus stages. yte diameter varied between 54.03 μ to 107.3 μ. Among the experimental

nimals 40% of the injected animals were at the stage I or pre-vitellogenic tage of development. While rest of them had translucent immature ovaries. eeding and swimming activities of these animals were normal, however, 40% f them moulted twice and rest moulted once during the experimental tenure.

Control I animals subjected to saline injections did not show any rogressive stages of ovarian development. Ovary was not visible externally hrough the dorsal exoskeleton in any of these animals. Ovaries appeared hin, translucent, white or flesh coloured and devoid of visible eggs. The nder developed ovarian condition was clearly evidenced by the low GSI value ariying between 0.9 to 2.1. Pre-vitellogenic ovaries were found in 20% f the control animals while 80% of the animals were found immature.

Similarly control II animals kept in the same conditions without any njections also did not show much progression in the gonadal maturation. ere also the maximum maturation observed was only up to the early itellogenic stage (stage II). Atleast 40% of these animals exhibited early turing ovarian condition. Ovary of these animals were visible externally hrough the dorsal exoskeleton as a comparatively thick band extending from he cephalic region to the end of the abdomen. Here the ovaries appeared ep yellow to greenish yellow in colour, firm and granular in texture with average GSI value of 5.3. The oocyte diameter varied from 185 to 206.5u. nother 40% of the experimental animals were found with white to flesh oloured pre-vitellogenic ovary with a GSI value of 2.4. The ovarian samples owed developing oocytes and pre-vitellogenic oocytes at chromatin nucleolus d peri-nucleolus stages. Their oocyte diameter varied between 140 to 210u. maining 20% animals had translucent immature ovaries with a GSI value of 37. Ovarian samples showed only developing oogonia and immature oocytes.

From these observations it is concluded that, ovarian maturation n be induced by the injection of crude extracts, of thoracic ganglion well as cerebral ganglion. Among these two ganglionic extracts thoracic nglion extract was found more potential than cerebral ganglion extract. wever, the ovarian maturation was found to be suppressed by the eyestalk tracts in the experimental animals.

**Effect of synthetic hormone injection on ovarian maturation**

**1. 17a-Progesterone**

Adult wild female prawns ranging in size from 196mm to 240mm were lected for the injections. Half the number of total prawns with immature aries were injected with 17alpha hydroxy progesterone and other half kept control. Immediately after the injections, prawns were found inactive r first few minutes and soon after they resumed their normal movements. allergic or abnormal effects were noticed in the behaviour due to the mone injection till the termination of the experiment.

Ovarian developmental stages observed at the termination of the periments are presented in table (8). Ovary of the initial samples before treatments were white to cream coloured and contained immature, pre tellogenic oocytes. At the termination of the experiment it was observed t 20% of the injected animals attained late vitellogenic (stage III) ovary. these animals ovary was visible externally through the dorsal exoskeleton, a dark thick band along the entire length of the body. The ovary in such mals, appeared green in colour, granular and firm in texture. The reased GSI value of 5.66 to 6.25 indicated the advanced ovarian stages.

Table 8

## EFFECT OF SYNTHETIC HORMONES INJECTIONS ON GONADAL MATURATION IN FEMALE PENAEUS MONODON

| S.No. | Total length | Body wieght (g) | Gonadal | GSI | colour and nature of the ovary | Oocyte diameter (u) | Maturity stages | | | | Spawni |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 0 | I | II | III | IV |
| **1. Effect of projesterone injections** | | | | | | | | | | | |
| 1. | 215 | 138.6 | 4.7 | 3.39 | Dark yellow granular & white | 185.4 | - | - | II | - | - |
| 2 | 240 | 144.5 | 8.9 | 6 | Green granular | 295.4 | - | - | - | III | - |
| 3 | 179 | 118.5 | 3.01 | 2.54 | Light yellow opaque and firm | 86.6 | - | I | - | - | - |
| 4 | 196 | 120.5 | 5.4 | 4.48 | Greenish yellow granular and firm | 256.4 | - | - | II | - | - |
| 5 | 225 | 136 | 3.08 | 2.56 | Cream thin and smooth | 108.8 | - | I | - | - | - |
| II Effect of estradiol injections | | | | | | | | | | | |
| 1 | 226 | 148 | 4.3 | 2.9 | Light yellow firm and granular | 256.4 | - | - | II | - | - |
| 2 | 196 | 146.5 | 2.8 | 1.91 | Transluscent thin and smooth | 26.5 | - | I | - | - | - |
| 3 | 215 | 136 | 2.8 | 2.05 | Cream opaque and granular | 87.6 | - | I | - | - | - |
| 4 | 230 | 151 | 3.1 | 2.05 | Cream opaque firm and granular | 143.8 | - | I | - | - | - |
| 5 | 228 | 147 | 5.3 | 3.60 | Greenish yellow firm and granular | 294.3 | - | - | II | - | - |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **III Control I Effect of ethanol injections** | | | | | | | | | | | | |
| 1 | 196 | 132.2 | 2.18 | 1.57 | Thin transparent and smooth | 43.4 | 0 | - | - | - | - | - |
| 2 | 234 | 145.6 | 3.18 | 2.32 | Cream granular and opaque | 103.4 | - | I | - | - | - | - |
| 3 | 186 | 136.7 | 1.98 | 1.44 | Thin transparent and smooth | 57.4 | 0 | - | - | - | - | - |
| 4 | 218 | 146 | 2.91 | 1.99 | White firm and opaque | 87.5 | - | I | - | - | - | - |
| 5 | 224 | 149 | 3.48 | 3.1 | Cream firm and opaque | 105.4 | - | I | - | - | - | - |
| **IV Control II without any injections** | | | | | | | | | | | | |
| 1 | 212 | 124.5 | 2.35 | 1.93 | Opaque white firm and granular | 94.5 | - | I | - | - | - | - |
| 2 | 189 | 131.6 | 5.34 | 4.05 | Yellow firm and granular | 198.5 | - | - | II | - | - | - |
| 3 | 199 | 124.5 | 2.97 | 2.38 | White granular and firm | 19.6 | - | I | - | - | - | - |
| 4 | 231 | 132.5 | 2.12 | 1.6 | Transluscent and smooth | 48.5 | 0 | - | - | - | - | - |
| 5 | 226 | 134.5 | 3.05 | 2.26 | Cream opaque granular and firm | 89.5 | - | I | - | - | - | - |

stologically it was observed that these ovarian samples contained tellogenic oocytes with highly eosinophilic yolky cytoplasm. Platelet ase oocytes with granular yolky materials and cortical rod phase oocytes th characteristic cortical bodies were the dominating features.

Among the injected animals, 40% developed only up to the early tellogenic (stage II) stage and ovary was not clearly visible through the rsal exoskeleton. The ovaries appeared deep yellow to greenish yellow colour, and firm and granular in texture. The average GSI value was 3.93. stological studies showed, that ovaries were composed of pre-vitellogenic d early vitellogenic oocytes with oocyte diameter varied between 185 to 6 µ. Rest of the 40% animals had pre-vitellogenic ovary. In this case ary was not visible through the dorsal exoskeleton, and it appeared white flesh coloured and smooth with a low GSI value of 2.5. Ovarian samples d developing oogonia, developing oocytes and pre-vitellogenic oocytes th oocyte diameter variying between 86 - 108 µ.

**2. Estradiol**

Adult wild animals ranging in size from 196 to 230 mm were jected with estradiol and kept for 30 days to assess its effects on gonadal uration. Immediately after the injections prawns were found to be passive r the first few minutes and soon after they resumed their normal movements. abnormal responses were noticed in the behaviour due to hormone injections. inistration of estradiol could not produce any marked ovarian development r the initial stages even after 30 days of experiment. Ovarian development ges observed in the experimental prawns are presented in the table (8). phological observations showed that the ovaries in such animals appeared te in colour and their ovarian index was 1.83± 0.34. Even though shrimps

ected with estradiol did not show significant enhancement in the ovarian ices over to that of control group of prawns. 20% of the injected prawns ibited early vitellogenic (stage II) ovary with an average GSI value 3.25. The ovary was not clearly visible externally through the dorsal keleton. Ovary of these animals was white to cream in colour and smooth firm in texture. Histological observations revealed that, ovarian samples these prawns were dominated by the early vitellogenic oocytes than that the pre-vitellogenic oocytes. The average size of the largest oocytes found to be about 255 µ. Remaining 50% of the estradiol injected animals at the pre-vitellogenic stage of ovarian development at the termination the experiment. In this case the ovary was not visible externally through dorsal exoskeleton. The ovary was white to cream in colour and smooth firm in texture. The largest oocytes of these ovarian samples were at stage of pre-vitellogenesis. The oocytes were small with little amounts cytoplasm. Their diameter varied between 87 to 143.8 µ. The feeding swimming behaviour of experimental prawns were normal during the tenure the experiment. The significant observation made was, the repeated ding of the exoskeleton by these experimental animals from 5th day rds. 80% of the injected animals moulted twice during the tenure of experiment while the remaining 20% moulted only once.

Control I animals injected with ethanol did not show any signs varian maturation. All the injected animals were at the immature or vitellogenic stage of development. The under developed ovarian condition evidenced by the low GSI value of about 1.9 to 3.4. Morphological ination showed that ovaries in such prawns appeared small and white in ur. Histological investigations revealed that, in control prawns ovary ained oogonia and primary oocytes of basophilic nature. The oocyte eter varied between 48.4 and 105.4 µ.

Control II animals kept along with the experimental animals but thout any injections, also did not show much progression in the ovarian velopmental stages. At the termination of the experiment 20% of the animals re at the pre-vitellogenic stage of gonadal development. In these animals vary was not clearly visible externally through the dorsal exoskeleton. varies appeared cream to yellow in colour, firm and granular in texture d their ovarian indices was 2.05. Ovarian samples contained more early tellogenic oocytes than pre- vitellogenic oocytes. The oocyte diameter ried between 114 to 128 $\mu$.

However, 60% of the control animals exhibited pre-vitellogenic ovaries. eir thin, white to flesh coloured ovarian tissues were not granular and e ovarian index varied between 1.93 to 2.30. The largest oocytes of ese ovaries were at the stage of pre-vitellogenesis and their average ameter was 94.2 $\mu$. Thin translucent immature ovaries were observed in 20% the control animals with a GSI value of 1.6. The average size of the rgest oocytes was about 48.5 $\mu$.

From these observations it was found that the ovarian maturation P. monodon was induced to some extent by the synthetic hormone 17-a ogesterone. From histological examinations of the ovarian samples, gnificant difference in the oocyte development was found between 17 a rogestrone treated and control group of animals. However, there was no gnificant induction of ovarian maturation in the estradiol injected group animals.

# DISCUSSION

In the past decade a vast amount of research has been directed toward the understanding and manipulation of reproduction in penaeid prawns. In many decapods including penaeids removal of eyestalks that contain the X-organ/sinus gland complex which produce and stores gonad inhibiting hormones has become a well recognized technique for inducing gonadal maturation (Adiyodi and Adiyodi,1975). The most commonly accepted theory is that a gonad inhibiting hormone (GIH) is produced in the neurosecretory complexes in the eyestalks. This hormone apparently occurs in nature in the non-breeding season and is absent or present only in low levels during the breeding season (Bomirski and Kleke, 1974; Kulkarni and Nagabhushanam, 1980). By inference then, the reluctance of most penaeids to develop mature ovary in captivity is a function of elevated levels of GIH, and eyestalk ablation lowers the high haemolymph titer of this GIH. Eyestalk ablation in crustaceans has been reviewed by Fingerman (1970); Adiyodi and Adiyodi (1977); Kleinholz and Keller (1979) and Charniaux-Cotton (1985); Fingerman (1987); Wyban et al. (1987) and Liao (1992).

A few instances of natural maturation and spawning of unablated and captive P. monodon in seawater ponds and tanks have been reported by Chen (1976) and Liao (1977) from Taiwan, by Primavera et al. (1978) from Philippines and by Aquacop (1979) from Tahiti. The most successful technique in captivity for inducing maturation of penaeids has been the removal of eyestalks. This method has been tried with varying degrees of success by pioneers Idyll (1971) and Caillouect (1977) in USA, Anstein and Beard (1975) in UK, Alikunhi et al (1975) in Indonesia, Aquacop, (1975) and (1977) in Polynesia, Wear and Santiago (1976), Santiago, (1977), and Primavera, (1978)

Philippines and Halder, (1978) in India. These results indicated that ilateral eyestalk ablation unilateral eyestalk ablation greatly enhances nadal maturation in penaeids like P. indicus, P .stylirostris, P. vannamei d P. setiferus and it is a prerequsite for hardy penaeids like, P. monodon quacop, 1979) Though P. monodon showed a good promise in view of the number good spawnings, the proportion of regression among females which have rted their ovarian development has been reported to be high (Aquacop, 79). However the use of eyestalk ablation to induce gonadal maturation P. monodon female has been done with success by many other workers like ikunhi et al. (1975), Wear and Saantiago (1976), Aquacop (1977, 1979, 83), Muthu and Laxminarayana (1977), Primavera (1978, 1979), Primavera al. (1979), Liao and Chen (1983), Millamena e al. (1985), Lin and Ting 986) and Menasveta et al. (1989). It is also reported that the stimulation gonadal maturation in P. monodon is dependent on the relative interaction environmental factors and the age (Laubier, 1978; Emmerson, 1980; 1983), rt from eyestalk factors.

Results of the experiments conducted at a salinity of 34%. using d animals in the present study showed that eyestalk ablation significantly ances ovarian development and spawning in P. monodon. Similarly, eriments conducted at the same salinity (S 34 %.) using animals collected m filtration ponds also showed that gonadal maturation has been enhanced eyestalk ablation, though the progress was slower in these animals than t observed in wild animals collected from the open sea.

Generally P. monodon appeared to take longer time to mature in tivity than other penaeids (Vide Alikunhi et al.1975; Aquacop, 1975). r and Santiago (1976) and Santiago (1977) reported that P. monodon took roximately 2 months to attain first maturity. However, Primavera (1978)

btained first spawnings in P. monodon 22 days after unilateral eyestalk blation under reduced light intensity and at a salinity of 34%. while Halder 1978), reported 38 days for gonadal maturation in the same species at a alinity of 25%.. It was reported that all of them used outdoor ponds or ges, presumably with high light intensity to mature ablated P. monodon. his is presumably why they took longer periods than reported by Primavera 1978) and Emmerson (1980) who usedindoor tanks with reduced light conditions. ard and Wickins (1980) also matured ablated P. monodon under reduced light tensity (40-70lux). Results of the experiments conducted in two different vironmental conditions using animals collected from two different sources ring the present study revealed that eyestalk ablation enhances gonadal turation in P. monodon . It has been noted that water quality also plays role in the full achievement of the process. During the present vestigation the shortest period observed from ablation to the onset of turation was 22 days and first spawning occurred after 25 days in wild imals collected from the open sea. Where as animals collected from ltration ponds took 30 days to reach the late-vitellogenic stage of ovarian velopment, spawning could not be achieved in these animals till the mination of the experiment after 30 days. However, the ovarian development found progressing in most of the experimental animals. Muthu and minarayana (1982) reported that P. monodon collected from filtration ponds k 66 days to mature. They concluded that frequent power failures leading disruption of the recirculatory system collapsed the water quality which ayed the maturation process.

In terms of the size of the animals, the rate of achievement of adal maturity in larger animals is much greater compared to the smaller wns during the present investigation and it is comparable with other

penaeid prawns. Chotikun (1988) and Tansutapanich et al. (1989) found that optimal age of maturation of pond reared penaeid prawns was at least 18 months. In the wild, P. monodon attained full maturity and spawning at 10 months (Motoh, 1981) and optimum prawn size reported in these studies was >100 g. Primavera (1978) reported that five month old P. monodon could mature and spawn after ablation, but produced poor quality larvae. These results indicated the need for older and larger females for successful production of PL from pond reared broodstock. In the present investigation animals of more than 200 g size matured faster than smaller animals.

