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STUDIES ON SOME ASPECTS OF THE REPRODUCTIVE PHYSIOLOGY OF *METAPENAEUS MONOCEROS* (FABRICIUS)

THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

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OF THE
CENTRAL INSTITUTE OF FISHERIES EDUCATION
(DEEMED UNIVERSITY)
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BY

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(Ph. D. 131)

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AUGUST 2005

Dedicated to.....

Daddy, Mummy & Jeena

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ICAR

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CERTIFICATE

Certified that the thesis entitled " **STUDIES ON SOME ASPECTS OF THE REPRODUCTIVE PHYSIOLOGY OF *METAPENAEUS MONOCEROS* (FABRICIUS)**" is a record of independent bonafide research work carried out by **Mr. Joice Abraham** during the period of study from September 2000 to August 2005 under our supervision for the degree of **Doctor of Philosophy in Fish and Fisheries Science (Mariculture)** and the thesis has not previously formed the basis for award of any degree, diploma, associateship, fellowship or any other similar title.

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I hereby declare that the thesis entitled **" STUDIES ON SOME ASPECTS OF THE REPRODUCTIVE PHYSIOLOGY OF *METAPENAEUS MONOCEROS* (FABRICIUS)"** is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

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COCHIN

JOICE ABRAHAM

सारांश

आम रूप से 'स्पेक्किल्ड श्रिंप' नाम से जान लिये जानेवाला *मेटापेनिअस मोनोसिरस* (फाब्रिकस) नामक झींगे का भारत के श्रिंप उद्योग में महत्वपूर्ण स्थान है । झींगा पालन उद्योग में उल्लेखनीय व्यतियान लाने में यह जाति सक्षम मानी जाती है । प्रकाश और इलक्ट्रॉन माइक्रोस्कोपी के ज़रिए इसकी अंडजनन (oogenesis) और शुक्रजनन (spermatogensis) प्रक्रियाओं का अध्ययन किया गया। अंडाशय (ovary) हेपाटोपानिक्रियास (hepatopancreas) हीमोलिम्फ़ (haemolymph) और पेशी ऊतक (muscle tissue) में होनेवाले जैवरासायनिक परिवर्तनों का विश्लेषण करके योक प्रोटीन विटलिन (yolk protein vitellin) का स्वभाव समझ लिया गया। अंडाशय विकास क्रम की पाँच दशाओं में होनेवाले आकृति परिवर्तनों को कोशिका विज्ञान से मिला लिया । अंड का व्यास अपरिपक्व दशा में 35.8 माइक्रोन और पूर्ण परिपक्व दशा में 230.56 माइक्रोन देखा गया । अधिकांश मोनोसीरस झींगों की अंडों में दिखाऐ पडनेवाले कोरटिकल बोडीस *एम.मोनोसीरस* में दिखाया नहीं पडा । अंडकों (oocytes) के विविध विकासात्मक दशाओं पर अत्यंत सूक्ष्म माइक्रोस्कोपी से किए निरीक्षणों ने सूचित किया कि कोशिकाद्रव्य (cytoplasm) में माइटोकॉन्ड्रिया, मुक्त राइबोसोम, रफ इन्डोप्लास्मिक रेटिनम और गोलगी बोडीस भारी मात्रा में होने पर पीतक का संश्लेषण (autosynthesis) होता है । शुक्राणुधर (spermatophore) रूपायन प्रक्रिया पर किए अध्ययन से व्यक्त हुआ कि वास डेफरन्स को ओढनेवाला कोलम्नार को एपिथीलियल कोशों से स्रवित होनेवाले द्रवों से शुक्राणुधर रूपायन हो जाता है । शुक्राणु समुच्चय को ओढने में प्रोक्सिमल वास डेफरन्स (PVR) का स्रव और शुक्राणुधर परत (spermatophoric layer) को ओढने में मीडियल वास डेफरन्स (MVD) के स्रव कार्य करते हैं । शुक्राणुओं की रचना पूर्ण हो जानेवाले टर्मिनल अम्प्यूल में, चिपकानेवाली गोलिकाएं रूपायित होती दिखायी पडी । जन्तु के प्रौढ हो जानेवाली दशाओं में उसके ऊतकों में होनेवाले जैवरासायनिक घटकों के परिवर्तनों पर किए गए निरीक्षणों ने सूचित किया कि पेशी ऊतक को छोडकर बाकी सभी की सान्द्रता में चाक्रिक परिवर्तन होता है । हेपाटोपानक्रियास से अंडाशय की ओर प्रोटीन का चलन बहुत कम था । पीतकोत्पत्ति (vitellogensis) के साथ हीमोलिम्फ प्रोटीन में घटती की प्रवणता दिखायी पडी । यह इसके अंडाशय में बदलने के कारण है । हेपाटोपानक्रियास से अंडाशय की ओर लिपिड का भागिक चलन दिखाया पडा । अंडाशय की बढती के साथ विटलिन में नियमित बढती दिखायी पडी । इस जाति में नेटिव विटलिन का मोलिकूलार भार 326 kDa देखा गया और यह कालश्यम का लिपोग्लैकोप्रोटीन बाउंड है । *एम. मोनोसीरस* की हैचरी तकनॉलजी दक्ष करने के लिए यह अध्ययन उपयोगी होगा जिसके प्रयोग करते हुए अब संवर्धन किए जानेवाली झींगा जातियों के समान इसका संवर्धन भी साध्य हो जाएगा ।