Results of the eyestalk ablation conducted in pond reared animals at low salinity during the present study, showed that it did not induce in gonadal maturation P. monodon; instead it induced repeated moulting. It was found that, general that the penaeid shrimps attained maturity faster when the salinity of the water was 30-33 ppt. The fact that juveniles of these species live in brackishwaters and usually migrate to the sea for spawning purposes suggests that salinity is one of the important factors that influences the maturation process (Muthu and Laxminarayana, 1982). This is strongly supported by the observations of Silas et al. (MS) that M. dobsoni could attain maturity in brackish water ponds when the salinity increased to 28-30 ppt (See review Muthu Laxminarayana, 1982). Even P. indicus of stage III maturity have been collected by George, (1974) from the brackishwater ponds during the high salinity months. Vasudevappa (1992) reported that M. dobsoni can mature through eyestalk ablation during high saline months in brackish waters. The only exception was reported by Halder (1978) who stated that ablated P. monodon attained maturity and spawned viable eggs in a brackishwater environment when the salinity was only 25‰.

Although the experiments carried out in the present study were on a limited scale, the results obtained do suggest that a system incorporating unilateral eyestalk ablation, high salinity; good water quality and reduced light intensity are sufficient to induce the maturation of female P. monodon in captivity. Regarding the effect of broodstock source on maturation, in this study it was found that pond reared and wild-caught broodstock performed comparably if the size and age are adequate. These findings are in accordance with the similar observations made on Srimudda (1987) and Menasveta et al. (1994) in the same species. Similar observations were reported by Rangnekar and Deshmukh (1968) in Scylla serrata; Bomiriski and Klek, (1974 and 1975) in Crangon crangon; and Nagabhushanam and Diwan, (1974) in Barytelphusa cunicularis, and Lysmata seticaudata.

Demonstrating hormone's effectiveness in inducing Vg synthesis in decapod crustacea is difficult because females with immature ovaries are strongly affected by a gonad inhibiting hormone (GIH) compared to vitellogenic females (Yano and Wyban, 1992). Moreover, the complete physiological process in decapod crustacea are under the control of different hormones secreted by neuroendocrine cells located in different ganglionic centers (Fingerman, 1970). Evidences have also been presented to show the presence of a gonad stimulating hormone (GSH), which stimulates ovarian maturation, in the thoracic ganglion of vitellogenic crab, shrimp and lobster (Otsu, 1960, Oyama, 1968, Hinsch and Bennet, 1979; Eastman-Recks and Fingerman, 1984; Takayanagi et al., 1986; Yano et al., 1988; Yano, 1992). Therefore, in the present study crude extracts of different ganglionic centers of vitellogenic females were injected and their effects on ovarian maturation in adult female P. monodon were studied.

During the present study it was found, that complete ovarian development and spawning was achieved in P. monodon injected with crude extracts of thoracic ganglion of vitellogenic female prawns, Penaeus semisulcatus. Otsu, (1960) reported that the accumulation of yolk granules in the oocytes was stimulated by repeated implantation of pieces of the thoracic ganglion of the immature female crab Potamon dehaani. Oyama (1968) in Thalamita crenata, Hinsch and Bennett, (1969) in Libinia emarginata, Nagabhushanam and Kulkarni (1982) in Parapenaeopsis hardwickii, Eastman-Recks and Fingerman (1984) in Uca pugilator and Takayanagi, et al. (1986) in Paratya compressa reported similar effects of the thoracic ganglion on ovarian maturation with in vivo and in vitro experiments. In addition to these findings, Yano et al. (1988) demonstrated that ovarian maturation of P. vannamei can be induced and accelerated by implantation of pieces of thoracic ganglion tissue prepared from female, Homarus americanus with developing ovaries. Again Yano, (1992) reported that injection of thoracic ganglion extracts stimulates Vg secretion in the sera of P. japonicus. 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 hydrodromous. 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. Among Yano and Wyban (1992) have observed that the administration of crude extracts of brain induced vitellogenesis in the oocytes of P. vannamei. Even though complete ovarian development or spawning was not encountered in P. monodon injected with crude extracts of brain, during the present investigation advanced stage of ovarian development and high rate of (80%) maturing shrimps were observed in this group.

These results indicated that the thoracic ganglion extract is more effective in the induction of ovarian maturation in adult female P. monodon. Even though the hormones from the brain of vitellogenic females also have a role in the induction of gonadal 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 after injection, was detected, in the fractions, corresponding to 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. Similarly, during the present study the ovarian maturation of P. monodon was found induced by the injection of both thoracic ganglion extract as well as brain extract. However, the induction was slower in brain extract injected prawns. These results imply the possibility that brain may be working for ovarian maturation through the thoracic ganglion. Yano and Wyban, (1992)

ported that brain probably has a hormone, which stimulates the releasing GSH from the thoracic ganglion in vitellogenic female shrimp and therefore, gonad-stimulating hormone-releasing hormone (GSH-RH) is nominated as a ssible hormone in the brain responsible for ovarian maturation in shrimp. th the crude extracts, ie. the cerebral ganglion extract and the thoracic nglion extract used in the present study were from the vitellogenic females P. semisulcatus. This indicates that ovarian maturation of adult P. nodon can be induced and accelerated in captivity by administrating crude tracts of thoracic ganglion as well as brain prepared from maturing females another species. Hormones secreted by the thoracic ganglion and brain maturing females, induces the gonadal maturation in penaeids, and these rmone are not species specific in activity in this particular groups of capods. Similar type of observations have been reported in other penaeids ke, P. japonicus and P. vannamei by Yano, (1988) and (1992). Yano, (1988) ported that ovarian maturation of P. vannamei can be induced and accelerated implantation of thoracic ganglion prepared from Homarus americanus. ilarly the ovarian maturation of P. vannamei was induced and accelerated injection of brain extracts prepared from maturing females of lobster americanus.

It has been demonstrated by many investigators that, in decapod staceans ovarian maturation and function appear to be regulated by ibitory hormonal factor produced in the X-organ sinus gland complex of estalk (Kulkarni and Nagabhushanam, 1980). Results of the present study P. monodon showed that ovarian maturation did not occur by eyestalk extract ections, on the contrary degeneration of the ovary was detected. However, stalk ablated animals showed a rapid acceleration of vitellogenesis and sequent increase in ovarian weight. A similar effect of eyestalk ablation

on vitellogenesis has been reported in Barytelphusa cunicularis (Nagabhushanam, and Diwan, 1974); Orchestia gammarellus and L. seticaudata (Charniaux-Cotton, 1975) and Parapenaeopsis hardwickii (Kulkarni and Nagabhushanam, 1980). Similarly Kulkarni and Nagabhusanam (1980) reported that eyestalk extract injections inhibited the development of ovary in Parapenaeopsis hardwickii. They reported that the maximum inhibitory activity was found when the extracts of eyestalks from post spawned animals were used. Again Nagabhushanam and Kulkarni, (1982) reported that inhibition of ovarian growth and vitellogenesis was found in the eyestalk less prawns administered with unboiled eyestalk extracts.

Although very little is known of steroid action on maturation, it has been postulated that steroid hormones can stimulate vitellogenesis in crustaceans. In crustaceans the steroids are apparently necessary for moulting (Stevenson et al., 1979) and reproduction (Adiyodi, 1978). The use of exogenous hormones to induce the gonadal maturation and spawning is not well established. However, various steroid hormones have been identified in the ovaries of decapod crustaceans, including estrogen (Hagerman et al., 1957); estrone (Fairs et al., 1990); the estrogenic compound 17 Beta-estradiol (Couch et al., 1987; Fairs et al., 1989) Progesterone and related compounds (Kanazawa and Teshima, 1971; Teshima and Kanazawa, 1971, 1973; Couch et al., 1987; Quinito et al., 1990 and 1987; Tsuminura, 1988; Tsukimura and Kamemoto, 1991; Yano, 1985 and 1987). In short, vertebrate steroid hormones seem to be present in crustacean tissues and exogenous applications of these hormones produce consistent effects with a role promoting ovarian maturation. It is likely that in future, the use and manipulation of steroid hormones in the regulation of penaeid maturation is a real possibility (Quackenbush, 1991). Junera et al. (1977) first proposed that a vitellogenin stimulating

varian hormone was present in the ovary of the amphipod, Orchestia mmarella. Since then progesterone (Kulkarni et al., 1985; Yano, 1985 and gabhushanam et al., 1982; Yano, 1987; Tsukimura and Kamemoto, 1988 and skela et al., 1992) and various estrogens including 17 Beta-estradiol ouch et al., 1987; Fairs et al., 1990; Sarojini et al., Ghosh and Ray, 92; Koskela et al., 1992 and Ghosh and Ray, 1994) have been considered the maturation process.

Administration of exogenous steroids (17alpha-hydroxy progesterone) used marked ovarian development in P. monodon during the present study, en compared to that of control animals. This observation is suggestive at, this hormone has got some potential to elicit active ovarian growth this penaeid prawn. Whereas in the estradiol injected shrimps marked arian development was not found. Kulkarni et al., (1980); and Kulkarni d Nagabhushanam (1987) were able to induce oogenesis in P. hardwickii jected with ions of 10 ug of progesterone/shrimp (9-10 cm body length) alternate days. In contrast Yano, (1985) reported that mortality was eat in M. ensis when they were injected with similar doses of (10ug) ogesterone or were subjected to repeated injections of the hormone. In ensis ovarian development and spawning were induced by a single, low se injection of progesterone (0.1 ug/g body wt.) (Yano, 1985). Sarojini al. (1985) also reported that administration of progesterone into immature male Macrobrachium kistnensis accelerated oogenesis. Nagabhushanam et . (1980 and 1982) reported that spawning has been induced in P. stylifera injecting a single dose of (50 ug/prawn, 80-85 mm) 17-hydroxy progesterone to the abdominal masculature at a lower temperature (20°C), at which this awn does not spawn naturally. Later on Yano, (1987) demonstrated that jection of 0.01 ug. 17-alpha-hydroxy progesterone/g body wt. is effective

stimulating secretion of large amount of Vg. into haemolymph of P. onicus. Conversion of progesterone into 17-alpha-hydroxy progesterone s been demonstrated in the ovaries of the crab, Portunus trituberculatus eshima and Kanazawa, 1971). Therefore based on these observations, it n be deduced that progesterone may serve for ovarian vitellogenesis as precursor of 17-alpha-hydroxy progesterone, which stimulates Vg. synthesis ano, 1987). Results of the present investigation in which 20 ug/g. body . of 17-alpha-hydroxy progesterone has been administered, caused the duction and acceleration of the ovarian maturation to a marked extent. e lack of full maturation and spawning may be due to the limited time and/or appropriate dosage. Optimal progesterone doses and injection sequences induce ovarian maturation and spawning may vary from species to species ano, 1985). It is however unclear whether 17-alpha-hydroxy progesterone ts directly or as a precursor to stimulate Vg. synthesis. Nagabhushanam al. (1982) suspected that 17-alpha-hydroxy progesterone and 17-Beta-hydroxy gesterone, bypasses the synthesis of steroid mediator. The possible nversion of injected steroid hormone into desired hormone which may be urally occurring in crustaceans, is also speculated (Nagabhushanam et ., 1987; Sarojini et al., 1990). In contrast, Tsukimura and Kamemoto 88) have reported that, neither 17-Beta-estradiol nor progesterone induced ovarian maturation in P. vannamei. Similarly, Anon (1992) failed to fectively enhance ovary development in pre-adult P. monodon using alpha-hydroxy progesterone and 17-Beta-estradiol. Koskela et al. (1992) e also reported a lack of response in ovarian maturation to alpha-hydroxy progesterone and 17-Beta-estradiol administered in P. ulentus. Administration of estradiol caused marked ovarian development r the control in crab Scylla serrata (Sarojini et al., 1990). Ghosh and

ay (1992 & 1994) found a dose-dependent increase in the concentration of wo lipogenic enzymes viz. glucose-6-phosphate dehydrogenase and malate ehydrogenase in the ovary of freshwater prawn M. rosenbergii, which are nown to be estrogen responsive in vertebrate systems. As in the present bservation in P. monodon, the lack of response in the induction of ovarian aturity was reported in estradiol injected penaeid prawn P. esculensis by oskela et al. (1992).

The water quality parameters during the present experimental studies ere within the acceptable level prescribed for maturation of penaeids by rimavera (1985), Lumare (1981), and Yano (1985) in P. japonicus and Beard nd Wickins, (1980) in P. monodon. It is indicated from the above studies hat the effect of GSH or GIH on induction or inhibition of vitellogenesis n penaeid shrimps is controlled by temperature, salinity and/or photoperiod. n general each animal species uses distinct environmental cues for timing f its reproduction, which has been detected by the neuronal structures and he complex neuroendocrine system activates or inhibits the process (Van erp, 1992). Each animal species uses distinct environmental cues for timing f its reproduction, has a full range of neuronal structures for perception f the signals, and uses a complex neuroendocrine system for transduction f the messages to the endocrine organs, which themselves produce factors egulating the activity of the organs involved in reproduction.

The inside wall of the tanks were painted in black during the esent study, since Emmerson (1980) reported that there is more ovary turation rate for P. indicus maintained in black walled tanks. The njuries caused by the collision of the shrimps P. setiferus and P. ylirostris against the walls of the tanks were minimized by using walls inted in black (Brown et al., 1979; 1980). The light intensity in the

experimental tanks was reduced by covering the tanks with black cloths since influence of photoperiod on maturation has been studied by Laubier and Laubier, (1979) in unablated P. japonicus and Lumare, (1979) in unilaterally eyestalk ablated P. kerathurus and found that best maturation and more spawnings were occurred in tanks where light intensity was less. Beard and Wickins, (1980); Emmerson, (1983; and Hiller, (1984) opined that maturation is faster in reduced light conditions in P. monodon.

From the observations it was concluded that among the different neuroendocrine and hormonal manipulations eyestalk ablation and thoracic ganglion extract injection are found to be the best methods the induction of gonadal maturity of P. monodon in captivity together with the appropriate age and size of the animal in good quality seawater. This indicates that when the environmental factors are favourable the GIH from the eyestalk become dormant and the GSH mainly from the thoracic ganglion takes the role for the gonadal development. Hormones from the brain also helps in the secretion of the GSH either directly or through thoracic ganglion. Among the two exogenous hormones tried 17alpha-hydroxy progesterone induced the ovarian development to great extent but not estradiol.

CHAPTER V

# STUDIES ON CRYOPRESERVATION

A *Reproductive quality of male* ***Penaeus monodon***

B *Cryopreservation studies*

# CHAPTER V

## STUDIES ON CRYOPRESERVATION

### INTRODUCTION

The prospects of shrimp culture had improved well during the past decade, as the biology and husbandry of these crustaceans have been more thoroughly investigated. Presently, the shrimp culture is progressing to the point where genetic programmes can begin to help increase the production target (Tave and Brown, 1981). Technological improvements in the areas of reproductive biology however are needed for the further development of shrimp aquaculture industry in our country. In sufficiently sound breeding programmes, it is often necessary to produce and identify full sib-families and/or half sib-families and to identify all parents. The current spawning techniques of placing many shrimps in large tanks is not adequate for such programmes. By establishing the modern techniques for the preservation and utilization of male genetic materials of known heritage, as in higher animals, for the future use, the additional efforts and costs for the maintenance of male broodstock in the hatcheries can be avoided. Cryopreservation of viable gametes from known heritage can solve these problems to a greater extent.

Although the shrimp species can be bred in captivity, there are many problems associated with maturation and insemination that have not yet been solved. The major problem often arising during the breeding programmes of penaeid prawns is the absence or loss of the stored spermatophores from the wild caught and laboratory matured shrimps. Another problem is the failure of shrimps to copulate in captivity. In order to solve these problems and simplify selection studies, Persyn (1977) developed a method of artificial

insemination in penaeid prawns. Artificial insemination involves manual extrusion and attachment of the spermatophores inside the thelycum of the female. This technique will allow the production of/and identification of the parent strains and progeny. This technique is necessary during the production of intra and inter specific hybrids, because behavioral and structural differences of thelyca among strains and species can serve as a pre-fertilization barrier (Aiken et al., 1984). Manual spermatophore transfer can circumvent these problems and enable the breeders to produce valuable hybrids.

Artificial insemination results in high yield, in terms of nauplii per spawn (Alfaro, 1993). Artificial insemination has been successively done in many decapod crustaceans by researchers like Sandifer and Lynn (1980) in P. monodon, Chow et al. (1982) in M. rosenbergii, Brey et al. (1983) in P. setiferus, Aiken et al. (1984) in P. monodon, Lin and Ting (1986) in P. monodon, Talbot et al. (1986) in H. americanus and Joshi and Diwan (1992) in M. idella in the laboratories. However, these technique is not practiced in most of the commercial hatcheries due to the unawareness of its high potentialities. The technique involves the extraction of spermatophores, and then its placement in the thelycum which is a complicated process and needs utmost care. A small mistake in the technique may lead to the failure of the whole attempt. One of the biggest problems in spermatophore transfer is the amount of time needed to position the spermatophore properly. Shrimps are easily stressed by short periods out of water and stressed condition for longer period leads to the unsuccessful attempt. Strong healthy undamaged spermatophores enables a quick artificial insemination. Usually the spermatophore extrusion method determines the condition of the spermatophores. Sandifer et al. (1984) suggested that, the use of electrical stimulation

for obtaining spermatophores is an adequate method for penaeid shrimps. This technique has been widely used by many scientists for the ejaculation of spermatophores from various decapod crustaceans like lobsters (Kooda-Cisco and Talbot, 1983) and shrimps (Sandifer et al., 1984; Harris and Sandifer, 1986; Rosas et al., 1993; Alfaro and Lozano, 1993).

The successful cryopreservation of gametes would open new perspectives in any culture operations. The ability to cryopreserve gametes and embryos is an important insurance for the research and breeding programmes. In a case of aquatic animals, the identification of parental strains and progeny, management of better quality and/or wild stocks for farming etc. are hurdles which are difficult to overcome. The ability to freeze the gametes of aquatic organisms and to store them for longer periods without deterioration, would be of considerable value in genetic improvement of aquacuture.

The earliest cells to be cryopreserved successfully were the bovine sperm in 1949 (Polge et al., 1949). This breakthrough allowed for storage of viable broodstock sperm and led to increased production. Sperm has been chosen for use in cryopreservation studies because of the ease in assessing viability either by motility or fertilization and their commercial importance. Studies on cryopreservation of vertebrate semen, especially that of mammals have grown considerably since 1960, but unfortunately similar studies on invertebrate semen have been sparse (Behlmer and Brown, 1984). Methods to cryopreserve gametes of aquatic animals are less developed, (Alderson Mc Neil, 1984; Bougrier and Rabenomanana, 1986) even though the sperm cryopreservation has been successfully done in a number of commercially important aquatic vertebrate, and invertebrate species in

the past few years. However, the reproducibility of sperm cryopreservation needs to be improved and sperm cryopreservation on a large, commercial scale are yet to be developed.

Many attempts have been made to cryopreserve the spermatozoa of aquatic vertebrates, mainly fishes. However, less attention has been paid on invertebrates. The various attempts of cryopreservation in invertebrates includes the reports of Sawada and Chang (1964) in honey bee; Dunn and Mc Lachlin (1979) in echinoderm sperm; Behlmer and Brown (1984) in Limulus polyphemus; Chow et al. (1982 & 1985) in M. rosenbergii; Ishida et al. (1986) in lobster sperm; Anchordouguy et al. (1987 & 1988) in Sicyonia injentis; Jayalectumie and Subramoniam, (1989) in Scylla serrata; Renard,(1992) in Crassostrea gigas; Bury and Olive (1993) in polychaetes; Joshi and Diwan (1992) in M. idella and Subramoniam and Newton (1993) in Peneaus indicus embryos. In site of the fact that among invertebrates, decapod crustaceans are the most important group, very little attention has been paid on the cryopreservation of gametes. The limited numbers of attempts in the cryopreservation of gametes in the decapod crustaceans may partially be due to the fact that, sperm of decapod crustaceans are an exemption from those of the other animals, as they are nonmotile. The non-motile sperm of decapod crustaceans are a richly diverse group, in terms of both morphology and the types of activational changes that precede fertilization (Griffin et al., 1988). Motolity assays employed with sperm of other animals are obviously inappropriate with the sperm of penaeiodean shrimps, as they are non-motile. So a reliable technique for the assessment of viability of the preserved spermatozoa is needed as a pre-requisite for cryopreservation studies. Over a century ago, reptantian sperm were shown to undergo extensive structural changes (Hallez, 1874) that could be induced either mechanically or chemically

(Koltzoff, 1906; Binford, 1913). These structural changes were later on recognized as the acrosome reaction by Berker and Austin (1963). The fine structural events of the reptantian acrosome reaction were examined during sperm-egg interaction in the blue crab, Callinectes sapidus by Brown (1966). Similar reactions have been described in more detail, in other reptantians by Pochon-Masson, (1968). However, Talbot and Chanmanon (1980) showed that, the acrosome reaction of reptantian sperm can be artificially induced with the divalent cation ionophore A 23187.

Pochon-Masson (1968) reported the existence of an acrosome also in the natantian sperm also and Kleve et al. (1980) described its structure in detail. The acrosome reaction in the natantian sperm has been observed by Yudin et al. (1979) during sperm-egg interaction. It has been also reported that, the unistellate and non-motile spermatozoa of the penaeiodean natantians do undergo an acrosome reaction (AR) at the time of sperm-egg interaction (Clark et al., 1981, 1984). Acrosome reaction in a sperm is a pre-requisite to fertilization and consists of exocytosis of the acrosomal vesicle, followed by the formation of an acrosomal filament (Griffin et al., 1987). The acrosome reaction of natantian sperm can be inducted in vitro by the egg-water and ionophore 23187 (Griffin et al., 1987). In decapod sperm the viability can be assured by the structural changes that take place during the acrosome reaction. Sperm viability of decapod sperm has been assessed through acrosome reaction by investigators like Talbot et al. (1976) in lobsters, Talbot and Chanmanon (1980) in H. americanus; Clark et al. (1981 & 1988) in S. injentis; Griffin et al. (1987, 1988, 1990) in S. injentis; Anchordouguy et al. (1987, 1988) in S. injentis.

Sperm of consistently high quality are an absolute requirement for cryopreservation (Aquacop, 1982 and Heitzmann et al., 1993). As spermatophore transfer, hybridization and sperm bank programme require high quality sperm (Bray et al., 1982 and Lin and Ting, 1986), the evaluation of the reproductive quality of males is essential as a pre-requisite for such studies. Although penaeid males have been assumed to maintain favourable reproductive performance in captivity, a decrease in sperm quality has been described in many penaeids by various investigators like Aquacop (1982) in P. vannamei, Chamberlain et al. (1983) in P. setiferus, Lin and Ting (1984) in P. monodon, Leung-Trujillo and Lawrence (1985) in P. vannamei and Gomez and Primavera (1994) in P. monodon. Consequently, satisfactory male broodstock management is routinely practiced in by our hatcheries, resulting in sub-optimal production of high quality sperm. Therefore an increased number of male brooders is necessary to get high fertilization rates, but this has a negative impact on production cost, and also on the quality of the larvae produced due to low sperm quality in the captive males. Moreover once the spermatophore has been transferred from a live male to female during mating it took a few day for the regeneration time for the spermatophores in male penaeids is fragmentary. Only two reports are available in connection with the spermatophore regeneration time in penaeids by Leung-Trujillo and Lawrence, (1991) in P. setiferus, P. vannamei and P. stylirostris and by Rosas et al. (1993) in P. setiferus. Far less published information is available regarding the time taken for the complete formation of this complex structure in penaeid shrimps under laboratory conditions (Leung-Trujillo and Lawrence, 1991). However rhythms and control of spermatophore production, and the effects of forced expulsion of spermatophores on its successive production and sperm quality remain unknown.

Similarly, there has been limited knowledge about the quality of the sperm during successive regeneration after extrusion of the spermatophore through electric stimuli (Leung-Trujillo and Lawrence, 1985; Bray et al., 1985; Pratoomchat et al., 1993; Alfaro, 1993 and Gomez and Primavera, 1993).

Although significant achievement has been made in female reproduction through eyestalk ablation, its effect on male reproduction has not been studied thoroughly. However, improvements in male reproductive performance has been reported recently in various penaeids like P. vannamei and P. setiferus by Leung-Trujillo and Lawrence (1987) and P. vannamei by Salvador (1988). Increased nauplii production, after artificial insemination using spermatophores extruded from eyestalk ablated males, has been reported in P. stylirostris by Rosas et al. (1993). However, reports of problems affecting the reproductive potential of several species of male penaeid shrimp, and the possibility that the lack of fertilization might be partially responsible for the persisting problems in the laboratory spawned animals, has prompted closer study of male shrimp fertility.

## MATERIAL AND METHODS

### Collection and maintenance of animals

Adult specimen of P. monodon (maleš) ranging in size from 115 to 0 mm in length, were collected from the sea as well as from the brackish ter ponds at Vypeen island near Cochin and brought to the laboratory in ve condition. Matured males were distinguished by the presence of united tasma and swollen white colour at the coxae of the last pereiopods. These imals were kept in 500 l capacity circular tanks, provided with a biological lter, till their utilization. The tanks were aerated with oil free air om an air compressor. Every day about 50% of the (S 30%.) water in the nks was siphoned out along with the sediments and faecal strands and placed with fresh filtered seawater without disturbing the prawns. The awns were fed ad libitum with clam meat.

### Acquisition of spermatophores

Three methods of extraction were employed for the acquisition of ermatophores for further studies. A) Micro surgery techniques B) Squeezing thod C) By electro ejaculation techniques. Among these three methods, e best method was followed thereafter.

### Micro-surgery technique

In this method spermatophores were taken out by carefully dissecting e coxal part of the 5th pereiopods. Animals were kept ventral side up d held carefully and strongly, so that the jerk could be prevented during e time of dissection. The gill irrigator in conjunction with a restraining vice (Tave and Brown, 1981) was used to reduce the stress during the ssections. Coxal muscle was cut open, making the terminal ampoule exposed

and by gentle squeezing, the spermatophores were extruded from it. Extreme care was taken to minimize the wound and loss of haemolymph. Dissections were made as quickly as possible to remove the spermatophores and the animals were than released in a separate tank containing filtered seawater provided with aeration. Wounded animals kept separately, fed with clam meat and observed for the regeneration of the spermatophores.

**b). Squeezing method**

Here spermatophores were squeezed out of genital pore by gentle pressure around the coxae of the fifth pereiopods. No wounds were made during the process of extraction. However, this method required skillful experience for extracting whole spermatophore undamaged. The same gill irrigator described above was used here also to reduce the stress during the extrusion. Animals which were used for the spermatophore extrusion were kept separately in tanks filled with fresh seawater, and observations were made on their survival and regeneration of the spermatophores.

**c). Electro-ejaculation techniques**

The electrical stimulation device was an improved model of that described by Sandifer and Lynn (1980). It consisted of an electrical transformer to reduce voltage, a rheostat, a volt meter, and two electrodes (fig. 199). The shrimps were held ventral side up in a plastic tray and the delivery tubes of the gill irrigator was placed in each branchial cavity, and a continuous flow of water bathed each set of gills. The electrodes were placed near the gonopores at the base of the 5th pareiopods and a stimulus of 6 to 12 V was applied, usually for 1 to 2 sec. The stimulus was expected to cause the musculature surrounding the terminal ampullae to contract, expelling a single spermatophore from each gonopore (fig. 200). The expelled

ermatophore (fig. 201) was removed with a wooden needle and used for rther studies. In cases where the spermatophores are partially expelled, was removed by applying gentle pressure at the coxal region for the plete ejection and the completely extruded spermatophores were taken out. certain cases partially expelled spermatophores were taken out using rceps with blunt ends to avoid the damage during its transfer.

Among the three methods used for the extrusion of spermatophores, it s found that electro-ejaculation was the best method, as it did not harm e animals nor the spermatophores (fig. 202). Therefore, this technique s followed for the acquisitions of spermatophores for the further vestigations in P. monodon.

**Experiments of spermatophore regeneration time in P. monodon**

To determine the time required for regeneration of spermatophore in n-ablated sexually mature males, existing spermatophores were removed from adults at the onset of the experimental trial. Baseline (control) ermatophore's weights, sperm count, morphology and viability were determined om a spermatophore sample (N=8). The methodology for sperm count and iability of sperm has been described in third experiment on effect of estalk ablation on sperm quality. Each day, terminal ampoules from two three randomly selected males were dissected, to assess the extent of ermatophore development if they did not ejaculate the spermatophores upon lectro-ejaculation. The sperm quality and quantity (if sperm were present) as assessed using the techniques of Leung-Trujillo and Lawrence, (1985). ermatophore maturity was assessed based on its morphology and colour. se of these criteria as determinants of spermatophore maturity were first eported by Persyn (1977). Experiments were terminated when the remaining xperimental animals extruded spermatophores upon electrical stimulation.

At the termination of the experiment animals which did not extrude their spermatophores, were dissected out and their maturity was assessed. Spermatophore morphology, appearance and weight along with sperm count, sperm morphology and viability were compared to the baseline (control) values. The experiment was repeated three times.

### 3. Effect of eyestalk ablation on sperm quality

The second series of experiments were planned to investigate the effect of unilateral eyestalk ablation on the sperm quality. Eyestalks of 30 animals were ablated using the method of Muthu & Laxminarayana, (1977) with an electro-cautery apparatus. After eyestalk eyestalk (4 to 5 days), the spermatophores were removed from animals and the extent of the development of new spermatophore was monitored on a daily basis as previously described.

The quality of the sperm cells obtained before and after regeneration was evaluated through spermatophore weight, sperm cell counts, percentage of normal and abnormal sperm as well as the viability of the sperm. Spermatophore weight was taken to a point nearest to 0.01 g. Sperm counts were performed by homogenizing the compound spermatophore in a glass tissue grinder, containing 3.0 ml of artificial seawater. The suspension was then mixed thoroughly by gentle shaking to ensure homogeneity, and the samples were then used for the sperm counting using a haemocytometer (the sample volume was 0.0001 ml.) (Leung-Trujillo and Lawrence, 1985).

Percentage of abnormal sperm was obtained by recording, over a transect of a microscope slide, number of normal sperm (spherical body and elongate spike) and number of abnormal sperm (malformed bodies and bent spikes) (Talbot et al., 1989). At least 100 cells for one of the categories were recorded and from this percentage of normal abnormal cells was calculated.

**Viability of spermatozoa**

Acrosome reactions, induced by gamete interaction were observed in sperm sociated with eggs spawned from inseminated females. The morphological anges that occurred at different intervals in the sperm were studied using phase-contrast microscope and was used as the standard for comparing the ructural changes that occurred during the *in vitro* acrosomal induction. e morphological changes that occurred during the *in vitro* acrosome reaction s been studied with the aid of three chemical compounds viz. Bromocalcium nophore A 23187, Valinomycin and Nigricin (three from Sigma) (Clark *et* . 1981) and a biological agent the egg-water(Griffin *et al.* 1987). The ructural changes that took place in the sperm cells were compared with e structural changes that were found in the *in vivo* studies made during e natural spawning. From these observations, the best inductors were ntified for the sperm of *P. monodon* and selected for the following periments. During the present investigation "egg-water" described by iffin *et al.* (1987), as well as the ionophore A 23187 (Talbot *et al.* 1980), s been identified as the best solutions for the induction of acrosomal lament during the *in vitro* acrosome reaction. The egg-water was extracted follows. Fully gravid female prawns were collected from wild and nsported to the laboratory in live condition. They were transferred to ass aquarium tanks containing clean filtered seawater provided with ration, and observed for spawning. When a female prawn showed spawning haviour it was removed from the glass tank and held in 1 l glass beaker th half (500 ml) filtered seawater (34 S%.) until eggs were released. gs were allowed to settle and after the negatively buoyant eggs settled the beaker bottom, about 80% of the seawater was drawn off and the eggs re swirled for five minutes with enough force to keep them in suspension. e eggs were again allowed to settle, and after settling the egg, the

remaining supernatant fluid now called 'egg-water' was removed by pipette and cleared by centrifugation for 15 min. at 15,000 rpm (4°C, 15 min.). This egg-water was divided into 5 ml aliquots and stored in liquid nitrogen, if not used immediately.

Extruded spermatophores were individually and gently homogenized to free the sperm cells, using a glass tissue grinder and filtered seawater (S %.). Tissue fragments were separated from the sperm supernatant by hand centrifugation. Assays were conducted in culture tubes of 3 ml capacity. Prior to the addition of sperm, 900 ul of egg water is added to each assay tube. Then immediately the supernatant was removed by a pipette and 100 ul of sperm cells were pipetted into the egg water, and mixed thoroughly. After 5 minutes of incubation, 100 ul of the sample was removed to another tube and fixed with a drop of 70% ethanol in seawater. The remainder of the sample is allowed to incubate for another 55 minutes. After 1 hr incubation, sperm were examined to determine the number of reacted sperm cells which had undergone acrosomal exocytosis as described by Clarck et al. (1981). Sperm cells were examined using a haemocytometer and phasecontrast microscope, with >100 sperm cells observed per count and three counts per replication. Sperm are scored for acrosomal status by viewing under 40 power (objective lense) using a phasecontrast microscope. To ensure accuracy, triplicate slides were prepared and scored from each aliquot. In addition, a portion of the sperm suspension was examined microscopically for normal and abnormal sperm cells. Abnormal cells were distinguished from normal by malformed bodies or by bent, short or missing spikes. Percentage of viable sperm was calculated as a percentage of normal sperm. Sperm quality of unilaterally eyestalk ablated P. monodon males during different regeneration were also studied in the same manner. Statistical analysis were made to analyze the data.

**Cryopreservation studies**

Sperm cells were isolated from the spermatophores by the same method as described in the earlier section. Isolated sperm were diluted in the extender solution to a concentration of 10 to 10 sperm/ml and incubated in an equal volume of cryoprotectant solution (50 ul) for 5 minutes at room temperature (28 °C), before initiation of the cooling process. Screening experiments were done using seven individual and six combinations of cryoprotectants for short durations. Cryoprotectants used for the screening experiments were glycerol (0.5 M), trehalose (0.25 M), dimethyl sulphoxide (DMSO) (5%), proline (0.5%), valine (0.5 M), sucrose (0.25 M) and ribose (0.5 M). Combinations of these with DMSO viz. DMSO (5%) + glycerol (0.5 M), DMSO (5%) + trehalose (0.25 M), DMSO (5%) + proline (0.5 M), DMSO (5%) + valine (0.5 M), DMSO (5%) + sucrose (0.25 M) and DMSO (5%) + ribose (0.5 M) were also tried. All solutions were made up in standard seawater. Three different temperatures at which the samples stored were 0°C, -30°C and -195°C. Freezing chamber of the refrigerator was used for 0°C, where as freezing chamber of the cryostat was used for -30°C. Samples preserved at -195°C were kept immersed in liquid nitrogen in closely sealed containers. In order to select the best cryoprotectants for the spermatozoa of P. monodon short duration experiments were carried out. Sperm samples were diluted with either of the above mentioned cryoprotectants, (individual and combinations) and the percentage viability were determined for each. Samples were preserved for 3 time intervals of one day, 5 days and 10 days for the screening experiments. Percentage of viability were determined for samples preserved in all the cryoprotectants for the three different temperatures as well as for the three different intervals. From the results cryoprotectants which gave the best results were selected and used for the

actual cryopreservation studies. The solutions selected were glycerol (0.5M), trehalose (0.25), DMSO (5%), DMSO (5%) + glycerol (0.5 M) and DMSO (5%) + trehalose (0.25 M). The same experimental protocol was used for both the screening and cryopreservation studies as follows.

Samples incubated with cryoprotectants were frozen in plastic cryovials at the precise cooling rates, using a freezing chamber of cryostat. The samples were cooled from room temperature to -30°C at a rate of 1°C per minute. Once the sample temperature reached -30°C, the samples were plunged into a liquid nitrogen bath, after incubating 5 minutes in its vapour.

Samples were thawed at room temperature (28°C). When the sperm suspensions were melted, the samples were diluted with 900 ul of standard seawater and centrifuged in refrigerated centrifuge for 3 min.(-10 °C). The supernatant was removed and the sperm pellet was resuspended in 100 ul of egg water which was collected and preserved as described above. Sperm samples were incubated in egg water for 1 hr. and thus the percentage of sperm that had undergone both phases of the acrosome reaction was determined using phase contrast microscopy. Frozen samples were compared to fresh, unfrozen samples which reacted immediately after isolation. Sperm viability after freeze/thaw is expressed as a percentage of unfrozen controls.

All results were compared by a multiple-way analysis of varience (M-ANOVA) on arcsin transformed data. Variance homogeneity was tested according to Bartlett's statistic. Newman-Keuls'test were used to classify the data when significant statistical differences were observed (Snedecor and Cochran, 1967).

# Results

## Reproductive quality of male Penaeus monodon

### lectro-ejaculation of the spermatophore

Spermatophore collection was made by the three methods described in terials and methods. Animals having a prominent white colour at the base the 5th pereiopod (around the coxae) were selected for spermatophore pulsion. Among the 3 methods tried, the best results were obtained with lectro-ejaculations. This technique did not appear to harm the animals d could be used to elicit spermatophore from the same animals again and ain. Spermatophores were extruded from a total of 90 animals. For this rpose the animals collected were divided into three groups of thirty imals. The spermatophores were collected by micro-dissections of the rminal ampoule from the first group and it gave the greatest number of permatophores at a time. Spermatophores were obtained from both sides of ll the dissected animals. But the major disadvantage of this method was t wounded the animal during dissection. Mortality rate was found high in his method (50%). Even though the animals were alive they were not found o regenerate the spermatophores easily due to the stress caused by the wound nd handling. Most of them were found moulted within 6 to 7 days of the issections. Even after the moulting, long periods were taken for egeneration of the spermatophores. Apart from this, infections were found t the regions of dissections making the animal incapable of regeneration f the spermatophores. In addition, sometimes some of the spermatophores ollected by this method had not completed their full development. However, he advantage of this method is that it can be used to collect the permatophores from the live as well as freshly died animals when large umbers of spermatophores are required.

The second method tried was the manual extrusion by mechanical force ie. by forcefully and gently pressing the coxal regions. Here the fully developed spermatophores were only extruded out. This method was not useful to eject the spermatophores from dead animals. However, in the case of live animals, mortality rate was high (78%). and the spermatophore extrusion rate was less compared to the other methods and only 34% of the animals extruded their spermatophores by this technique.

The best results were obtained by the third method, viz. the electro-ejaculation. The main requirement for this method was an electro-ejaculator (fig. 199) with an electrical transformer for reducing the out put current to a maximum of 12V. The extrusion was found induced when the out put current reached 12V (fig. 200). In all the trials, it was found that the fully formed spermatophores were extruded out by more than 80% of the animals tried. Spermatophore extrusion was successfully induced in 25 out of 30 shrimps tried. Mortality rate was only 10% in this method. All the 90% of animals were maintained live condition after the extrusion and observed for regeneration of the spermatophores. Spermatophore regeneration was noted in all the live animals. A second trial of extrusion was made after 10 days of the 1st extrusion and more than 60% of the experimental animals gave positive results. Rest of the animals also extruded their spermatophores on consecutive days. In 11 cases spermatophores were extruded out from both the gonopores in one trial and in other cases, only one side responded positively. In most of these cases in which both spermatophores were extruded, the extrusion was simultaneous and it happened at the 1st shock itself. In the rest few cases, one of the spermatophores came out first during the first shock and the next came after some time on the second or third shock. A decrease in the size was noted in these regenerated spermatophores. From all the trials it was noted that, spermatophore

extrusion was a rapid process. The ejection was taking place by the rapid contraction of the muscles of the terminal ampoule, as well as coxal muscles which encompassed the spermatophores. Its actual passage through the gonopores took only 2-5 seconds. Immediate response was observed when the current reached 12V. Animals with a prominent white colour at the gonopores, only did complete expulsion of the spermatophores within 1-2 seconds. Even though electrical stimulation did not cause the complete expulsion of the spermatophores in all cases, almost 45% of the animals expelled either one or both of their the spermatophores as a whole. In case of others in which the spermatophores expelled were only partially they could be removed easily with forceps without disturbing the animals further. Nearly 40% of survival was observed even after the 2nd turn of electro-ejaculation. However, electrical stimulation could not induce 10% of the experimental animals to extrude its spermatophores. Some of these animals which failed to extrude their spermatophores, acquired a gonopore infection during the experiment. It was found that further spermatophore extrusion was blocked from these infected gonopores. Gonopore infection was also observed in some of the spermatophore extruded animals. It was noted that infections were found in animals which were subjected to electric stimulation more than twice.

Single short stimuli usually appeared to cause little or no permanent injury or mortality to the animals. Still mortality in few numbers was found due to the contraction of the abdominal muscles. Powerful strokes of the animals at the time of electric stimulation was the cause for this contraction of the abdominal muscles. After the electric stimuli these animals were seen bend due to the contraction of the muscles, and those which failed to attain the normal state were found dead after 1 or 2hrs. In spite of all these, 80 to 90 % of survival was observed in animals where electrical stimulation was given for the spermatophore extrusion.

## Spermatophore regeneration and effect of eyestalk ablation

In order to find out the spermatophore regeneration time in P. monodon nd to check whether eyestalk ablation has any effect on spermatophore egeneration, 3 sets of experiments were carried out. All the experimental ets were comprised of twenty animals each. Half the number of total prawns n each group were subjected to unilateral eyestalk ablation and the remaining alf without ablation, kept as control. Spermatophore regeneration time n unilateral eyestalk ablated and unablated male has been given in the table 9). Production of mature spermatophores in the terminal ampoules were bserved only after 9 to 12 days in unablated males. Where as unilaterally yestalk ablated animals extruded mature spermatophores within 6 to 8 days y electro-ejaculation.

| Groups | Timings of spermatophore development (days) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Eyestalk ablated | (I) | (II) | (---III---) | | | | | (----IV----) | | | | |
| Non ablated | (--I--) | | (----II----) | | | (-----III-----) | | | | (-------IV-------) | | |

Stage I- Undeveloped Stage II- Early developing
Stage III- Late developing Stage IV- Mature

Table 9: Schematic presentation of spermatophore development stages (at terminal ampoule level) for adult male P. monodon in eyestalk ablated and non ablated animals during captivity

In order to study the transitional stages of development in the permatophores, randomly selected one or two animals from both the eyestalk blated and unablated sets were subjected to electro-ejaculation. If the nimals did not ejaculate the spermatophores, they were then sacrificed nd their terminal ampoules were dissected out for the assessment of the xtent of spermatophore formation. Dissections made in the random samples f the experimental groups revealed that, complete development of the permatophores required more than 9 days in non-ablated animals, however, he same required only 6 to 8 days, in unilaterally eyestalk ablated animals. he maximum time taken for the regeneration and extrusion of fully developed permatophores in ablated group was 8 days whereas it was 12 days in on-ablated group. Among the unablated animals 40% extruded fully formed permatophores on the 9th day and 30% on the 10th day. All the animals of he ablated group extruded their regenerated spermatophores by 8th day itself, hereas non-ablated animals extruded only by 12th day.

Present investigations during regeneration of the spermatophores in P. onodon revealed that, the final moulting of spermatophore is a complicated rocess which takes place inside the terminal ampoule. Various steps in he process of spermatophore formation and also the time taken for each tep has been identified. Based on the structure, colour and shape the evelopmental stages of the spermatophores inside the terminal ampoule of P. monodon, development of spermatophores were divided into 4 stages viz. nder developed (stage I), early developing (stage II), late developing (stage II) and fully developed (stage IV). Spermatophore maturity was assessed ased on the colour, and morphological characters. Fully developed permatophores appeared white in colour with a well developed wing. The usculature of this bag like spermatophores became well developed. Such ully developed spermatophores, with well developed musculature are found

be ejected immediately upon electrical stimulation. The terminal ampoules the prawns dissected on the next day of ejaculation were found almost pty in both the eyestalk ablated and unablated groups. Stage I or derdeveloped spermatophores were observed inside the terminal ampoule on he 2nd day of extrusion in both the groups. The terminal ampoule at this me contained only some transparent but thick substances with a fluid nsistency. No sperm were observed among this fluid matrix. Unablated imals dissected on the third day also contained the same material. Whereas he terminal ampoule of the ablated animals on the third day contained permatophore materials in early developing stage (Stage II). In this case t was noted that the transparent fluid matrix observed in the previous stage came milky white in colour. This milky white fluid was highly viscous n nature. Small thin pieces of hardened rod like materials were also found n this viscous fluid material. The hardened rods were about 1 to 3 mm in ngth and 1 to 1.5 mm in width. These contents did not exhibit any rganization or presence of sperm or spermatophores inside the terminal mpoule. First evidence of the recognizable spermatophores has been found n the terminal ampoule on IVth day of the initial extrusion of spermatophore n ablated animals, whereas in non ablated animals the same was observed nly on the 6th day of 1st extrusion. Even though the electrical stimulation uld not ejaculate out, this stage of development of the spermatophore was dentified as late developing or stage III. During this stage of development he terminal ampoule contained a semi cylindrical sperm sac, with millions f sperm inside. These unhardened spermatophores were not fully equipped ith the membraneous parachute like wings. The spermatophores were found o be covered by an extremely thin transparent membrane. On the following ays in both the groups of eyestalk ablated and non-ablated animals partially odified wing like structures were encountered. The white viscous fluid

consistency found around the spermatophore was not so prominent indicating that, this material had transformed into the wing. Development of the wing as well as the hardening of the spermatophore was found only on the 5th day in ablated and on the 8th day in non ablated males. Electro-ejaculation of the fully formed spermatophores could be possible on the 6th day onwards in ablated group. Where as in non ablated group, this happened only on the 9th day onwards. This fully formed spermatophore was identified as the stage IV or mature spermatophore. The prominent white colour of the spermatophore was easily recognizable during this stage, at the basal coxopodite of the 5th pair of pereiopods externally through the exoskeleton. Here the white viscous material was not at all apparent, instead, a colourless transparent viscous fluid was found around the spermatophore. All the remaining animals in the ablated group extruded their spermatophores by the 8th day. However, the non-ablated animals started their spermatophore extrusion only from 9th day onwards and completed by 12th day.

## II. Effect of regeneration on the sperm quality of eyestalk ablated and non ablated animals

Various parameters selected for the assessment of sperm quality were the weight of the spermatophore, sperm count, percentage of normal and abnormal sperm and percentage of viable sperm. An evaluation of the eyestalk ablation on the quality of regenerated spermatophores and sperm is presented in the fig.(203).

### . Spermatophore weight

It was interesting to note that in the first group of unablated animals, the spermatophore's weight has been found reducing in successive regenerations. The overall mean weight of the spermatophores of control

hrimp was 0.56gm (fig. 203). At the termination of the experiments all he shrimps in both eyestalk-ablated and non-ablated animals extruded mature, ell developed spermatophores with no evidence of any discolouration or eterioration. All the eyestalk ablated males had significantly larger permatophores (0.58,0.59 and 0.60 g) than those of the non-ablated control roup (0.50, 0.47, and 0.45 g) during the 1st 2nd and 3rd regenerations espectively.

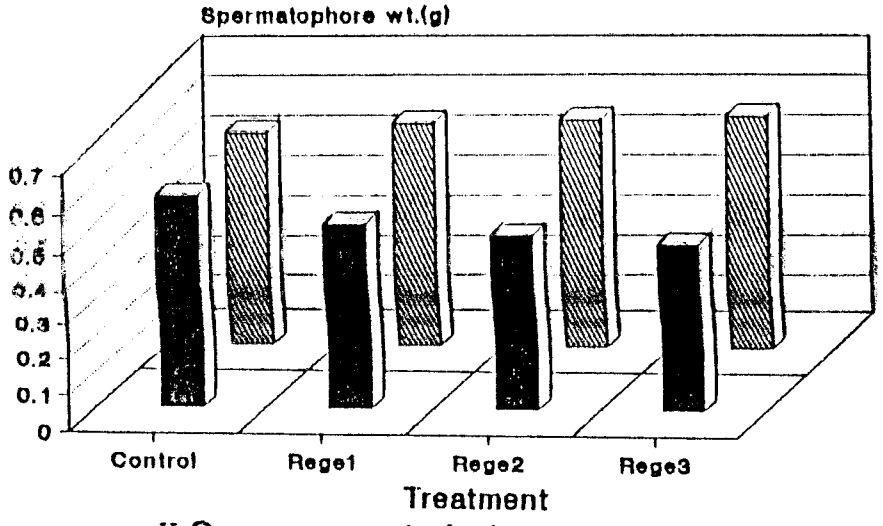
**perm count**

The mean sperm count at initiation of the experiment was 128 million ells (fig. 203). Unilaterally eyestalk ablated males exhibited a mean sperm ount of 146 millions cells at the 3rd regeneration time (fig. 203) which as significantly higher than those for the control (53.3 million cells) n the 3rd regeneration time. The sperm count was found increasing 128,132.3, 125.6, 144.6 respectively) in successive regenerations, according o the increase in the spermatophore weight in each spermatophore, in the yestalk ablated prawns during the successive regenerations. Whereas in he case of spermatophore regenerated by the non-ablated animals, reduction f the spermatophore weight resulted in considerable reduction of the total perm count (128, 77.7, 71, 53.3) in control, 1st, 2nd and 3rd regenerations ig (203).

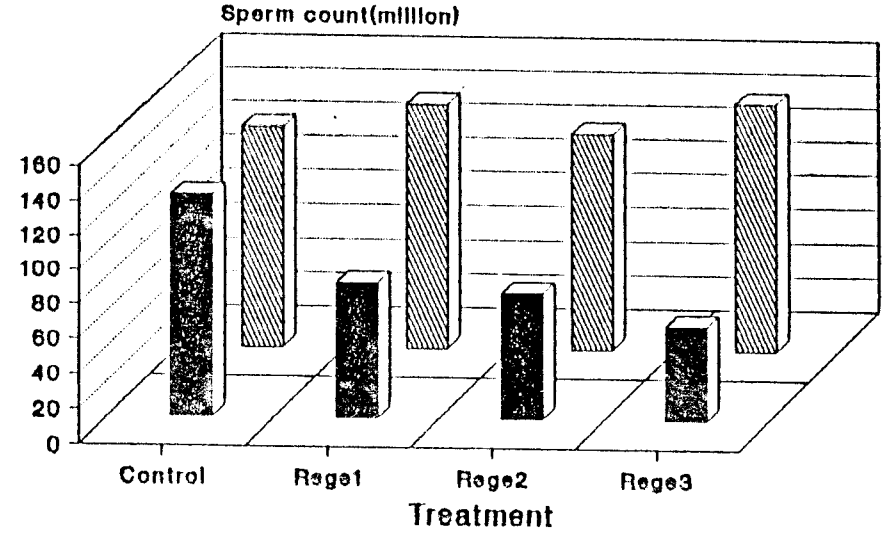
**perm morphology**

Normally the sperm of P. monodon as viewed through the phase-contrast icroscope has a round posterior main body and a central cap region connected o a long anterior spike. Abnormally appearing sperm have a round body with issing spike, round body with short or bent spike, malformed body or a mbination of more than one of the above said deformity. Generally it was

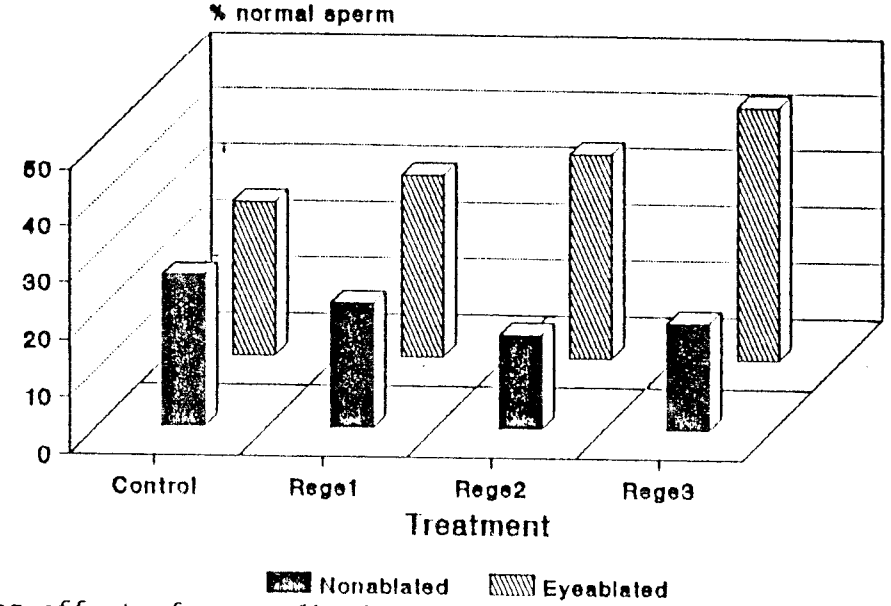
I Spermatophore wt. during regeneration




II Sperm count during regeneration




III % normal sperm during regeneration




Figures showing effect of eyestalk abation on the reproductive quality of male P. monodon (I-spermatophore weight, II-sperm count, III-percentage of normal sperm)

observed that, sperm without spike or deformed spike dominated over other malformations. The percent normal values for the initial baseline samples averaged 27 and 26.4 respectively in eyestalk ablated and non-ablated groups. The values increased from 27 (control) to 44.71 in the eyestalk ablated prawns at the 3rd regeneration (fig. 161). In the successive regenerations, the percentage of normal sperm were 32.06, 36.13 and 44.71 in 1st, 2nd, and 3rd regenerations respectively in eyestalk ablated prawns. Whereas in non-ablated animals the percentage of normal sperm had decreased from 26.4 to 18.7 at the 3rd regeneration. The percentage of normal sperm was 21.73, 16.33 and 18.7 during 1st, 2nd and 3rd regenerations respectively.

**Sperm viability**

The sperm of P. monodon are non-motile and therefore by observing the motility of the sperm it is not possible to assess the viability. Therefore, structural modifications taking place during the fertilization were observed under phasecontrast microscope. Normally sperm of P. monodon are unistellate, non-motile and roughly ovoid in outline (fig. 204,205). It possessed the already described parts like a) posterior main body, b) central cap region and c) anterior spike. The central cap region of the sperm included both the subacrosome and the majority of the acrosomal vesicle. The anterior spike is also a component of the acrosomal vesicle.

The in vitro fertilization of the freshly extracted ovum with the isolated sperm, exhibited dramatic and drastic morphological changes in the sperm cells from the above described structures. These structural transitions occurring in the sperm cells found under phasecontrast microscope revealed the following events. It was noticed, that at the time of contact of the sperm cells with egg, the former got bound with the tip of the anterior spike to the eggs vitelline envelop and in a fraction of second, it underwent

picturisation of the events that occurred upon ionophore contact. The biological agent, "the egg-water" also activated the sperm's acrosome reaction to the same extent as that of the natural process. The egg water at -196°C with a protein content of 50 ug/ml gave almost the same result to that found in the natural fertilization time. Although the different agents were tried for induction of AR of sperm, the "egg-water" and divalent cation A 23187 were found to be the best solution, and therefore they were selected for the assessment of viability, before and after the preservation of spermatozoa in short and long term trials, in the present study.

**B. Cryopreservation studies**

Cryopreservation studies were made after the screening of cryoprotectants at 3 different temperatures, for 3 short intervals. The temperatures selected were 0°C, -30°C, -195°C and the intervals were 1 day, 5 days and 10 days. A total of 11 solutions (6 individual and 5 combinations of the individual cryoprotectants) were used for the screening experiments. Six solutions (glycerol, trehalose, sucrose, proline, valine, ribose and DMSO), which were known to maintain bilayer integrity and protein function during freeze/thaw, were used in an effort to cryopreserve the shrimp sperm. Using the in vitro acrosome reaction for the viability assay, viabiliy was determined in each experimental trials. From the cryoprotectants tested, 5 (3 individual and 2 combinations) were selected for further cryopreservation studies.

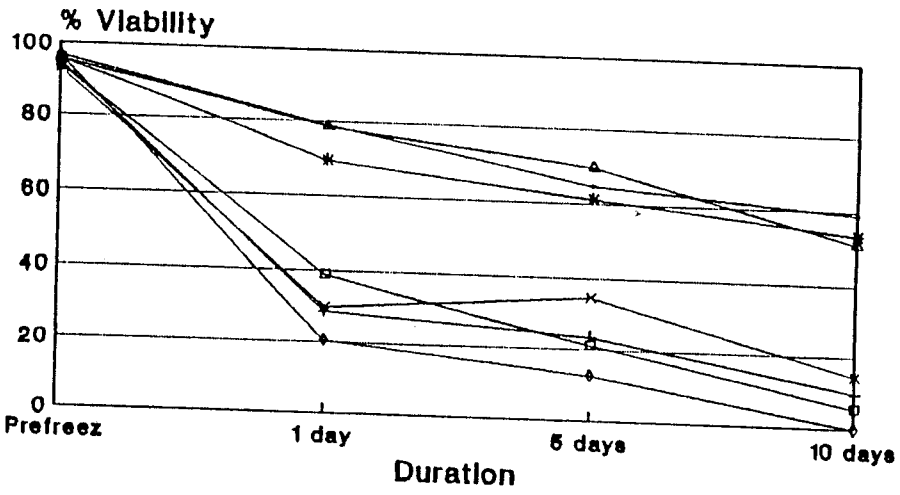
**Screening of cryoprotectants**

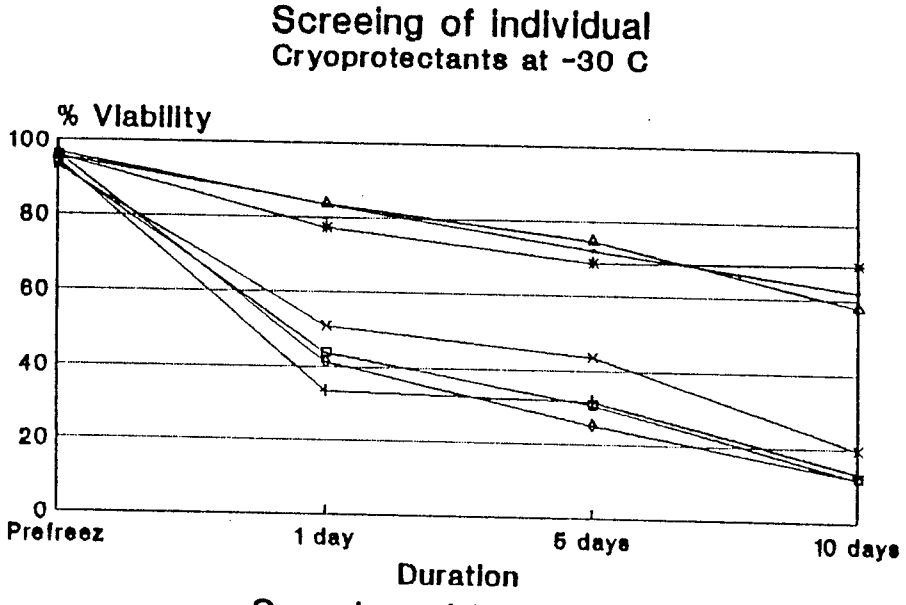
Results of the screening experiments conducted for 3 different periods viz. 1 day, 5 days and 10 days, at 3 different temperatures were presented in the (fig. 210). Among the individual cryoprotectants used for the

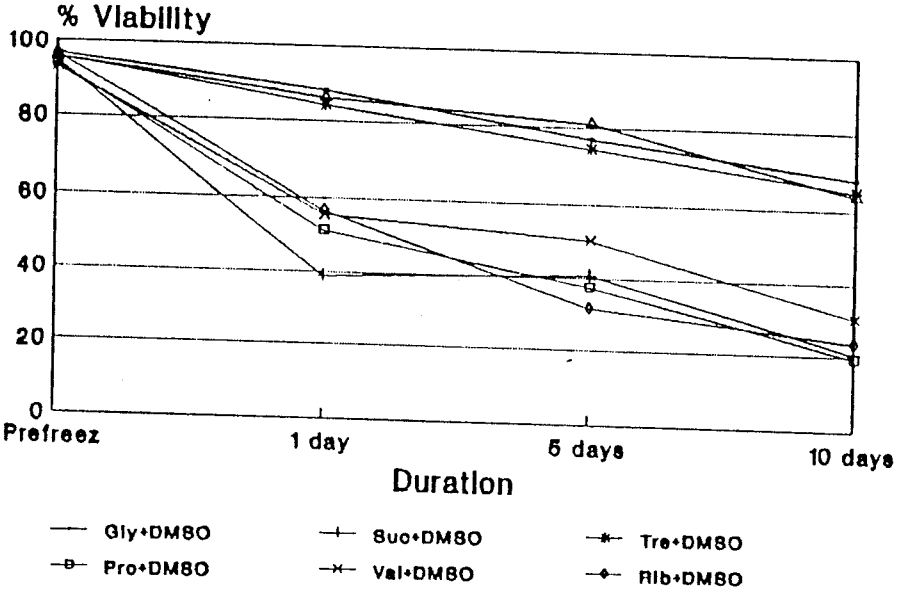
activation. The first manifestation of sperm activation was the loss of the anterior spike by which the sperm were bound with the egg (fig. 206,207). It was observed that, the loss of spike resulted in the externalization or ejection of the acrosomal vesicle (fig. 208,209). Sperm that had undergone exocytosis suddenly penetrated the egg membrane and further changes after penetration was not be visible through the light microscopy. However, sperm which did not penetrate the egg's vitelline membrane, also underwent the same activational changes as that of the penetrated ones. Sperm which has undergone this acrosomal reaction only were found to fertilize the eggs in vitro. From these studies it was observed that, the acrosome reaction is a prerequisite for fertilization.

Therefore, in order to determine the viability and fertilizing capacity of sperm, they were subjected to the in vitro acrosome reaction and were assessed for the percentage viability by observing the structural changes that occur during the acrosome reaction. Various inductors used for the artificial induction of acrosome reactions include a divalent cation viz. Bromo calcium ionophore A 23187, two monovalent ions ie. Valinomycin and Nigricin and the natural biological solution, the egg-water. Among them, the divalent cation bromo calcium ionophore A 23187 and the egg-water gave the best results, though valinomycin and nigricin also had induced the acrosomal filament formation. Even though the loss of spike and exocytosis was observed within short fractions of interval by these two monovalent ions, they took longer time for the formation of the acrosomal filaments. The number of sperm that underwent acrosome reaction by these monovalent agents was found to be lesser.

It was observed that the ionophore treatment affected only the acrosomal region of the sperm and caused dramatic structural changes that mimicked the natural acrosome reaction. Phasecontrast microscope enabled a clear



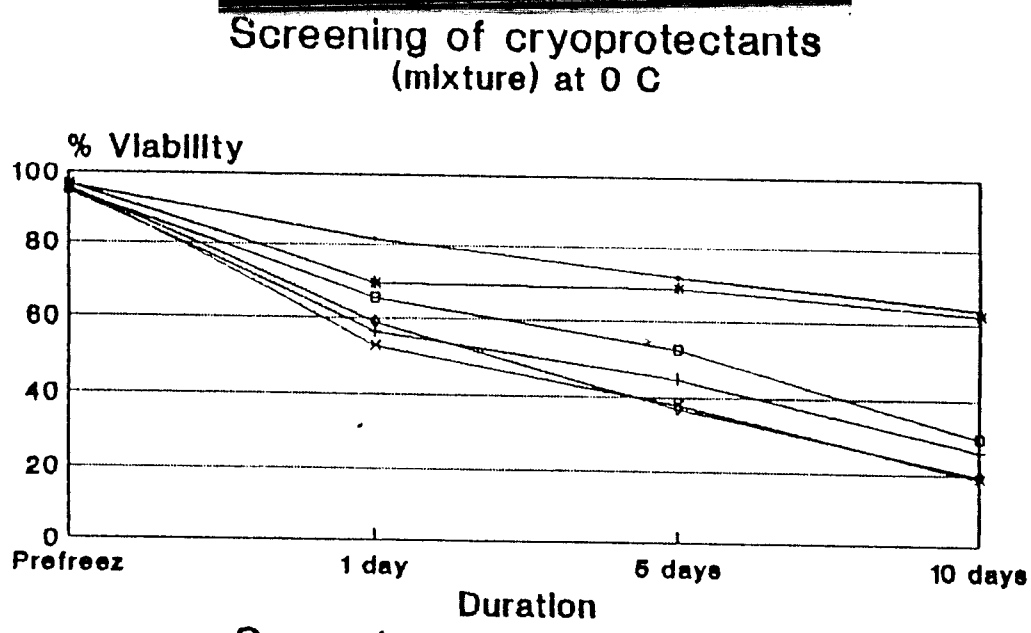

Figures showing the results of the screeing experiments for the cryoprotectants at different temperatures (I - 0 °C, II - -30 °C, III- -195 °C)

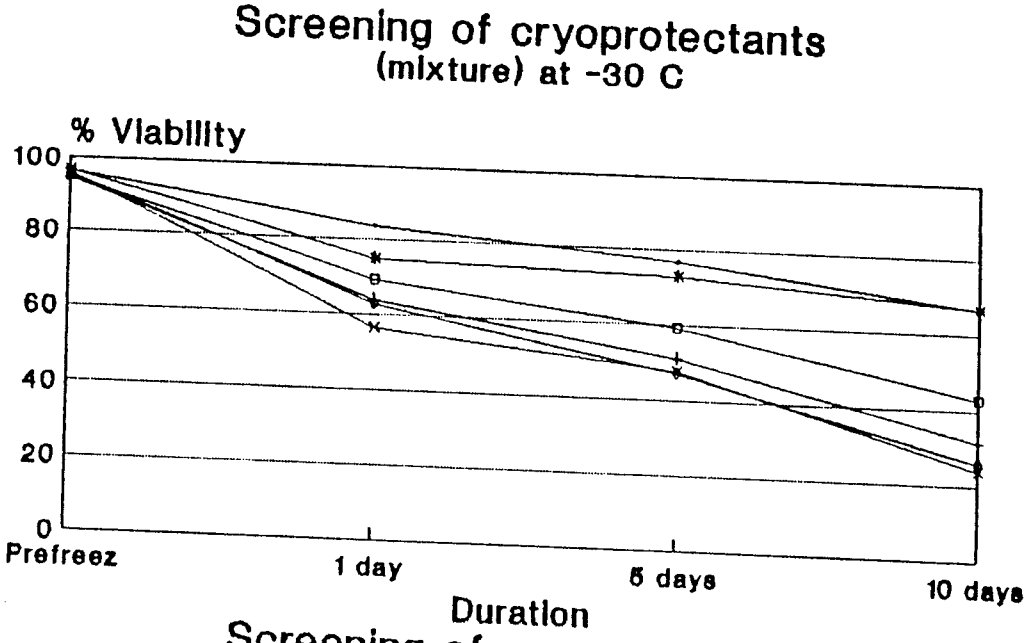
screening studies, 3 of them gave more than 50% of viability after 10 days of preservation. Glycerol gave the best result (68.16% at -196 °C), followed by trehalose (64.93% at -196 °C) and DMSO (63.70% at -196 °C). Among the 3 different temperatures tried -196 °C (liquid nitrogen) gave the highest percentage of viable sperm during the screening experiments. Viability of the spermatozoa after 10 days in 0.5 M glycerol was found to be about 59%, 62% and 68% respectively at 0°C, -30°C and -196 °C with reference to the 97% of viability in unfrozen samples. The percentages of viable sperm were about 53%, 59% and 66% respectively at 0°C, -30°C and -196 °C in DMSO, after 10 days of preservation. Whereas, the remaining 4 solutions viz. proline, valine, sucrose and ribose did not preserve the sperm, as evidenced from the drastic decline in the percentage of viability sperm after the 1 st day itself. In all these solutions percentage of viable sperm was found less than 20% after the 10 days of the experiments. Reduction in the sperm viability was evident in all the three temperatures selected. Even though the percentage viability was found less, the shape and size of the sperm cells were found similar to those of the viable sperm even in these cryoprotectants. Individual sperm with its spike was observed as such. Sperm cells preserved in sea water at -195°C underwent acrosome reaction after one day. However, the morphology differed. The size of the body was reduced and deformed. The control samples kept along with the experimental samples but without any cryoprotectant did not show acrosomal filament formation in the sperm cells. The sperm cells were not found intact even after 1 day in 0°C or -30°C. Instead of intact individual sperm cells, clusters of small sperm cells were observed in the control sample. Clustering of the sperm cells was the most dominating character noticed in the control samples. From these observations it was found that the three solutions viz.

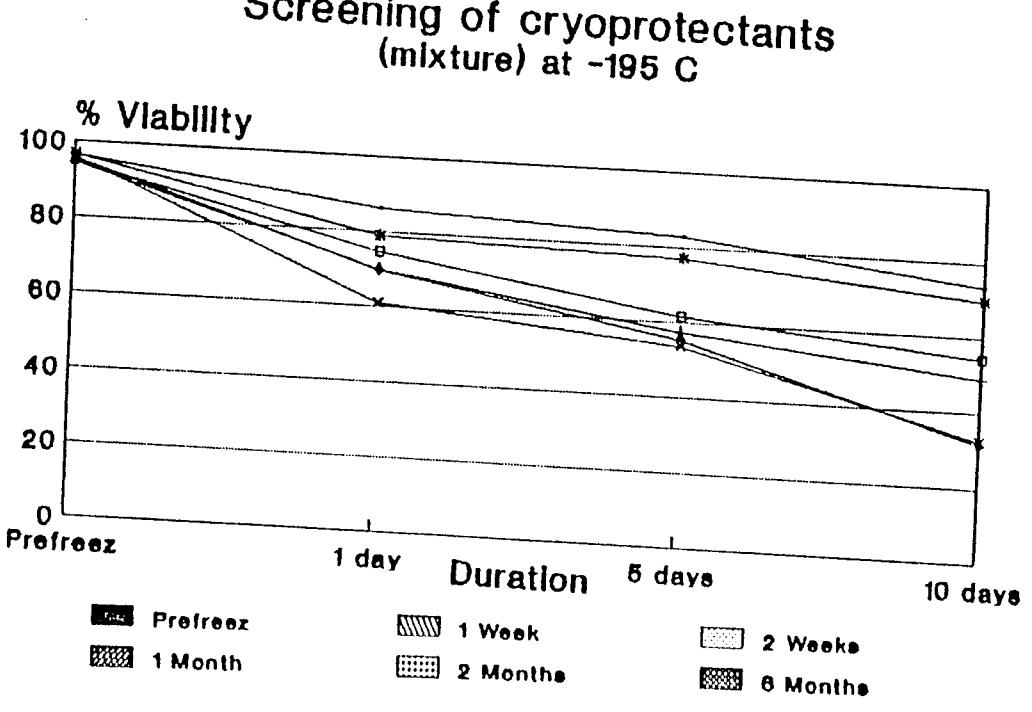
glycerol, trehalose and DMSO were better and preserved more viable sperm after 10 days of experiment and thus they were selected for the cryopreservation studies.

Six combinations of DMSO with the above said solutions were also used for the screening experiments. The experiments were conducted for the same 3 intervals of 1 day, 5 days and 10 days duration, and at the same temperatures viz. 0°C, -30°C and -196°C. The results were presented in the (fig. 211). Even though DMSO alone gave good results after 10 days, most of the tested combinations of DMSO did not give good results. Among the six combinations tried 3 of them, DMSO (5%) + glycerol (0.5 M), (74% at -196°C) followed by DMSO (5%) + trehalose (0.25 M) , (70% at -196°C) and DMSO (5%)+ proline (0.25 M), (54% at -196 °C) gave more than 50% of viability, after 10 days of the experimental trials. Among them, two gave nearly 70% of sperm survival after 10 days of preservation at -196 °C. Percentage viability of spermatozoa after the 10 days preservation in DMSO (5%) + glycerol (0.5 M) was 63.78%, 66.56% and 73.61% (respectively at 0°C, -30°C and -196 °C) and DMSO (5%) + trehalose (0.25 M) was 62.28%, 66.81% and 69.81% (respectively in 0°C, -30°C and -196 °C). Even though the (0.25 M) trehalose + (5%) DMSO had given 54% viable sperm at -196 °C, the number of viable sperm cells were very less at -30°C (42.82) and 0°C (29.79°C). The decline was sharp after 5 days and it was not selected for the long term preservation studies. The other 3 combinations of DMSO viz. valine + DMSO, sucrose + DMSO and ribose + DMSO gave a viability of only less than 50% after the 10 days preservation period and therefore discarded from the long term studies.

On the basis of these two sets of screening experiments, 5 solutions which had given the highest percentage of viable sperm in all the three temperatures tried, were selected finally for the short term and long term




Figures showing the results of the screeing experiments for the cryoprotectants at different temperatures (I - 0 °C, II- -30 °C, III- -195 °C)

cryopreservation studies. The solutions selected were glycerol (0.5 M), trehalose (0.25 M), DMSO (5%), DMSO (5%) + glycerol(0.5 M) (equal proportions) and trehalose (0.25 M) + DMSO (5%) (equal proportions). Experiments were carried out at the same three temperatures of 0°C, -30°C and -196 °C.

**Short-term cryopreservation studies**

Results of the cryopreservation experiments conducted at 0 °C for 3 different periods of one week, two weeks and one month are depicted in the table (10). It was observed that at 0 °C DMSO + Glycerol gave the highest percentage of viable sperm (65.93, 61.36 and 42.68%) after the three durations of one week, two weeks and one month respectively, followed by DMSO + trehalose which gave 63.81, 54.44 and 40.96% respectively. In this experiment, DMSO offered the least protection as 52.2, 47.92 and 29.90 respectively after one week, two week and one months duration of study. Variations in the percentage of viable sperm with reference to the unfrozen samples were clear from the fig (212). It was also evident from the figure that among the three temperatures used, -195°C was the best temperature for long term preservation of sperm. Percentage of viability was above 50% till the end of 2 weeks period in 0°C. The percentage declined drastically and it became negligible after one month. Even though sperm exhibited acrosome reaction, its shape and size was found varying. Clumping of spermatozoans were also observed in many cases even at the end of two week period at 0°C. But no structural changes or clumping of sperm were observed at the end of one week preservation at 0°C.

Percentage viability of the sperm cells preserved at -30 °C for the three periods of 1 week, 2 weeks, and 1 month are shown in the (table) and it is evident from the table that, here also DMSO + glycerol offered the best protection to the cryopreserved sperm cells, followed by DMSO +

trehalose. The percentage viability was 69.00, 65.32 and 53.63 and 66.71, 58.55 and 50.25 respectively for the 3 durations of one week, two weeks and one month. Here also DMSO offered the least protection but the percentage of viable cells were higher than that at 0 °C. It offered the percentage viability of 57.31, 52.25 and 32.87 respectively for the three ascending durations.

The highest percentage viability was observed in the samples preserved at -195 °C, the percentage of viable sperm cells are shown in the table (10). From the values it is evident that the highest percentage of viability was in samples preserved in DMSO + glycerol (74.27, 72.02 and 62.66%) respectively in three different periods, immediately followed by DMSO + trehalose (72.01, 62.46 and 58.45%) respectively. Here trehalose offered the least percentage of viable sperm (64.28, 52.20 and 42.10) in all the three durations. Still it was noted that, these percentages are higher compared to the percentage viability found in other temperatures of 0 °C and -30 °C for the same periods.

**Long-term cryopreservation studies**

Long term cryopreservation studies conducted at 0 °C for two periods of two months and 6 months were presented in the table (11) and from that it was found that temperature of 0 °C is not a good temperature for the long preservation of sperm. The percentage viability was still above 50% at the two intervals studied ie. 2 months and 6 months. The percentage of viable sperm in various cryoprotectants after the two periods of 2 and 6 months are shown in the table (11). The highest percentage of survival was obtained from samples preserved in DMSO + glycerol, followed by glycerol for the two periods of study. DMSO + glycerol gave a percentage viability of 56.74 and and 53. 95 respectively, after 2 and 6 months of preservation at -195 °C.

| S. No | Cryoprotectants | Conc. | PF | % viability of sperms through acrosome reaction | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Duration | one week | | | 2 weeks | | | 1 month | |
| | | | | 0°C | -30°C | -195°C | 0°C | -30°C | -195°C | 0°C | -30°C | -195°C |
| 1 | Glycerol | 0.5m | 94.7 | 60.44 | 63.92 | 70.50 | 48.82 | 53.23 | 68.81 | 41.36 | 49.92 | 55.42 |
| 2 | Trehalose | .25m | 94.38 | 54.77 | 57.29 | 64.28 | 45.63 | 48.9 | 52.2 | 36.34 | 38.64 | 42.1 |
| 3 | DMSO | 5% | 93.27 | 52.2 | 57.3 | 64.85 | 47.92 | 52.25 | 58.17 | 29.9 | 32.87 | 52.71 |
| 4 | DMSO + Glycerol | 5%+0.5m | 95.76 | 65.93 | 69 | 74.27 | 61.36 | 65.32 | 72.02 | 42.68 | 53.63 | 62.66 |
| 5 | DMSO + Trehalose | 5%+.25m | 96.62 | 63.81 | 66.71 | 72.01 | 54.44 | 58.55 | 62.46 | 40.46 | 50.25 | 58.45 |

Table 11.

## LONG TERM CRYOPRESERVATION EXPERIMENTS

| S. No | Cryoprotectants | Conc. | PF | % viability of sperms through acrosome reaction | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Duration | Two months | | | 6 months | |
| | | | | 0°C | -30°C | -195°C | 0°C | -30°C | -195°C |
| 1 | Glycerol | 0.5m | 94.7 | 18.31 | 30.50 | 51.46 | 6.89 | 14.72 | 60.74 |
| 2 | Trehalose | 0.25m | 94.38 | 9.43 | 21.18 | 34.65 | - | 19.18 | 22.99 |
| 3 | DMSO | 5% | 93.27 | 22.08 | 27.1 | 46.67 | 6.75 | 26.09 | 43.02 |
| 4 | DMSO + Glycerol | 5%+0.5m | 95.76 | 30.43 | 38.68 | 56.74 | 18.59 | 31.20 | 53.95 |
| 5 | DMSO + Trehalose | 5%+0.25m | 96.62 | 26.14 | 32.08 | 48.18 | 8.98 | 19.78 | 40.90 |

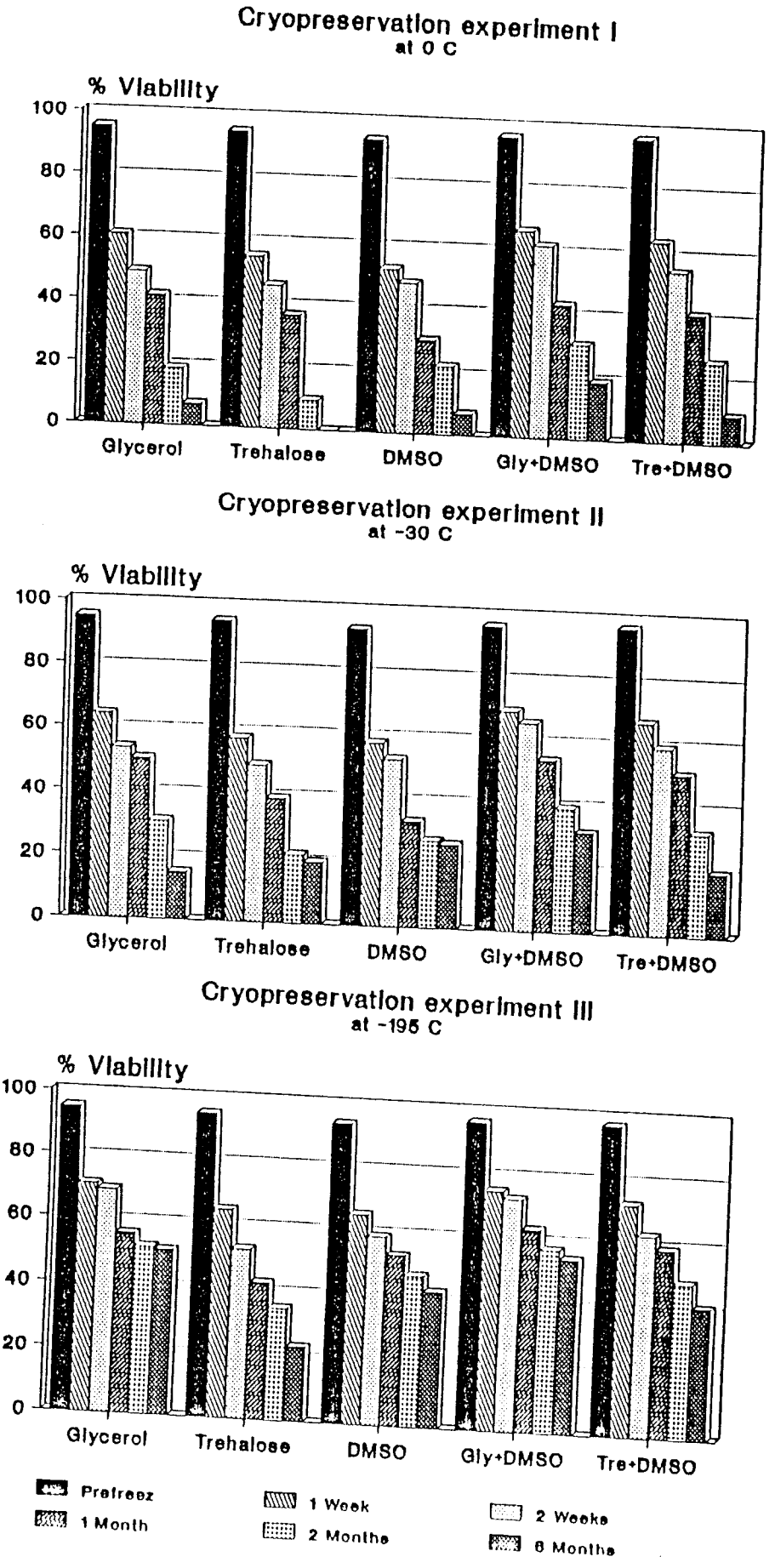
The percentage viability in glycerol was 51.46 and 50.74 after 2 and 6 months respectively. The lowest viability was observed in samples preserved in trehalose, which was 34.65 and 22. 99% after the two periods of 2 months and 6 months respectively.

Whereas -30 °C offered more protection to the frozen sperm cells in all the cryoprotectants till the end of 1 month. However, drastic decline in viability was observed in the two months preserved samples. Maximum number of viable sperm (56.74%) were found in the sample preserved in glycerol + DMSO at the end of 2 months. The lowest percentage observed was 34.65% in samples frozen in trehalose (0.25 M). The percentage of viability was negligible in 6 months preserved samples at -30°C. Here the size of the sperm was found much reduced and most of them were abnormal in appearance. Clumping of sperm were found in all the 6 months preserved samples.

The samples preserved at -195°C did not show much reduction in the percentage viablity till the end of the experimental tenure of 6 months. The maximum viability observed was 53.95% in samples preserved in glycerol + DMSO (0.5 M + 5%), followed by 0.5 M glycerol (50.74%) and 5% DMSO (43.02%). Trehalose showed the lowest percentage of viable sperm (22.99%) after the 6 months period. However, the combination of trehalose + DMSO showed a better percentage of viability of 40.90% even after 6 months preservation period.

From these investigations it was found that, among the compounds used trehalose offered the least protection for all the temperatures selected for short and long periods. However, it was noted that when the samples were frozen in mixture of trehalose + DMSO, the percentage viability found increased.

Among the solutions tried, glycerol + DMSO at equal volumes offered the best protection for short and long term preservation of P. monodon sperm, followed by glycerol. It was noticed that no clumping of sperm cells or




Figures showing the results of the cryopreservation experiments at different temperatures during different duration (I - 0 °C, II- -30 °C, III- -195 °C)

deformation in structure were observed in any of the samples preserved in liquid nitrogen (-195°C). The size and morphological features of the cells were intact in all the samples preserved in -195°C, except for the trehalose. Sperm cells preserved in the trehalose exhibited a little reduction in their cell size after 6 months of preservation. Abnormality of the cells was also noticed in some cells. Reduction in the cell size was not observed till the end of one month in the trehalose. Reduction observed after two months was also not easily recognizable through light microscopy. But it was easily recognizable after six months preservation period. All the other compounds used, offered better protection to the stored sperm cells till the end of the experimental tenure of six months.

## DISCUSSION

In nature during mating and insemination of aquatic organisms there is no control over the progeny. On the contrary, by adopting artificial insemination technique, selective breeding can be achieved. One of the basic requirements for this, is the ready availability of gametes for the manipulations. Artificial insemination and cryopreservation of the viable gametes are very much important in controlled breeding of the quality parents. In decapod crustaceans, all these above said biotechnological processes requires spermatophores, hence acquisition of the spermatophores is very much important. Different spermatophore extraction methods have been studied by many investigators in various decapod crustaceans which include dissection method, squeezing method, extraction method, cannulation method and electro ejaculation techniques (Sandifer and Lynn, 1980 and Lin and Ting, 1984). Three different techniques and their effects, both on spermatophores as well as its donors, were studied during the present investigation. From the observations it was found that various methods have its own merits and demerits and each of which was described in the results. Dissection and extraction was the earliest technique used for the removal of spermatophores (King, 1964 and Lin and Ting, 1986). The main disadvantage of this method was that the animal has to be sacrificed for the spermatophore extrusion. During the present study, a modified micro surgery method, was tried by using a gill irrigator and micro scissor. It was found that though it does not kill the animal the surgery wounded the animal. It was observed that for the regeneration of spermatophores in these animals, long period is required, as the healing of the wound has to take place first. Even though the spermatophores were obtained from both left and right terminal ampoules of all the animals, all of them may not be fully developed. The second method often employed for the spermatophore extraction was the squeezing method;

which does not require the sacrificing of the animal; however it require skill for the complete extraction of the whole spermatophore without damages (Lin and Ting, 1986; Alfaro, 1993 and Heitzmann et al., 1993). Whereas the electro-ejaculation (Sandifer and Lynn, 1980) of spermatophore has been reported as the most effective and simple method of spermatophore retrieval in penaeid prawns. Later electro-ejaculation technique has been refined by Talbot et al., (1983) in Homarus americanus and Sandifer et al., (1984) in Penaeus sp.. The gill irrigator of Tave and Brown (1981) ensured the continuous supply of water to branchial cavity during extrusion, so that the stress of animal due to electric stimulation could be reduced.

Similar method of spermatophore extrusion has been employed by many investigators after Sandifer and Lynn, (1980); for the acquisition of spermatophores for artificial insemination, evaluation of reproductive quality of males and also for sperm preservation experiments in various decapod crustaceans. Various attempts on artificial insemination by different researchers like Sandifer and Lynn, (1980) in Macrobrachium rosenbergii; Lumare, (1982) in P. japonicus; Bray et al., (1982) in P. setiferus; Kooda-cisco and Talbot, (1983) in Homarus americanus; Aiken et al., (1984) in P. monodon; Talbot et al., (1986) in H. orientalis; Joshi and Diwan, (1992) in M. idella showed that the discovery of electro-ejaculation technique to extrude spermatophores in males has simplified the artificial insemination to a greater extent.

Spermatophores were extruded for assessing the reproductive quality of males in various decapods like lobsters (Talbot et al., 1983); Penaeids like Penaeus vannamei, (Leung-Trujillo and Lawrence, 1985, Alfaro and Lozano, 1993, Heitzmann, et al., 1993) P. setiferus (Bray et al., 1985); P. setiferus, P. vannamei and P. stylirostris (Leung-Trujillo and Lawrence, 1991); P. monodon (Pratoomchat et al., 1993); P. monodon (Gomes and Primavera, 1993); P. stylirostris (Alfaro, 1993); and P. setiferus and

P. vannamei (Rosas et al., 1993) and in other prawns like Macrobrachium rosenbergii (Harris and Sandifer, 1986) by electro-ejaculation techniques.

There has been little published work on the time required for spermatophore development in various decapods. Lin and Ting (1984) reported requirement of 7 to 11 days for spermatophore generation in P. monodon after applying various artificial spermatophore extraction methods. However, the authors did not comment whether the extraction techniques used, affected the spermatophore development time as well as its quality. In the present study, nearly 60% of the electro-stimulated individuals displayed spermatophore regeneration in an average of 10 days, however it took 11 to 12 days in the rest of the 40% animals. Leung-Trujillo and Lawrence (1991) recorded a period for full regeneration of spermatophore from 5 to 7 days in P. setiferus. Whereas Rosas et al., (1993) reported that 20% of the animals displayed spermatophore regeneration in 3 days after electro -ejaculation, even though 80% of the animals did the same only in 7 days. They further reported that the slight discrepancy between the spermatophore regeneration time recorded by them and those given by Leung-Trujillo and Lawrence (1991) for P. setiferus might be due to the population differences.

The evaluation of reproductive quality in male penaeid shrimp was first reported by Leung-Trujillo and Lawrence, (1985) who determined spermatophore weight, sperm count, and percentages of live and abnormal sperm for evaluating the effect of eyestalk ablation in P. vannamei. Using the same parameters, Leung-Trujillo and Lawrence, (1987a) reported a decline in sperm quality in P. setiferus held in captivity. Bray et al., (1985) used the same approach to evaluate the effect of water temparature, EDTA and vibrio bacterin on sperm quality in captive P. setiferus. The same method was also followed by Alfaro, (1993) for the reproductive quality evaluation of male P. stylirostris.

Rapid deterioration of the spermatophore weight and sperm quality was observed in the regenerated spermatophores of P. monodon after electro ejaculations during the 50 days of the experiment, in the present study. Similar trends were reported by Leung-Trujillo and Lawrence, (1987) and Talbot et al., (1988 & 1989) in P. setiferus during 35 days of captivity. This deterioration was attributed to degeneration of the reproductive tract in males, associated with bacterial infection (Talbot et al, 1988). Melanization has been reported as a problem for P. stylirostris (Alfaro, 1993) whereas Chamberlain et al., (1983) found a higher incidence of melanization in manually ejaculated shrimp. Harris and sandifer, (1986) reported that although the electro-ejaculation procedure permitted repeated spermatophore expulsion, the frequent application of electrical stimulation induced, melanization of the reproductive tissues in M. rosenbergii. Alfaro, (1993) reported an increase in sperm count and reduction in abnormalities in regenerated spermatophores in P. stylirostris. However, further he mentioned that melanization has not been observed in any of the experimental animals. In the present study melanization was found only in males where repeated electric-stimulation has been given for the ejaculation.

Eyestalk ablation has been used to induce precocious spermatophore production in sexually immature male penaeids (Alikunhi et al., 1975). Eyestalk ablation has also induced increase in gonad size in P. setiferus (Lawrence et al., 1979) and mating frequency in P. vannamei (Chamberlain and Lawrence, 1981b). However, in the present study it was found that eyestalk ablation considerably reduced the spermatophore regeneration time from 9 to 12 days (non-ablated) to 6 to 8 days in ablated males. Leung-Trujillo and Lawrence, (1991) reported that spermatophore production seeme to be controlled by the X-organ sinus gland complex, as ablated P. setiferus produced new spermatophore almost twice as fast as unablated males.

The results of the present study indicates that unilateral eyestalk ablation improved sperm quantity and sperm quality (larger spermatophore, increased sperm count, less abnormal sperm) in P. monodon. These findings are similar to the the findings of Leung-Trujillo and Lawrence, (1985) who observed that ablation increased sperm count without affecting sperm quality in P. setiferus. Similarly, Gomes and Primavera (1993) reported that unilateral eyestalk ablation improved the sperm quality in terms of less abnormal sperm, bigger head and longer spike in P. monodon. In contrast to this, Pratoomchat et al., (1993) reported that eyestalk ablation did not increase the spermatophore size or sperm quality, although re-ejaculation significantly increased mortality of ablated males in P. monodon.

The percentage of viable sperm during the present study was determined through the acrosome reaction described by Griffin et al., (1988). Functionally P. monodon sperm can be divided into three regions 1. Nucleus 2. The acrosomal vesicle and 3. The sub-acrosome. Similar observations were made in other penaeids like S. injentis by Griffin et al. (1988) and in P. indicus by Mohammed and Diwan (1994). Kleve et al. (1980) reported that the acrosomal vesicle is a complex membrane bound entity, that encircles the central cap region including the anterior spike and the sub-acrosomal components are found within the core of the central cap region. The acrosome reaction in P. monodon is composed of both the exocytosis of the acrosomal vesicle and the generation of the acrosomal filament. Induction of the two phases of the AR in P. monodon is temporally separated and sequential in in-vivo and in-vitro. In-vivo upon binding to the ova, sperm undergo acrosomal exocytosis and 20 to 30 minutes later only, the formation of the acrosomal filament takes place. Similar observations were described by Clark et al. (1984) in S. ingentis. Griffin et al. (1988) reported that, the two phases of AR in sperm of S. injentis, removed from female seminal receptacle were also temporally separated and sequential.

In the present study viability assays of the sperm were made through the acrosome reaction as described by Clark et al., (1981). Three different chemical compounds viz. Bromocalcium ionophore A 23187, Valinomycin and Nigricin were found inducing the in-vitro acrosome reaction in the sperm of P. monodon, in addition to the biological solution viz. egg water collected according to the method and discribed by Griffin et al., (1987). Among the different above mentioned inducers "Egg water" gave the best result followed by ionophore A 23187 as in in-vivo conditions during natural spawning. The structural details were found similar to those observed in the sperm of P. monodon during the AR, closely resembling with that of the previous observations made in the natantians by different investigators like Clark et al. (1984) and Anchordougy et al. 1988 in S. ingentis. The ultrastructure of the acrosome reaction of natantian sperm during the sperm-egg interactions was described by Yudin et al., (1979) sperm of P. monodon. Clark et al. (1981 & 1984) reported that, the acrosome reaction of the natantian's sperm can be induced by the ionophore A 23187 and egg-water respectively to the same extent as in vivo conditions. Clark et al., (1981) summarised the results of various known acrosome inducers S. injentis sperm and found that most of the arificial medium used by earlier researchers as acrosome reaction inducers were ineffective in S. injentis sperm, and mentioned further that the divalent cation A 23187 was the only substance that induced the acrosome reaction among the different solutions tested. Griffin et al., (1987) reported that, egg water can induce the complete acrosome reaction including both acrosomal exocytosis and formation of the acrosomal filament equally to that of the natural condition. In vitro induction of acrosomal filament in the natantian sperm with egg water has been achieved by many investigators (Griffin et al., 1987, 1988; Griffin and Clark, 1990; Anchordouguy et al., 1988 and Clark and Griffin 1993 and in P. monodon by Pratoomchat et al., 1993). Therefore, viability studies

in the following experiments were carried out by the acrosome reaction induced by "egg-water" apart from ionophore A 23187. The morphologically identifiable structural changes during the acrosome reaction, made the viability studies easier which formed the basic information for further manipulations of the sperm.

Cryopreservation of gametes is widely practiced in animal husbandry and controlled breeding progammes. However, there has been very few reports on the cryopreservation of the crustacean sperm. Results of the present study showed that many factors are significant in the cryopreservation of penaeoidean sperm. The temperature at which the sperm samples are stored, as well as the cryoprotectants used for the dilution of sperm, have definite and significant roles on the achievement of viability of preserved sperm in P. monodon. The screening experiments carried out for short periods using different cryoprotectants at 3 different temperatures showed wide variation in sperm viability. From the results, it was found that -196 °C is the best temperature for the storage of intact viable sperm for long periods than the other two temperatures of -30°C and 0°C. In P. monodon, the viability of the sperm preserved at -196 °C varied between 73% to 20% in samples preserved in different cryoprotectants. The decrease in viability was less in certain cryoprotectants and higher in others. Leung (1991) reported that the storage temperature should be -130°C or below for long term preservations of live cells because the glass transformation temperature for pure water was found to be -130 °C. Successful preservation of spermatophores of shrimp in glycerol at -196 °C was reported by Chow et al., (1985). Anchordouguy et al., (1988) reported that, sperm samples of S. injentis stored at -196°C for 1 month showed no decrease in viability upon thawing. In Scylla serrata the viability of the sperm in 30 days of storage was fairly high, ie. 95% in samples stored at -195 °C (Jayalectumie and Subramoniam 1989). In the present study, it was found that the sperm samples frozen and stored at -30

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°C and 0 °C gradually lost their ability to undergo the acrosome reaction over a two week period. By the end of one month most of the sperm cells became clumped and formed sticky mass.

In S. injentis, Anchordouguy et al.,. (1988) tested different cooling rates and it was reported that a cooling rate of 1°C/min. was the best of the rates tested. The same cooling rate of 1°C/min. was used for the present investigation as it is found the best rate in many other animals too. Results of the screening study in present investigation indicated that the viability of the preserved sperm cells vary greatly in different cryoprotectants even at -196 °C. The decrease in viability was higher in other two temperatures tried (-30°C and 0°C) for all the cryoprotectants used. Temperature is a critical factor in cryopreservation. This is indicated by the wide variation in the percentage viability ie. from 78% to 20% at the same temperature of -196°C. This difference was more in other temperatures (30°C and 0°C) during the same interval of 10 days.

Among the thirteen cryoprotectants used, only 5 in which the viability of the sperm were about 50% upon thawing were selected for the cryopreservation studies. Out of the 5 cryoprotectans selected after the screening study, glycerol (5%) + DMSO gave the best results followed by glycerol, even after 6 months of preservation. Trehalose offered the least protection of the compounds tested. Glycerol was first reported as effective in protecting sperm from freeze thaw damage, by Rostand (1946) and Polge et al., (1949). Polge et al. (1949) first showed that, the spermatozoa could be frozen and thawed without losing motility if glycerol was introduced in their suspending medium. Glycerol has been used widely in the cryopreservation of viable fish milt (Horton and Ott, 1976). Similarly, glycerol has been used as a cryoprotectant in most of the sperm preservation experiments in invertebrates. Successful preservation of spermatophores of shrimp in glycerol at -196°C has also been reported by chow et al., 1985.

Similar results were obtained by Behlmer and Brown, (1984) for Limulus spermatozoa. When DMSO was used for the spermatophore preservation in Macrobrachium rosenbergii the post thawing survival was nil (Chow et al. 1985). While on the contrary, Anchordouguy et al., (1988) reported that sperm survival was higher in S. injentis when glycerol was used as cryoprotectant for the preservation of sperm even though DMSO gave the highest percentage of survival. In Scylla serrata glycerol and DMSO + trehalose gave highest sperm survival (Jayalectumie and Subramoniam, 1989).

In the present study it was found that, the percentage viability was higher in samples preserved in 5% DMSO even after 6 months. The maximum effect was in 5% DMSO; higher and lower concentrations of this yielded inconsistent results. Similarly higher percentage viability was obtained when the samples were preserved for 6 months in DMSO (5%) + trehalose (0.25M). Anchordouguy et al., (1988) reported that the highest percentage viability was obtained from samples preserved in 5% DMSO. However, it was reported in the same animal that, combining 5% DMSO with other cryoprotectants like trehalose, proline, sucrose and glycerol in the freezing medium did not alter the sperm viability in the marine shrimp S. injentis (Anchordogoury et al 1980). Whereas in S. serrata DMSO + trehalose gave high sperm survival but trehalose was not an efficient cryoprotectant when it is used alone (Jayalectumie and Subramoiam 1989). Similarly trehalose offered the least protection in the preservation of S. injentis sperm (Anchordoguy et al. 1994). During the present investigation also the lowest sperm survival was found in samples preserved in trehalose.

Results of the present investigation indicate that the spermatozoa of P. monodon can be cryopreserved upto 6 months without causing significant decrease in their viability in any of the three solutions viz. Glycerol, DMSO + Glycerol, and DMSO + Trehalose at -195°C. The temperature of liquid nitrogen is ideal for the long term preservation of sperm of P. monodon.

# SUMMARY & CONCLUSION

## SUMMARY AND CONCLUSION

Endocrine control of reproduction in the penaeid shrimp P. monodon has been investigated in detail by adopting a comprehensive approach to the problem. The major aspects of the study included in depth investigations of the cytological details of the reproductive and neuroendocrine organs in correlation with the process of gonadal maturation. Based on the conclusions drawn from such ultrastructural studies various endocrine manipulations were carried out to see their effects on gonadal maturation. Besides that studies on the reproductive quality of male P. monodon and the cryopreservation of spermatophores form a part of the present investigation.

The shrimp P. monodon used in the present study were collected from the offshore waters of Cochin, Madras and Mandapam and from the culture ponds of Vypeen Island near Cochin (Kerala). The entire investigation on the cytological aspects were carried out using standard histological and electron-microscopic methods. Endocrine manipulations and cryopreservation studies were also carried out using the standard methods. The salient features of the present investigation are given below.

The female reproductive system of P. monodon consists of the internal organs, the paired, partly fused ovaries extending the entire length of the body and the paired oviduct open out through the genital pore situated at the base of the third periopod and the external organ the single thelycum. Histological investigations revealed that the reproductive cycle of P. monodon includes a series of events starting from the activation of the primordial germ cells to the differentiation of highly yolk equipped ova. Based on the chain of cytoplasmic and nuclear transformations in the oocytes, the highly complicated process of oogenesis was classified into five different stages (stage I - V) considering the immature stage as the 0-stage viz. Immature (stage 0), pre-vitellogenic (stage I), early-vitellogenic (stageII),

late-vitellogenic (stage III), mature (stage IV), and spent (stage V). An active zone of proliferation with clusters of developing oogonial cells are the characteristic features of the immature ovary. Ultrastructurally the oogonial cells possessed a large nucleus and an electron-lucent cytoplasm with very few cell organelles. Pre-vitellogenic ovary contained basophilic oocytes at two stages of development, 1. **oocytes at the chromatin nucleolus stage** and 2. **oocytes at the peri-nucleolus stage.** Ultrastructurally the cytoplasm of these oocytes contained considerable numbers of sub-cellular organelles like robosomes, mitochondria, Golgi complex and rough and smooth reticular elements. Two sub-stages **(Cisternal and Platelet phase)** were identified in the early vitellogenic oocytes through light and electron-microscopy. Ultrastructurally the cytoplasm of the **cisternal phase oocytes** contained numerous active cell organelles and developing electron-lucent to dense vesicles. However, the cytoplasm of the **platelet phase oocytes** contained an abundant supply of yolk materials and vesicular bodies apart from the active cell organelles. Late-vitellogenic oocytes were characterized by the appearance of radially arranged club shaped structures, the cortical rods in their entire ooplasm. Ultrastructurally the matrix of these cortical rods were found packed with some "feathery" structures. The cytoplasm between these cortical rods, possessed yolk spheres, granules and pinocytotic vesicles. Active cell organelles were not apparent. However, follicle cells around these oocytes contained highly active cell organelles. In the mature oocytes, the entire cytoplasm was found filled with "feathery" matrix. The follicle cells of the mature oocytes were found detached from the oolemma at its later stages just before spawning. Regarding the site of vitellogenesis in P. monodon it was found during the present study that both intra-oocytic and extra-oocytic sources are present. Intra-oocytic synthesis of yolk was prominent during the early stages of

development, however, intake of extra-oocytic yolk was apparent in the oocytes at the later stages of development.

The male reproductive system of P. monodon is comprised of the internal organs, the paired testes and paired vas deferentia terminating in ampoules containing spermatophores and opens out through the gonopores at the coxae of the fifth pereiopod and the external organs the petasma and appendix musculina. The process of spermatogenesis gets initiated with the formation of gonial cells from the germinal zone. This gonial cells after passing through several transitions like spermatocytes and spermatids get transformed into spermatozoa. Ultrastructurally, it was found that the most complicated process during spermatogenesis is the acrosome formation. Ultrastructurally it was found that is the sperm of P. monodon is composed of a spherical main body partially encompassed by a morphologically diverse cap region containing acrosomal complex from which extended a single spike.

Light microscopic investigations of the vas deferens of P. monodon revealed that each part of this duct is highly modified and have specific roles in the production of the highly evolved spermtophores. Ultrastructural investigations revealed that the secretory epithelial cells lining the inner lumen secretes amorphous materials of different nature by exocytosis as well as apocrine mode of secretion and this materials constitute the different spermatophoric layers.

Light microscopical investigations of different neuroendocrine centers of P. monodon revealed different types of neurosecretory cells (NSCs). Based on the size, general shape of the cell body and presence or absence of vacuoles in the cytoplasm four different cell types were tentatively identified in these ganglionic centers. Light microscopical and ultrastructural studies of these NSCs revealed 3 distinct phases in their secretory cycle viz. Quiescent or resting phase (Q- phase), Vacuolar phase (V- phase), and Secretory phase (S- phase). Drastic ultrastructural changes

were noticed in these NSCs at different phases of their secretory cycle. Histological investigations of different ganglionic centers showed that the NSCs are found in distinct cell groups.

Investigations in the eyestalk neurosecretory system of P. monodon showed three NSC groups viz. MEGXO, MTGXO I, and MTGXO II with 3 types of NSCs viz. A, B and C cells. The largest type of GN cells found in other ganlionic centers were totally absent in the eyestalk. Ultrastructurally 5 different types of NS granules were observed in the sinus gland of P. monodon. Light and electron-microscopical studies of the various NSCs located in different ganglionic centers during the process of gonadal maturation showed concomitant changes in the secretory activity of these cells during different stages of gonadal maturation. In the immature animals the NSCs especially A & B cells of eyestalk were found highly active indicating the secretion of gonad inhibiting factors. Where as during early stages of gonadal maturation GN and A type NSCs of sub-oesophageal and thoracic ganglia were found highly active during the subsequent stages of oogenesis, indicating its secretion and synthesis of gonad stimulating factors. GN & A cells of brain are found to secrete some factors which in tern activate the thoracic and sub-oesophageal ganglia to secrete the gonad stimulating factors.

In order to study the effects of various endocrine manipulations on gonadal maturation in P. monodon three sets of endocrine manipulations were conducted viz. unilateral eyestalk ablation, crude ganglionic extract injections and synthetic hormone injections. Three series of eyestalk ablation experiments were conducted at two different salinities viz. full strength seawater (S 34%.) and at low saline condition (S 25%.) using animals from wild and culture ponds. From these studies it is found that a system incorporating unilateral eyestalk ablation, high salinity (34%.) good water quality and reduced light intensity are necessary to induce the maturation of female P. monodon in captivity. Regarding the effect of broodstock sources

on maturation, it is found that the performance of maturation in pond reared and wild caught P. monodon was even comparable if the size and the age are adequate.

Among the three ganglionic extracts injected viz. cerebral, thoracic and optic ganglion, injections of crude thoracic ganglion extract was found to be the best for the induction of gonadal maturation and spawning. Cerebral ganglion injection also enhanced ovarian maturation but at a slower rate. Among the two synthetic hormones tried viz. 17- alpha progesterone and Estradiol the former induced the ovarian maturation than the later, however, both could not induce the full maturation in captivity during the present study. From the investigation it was concluded that among the different neuroendocrine and hormonal manipulations, eyestalk ablation and thoracic ganglion extract injection were identified as the best methods for the induction of gonadal maturity and spwaning of P. monodon in captivity.

Reproductive quality of the male P. monodon assessed during captivity showed a gradual reduction in terms of weight of the spermatophore, sperm count and percentage of viable sperm and extended the spermatophore regeneration time. However, in eyestalk ablated males there was no such reduction in the quality of the sperm and the spermatophore regeneration time was faster. From these studies it was concluded that the same neuroedocrine factors are responsible for the control of reproduction in male penaeids.

Cryopreservation studies carried out during the present investigation showed that among the different cryoprotectants tried DMSO (5%) + Glycerol (0.5 M) at (v/v) offered the best protection to the sperm of P. monodon during short term and long term preservation. Similarly among the three temperatures tried -195 °C (temperature of liquid nitrogen) was detected as the best temperature for short term and long term preservation's of sperm. There was no clumping of sperm cells or deformations in morphological appearance

when the samples were preserved at -195 °C. The size and morphological features of the sperm cells were intact in all the 4 cryoprotectants studied except trehalose at -195 °C. Glycerol (5 M) + DMSO (5%) and Glycerol (5 M) offered the best protection to the cryopreserved sperm cells. Trehalose offered the least protection to the preserved sperm cells. Mixture of DMSO (5%) + Trehalose (0.25 M) and DMSO gave comparatively good results during short-term preservation's but long-term preservation's the results were not suggestive.

In conclusion, it could be seen from the present investigation that the reproduction in the penaeid shrimp P. monodon is under the control of neuroendocrine factors produced from the NSCs of various ganglionic centers. Female reproduction is found to be highly complicated process and required high amount of energy for the complete development of the ovaries. Therefore, the neuroendocrine mechanism uses various distinct environmental cues for the timing of reproduction, so that the excess energy loss for the gonad development at unfavourable conditions can be prevented. When environmental factors are unfavourable the NSCs of the optic ganglia became active and it produce a GIH and thus the development of the gonad is being arrested by this hormone. However, when the environment becomes favourable the NSCs of the optic ganglia became dormant and concomitantly the GN & A cells of brain became active and under this influence of the GN & A cells of sub-oesophageal and thoracic ganglia in tern produce GSH which accelerated the gonadal development. It was also found that these endocrine factors are not species specific.

From the cryopreservation studies conducted, it was concluded that the male gametes (spermatozoa) of P. monodon can be cryopreserved up to 6 months at -195 °C in DMSO (5%) + Glycerol (0.5 M) (v/v) without any drastic changes in viability.

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