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ABSTRACT

Metapenaeus monoceros (Fabricius), also known as 'Speckled shrimp', is one of the commercially important species contributing substantially to the shrimp fisheries of India and is a potential candidate species for diversification of shrimp culture. The processes of oogenesis and spermatogenesis of the species were studied using light and electron microscopy. Biochemical changes taking place in ovary, hepatopancreas, haemolymph and muscle tissue were analysed and the yolk protein vitellin was characterized. The five stages of ovarian development based on gross morphology were related to those based on cytological changes. The mean ova diameter increased from 35.68 µm in immature stage to 230.56 µm in fully mature stage. Cortical bodies, characteristic of fully mature ova of most penaeids were absent in *M.monoceros*. Ultrastructure of oocytes at various developmental stages showed that the mode of synthesis of yolk is mostly autosynthetic with the cytoplasm abundant in mitochondria, free ribosomes, rough endoplasmic retiulum and golgi bodies. The process of spermatophore formation which took place with the help of the secretory activity of columnar epithelial cells lining the vas deferens was demonstrated. Secretions of the proximal vas deferens aided in packing the sperm mass and those of the Spermatophoric duct of median vas deferens formed the primary spermatophoric layer. The accessory duct secreted accessory structures associated with the spermatophore while secretions of the epithelial cells lining the distal vas deferens formed the secondary spermatophoric layer. Adhesive globules were formed in the terminal ampoule, where the final moulding of spermatophore took place. Spermatozoa in this species were unistellate. Variations in the biochemical constituents in different tissues showed cyclic changes in their concentration during the various maturity stages except in the muscle tissue. Mobilization of proteins from the hepatopancreas to ovary was minimal. Haemolymph proteins showed a declining trend with vitellogenesis, which is due to the transfer to ovary. A partial mobilization of lipids from the hepatopancreas to the ovary was noticed. Native polyacrylamide gel electrophoresis of ovarian tissue revealed that vitellin showed a steady increase in the level of expression with progression in maturity. Native vitellin in this species has a molecular weight of 326 kD and is a lipo-glycoprotein bound to calcium.

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Introduction

1. INTRODUCTION

In India export of shrimps has been consistently fetching foreign exchange to the tune of Rs. 4000 crores during the last few years. Wild caught shrimp account for about a quarter of this, the rest coming from aquaculture (MPEDA, 2004). Exploitation of shrimp from conventional fishing grounds has already exceeded the potential yield level and this resource is in a state of over-exploitation. Hence aquaculture is the only avenue to pursue for realizing any further increase in shrimp production.

India has 1.2 mha of potential brackish water area suitable for aquaculture, of which only 1.5 lakh ha (just above 10 %) is presently utilized, that too for traditional and extensive shrimp farming (Bhojan *et al.*, 2004). Shrimp aquaculture at present in our country is synonymous with that of tiger shrimp *Penaeus monodon* and to a lesser extent to *Penaeus indicus*. Outbreak of shrimp viral diseases in the mid 90's forced farmers to switch over from semi-intensive to extensive type of culture, which has reduced the productivity from 823 kg/ha in 1994-95 to 600 kg/ha since then (Rao and Ravichandran, 2001). Most of the problems faced in shrimp farming sector is due to the concentration totally on a single species neglecting the biological maxim that diversified species utilization adds to sustainability of the culture system. This situation warrants concerted efforts from all stakeholders to bring more species of shrimps under the umbrella of culture system.

Metapenaeus monoceros (Fabricius, 1798) (Plate 1) commonly called as 'speckled shrimp' is one of the commercially important species found along both the coasts of India. With an average annual landing of around 10,000 tons, it accounts for 7-10 % of the total penaeid shrimp catch of the country (Mohamed, 1967; Sukumaran *et al.*, 1993). Living in shallow waters down to 60 meters, mostly between 10 and 30 meters, the species prefers sandy mud bottoms and

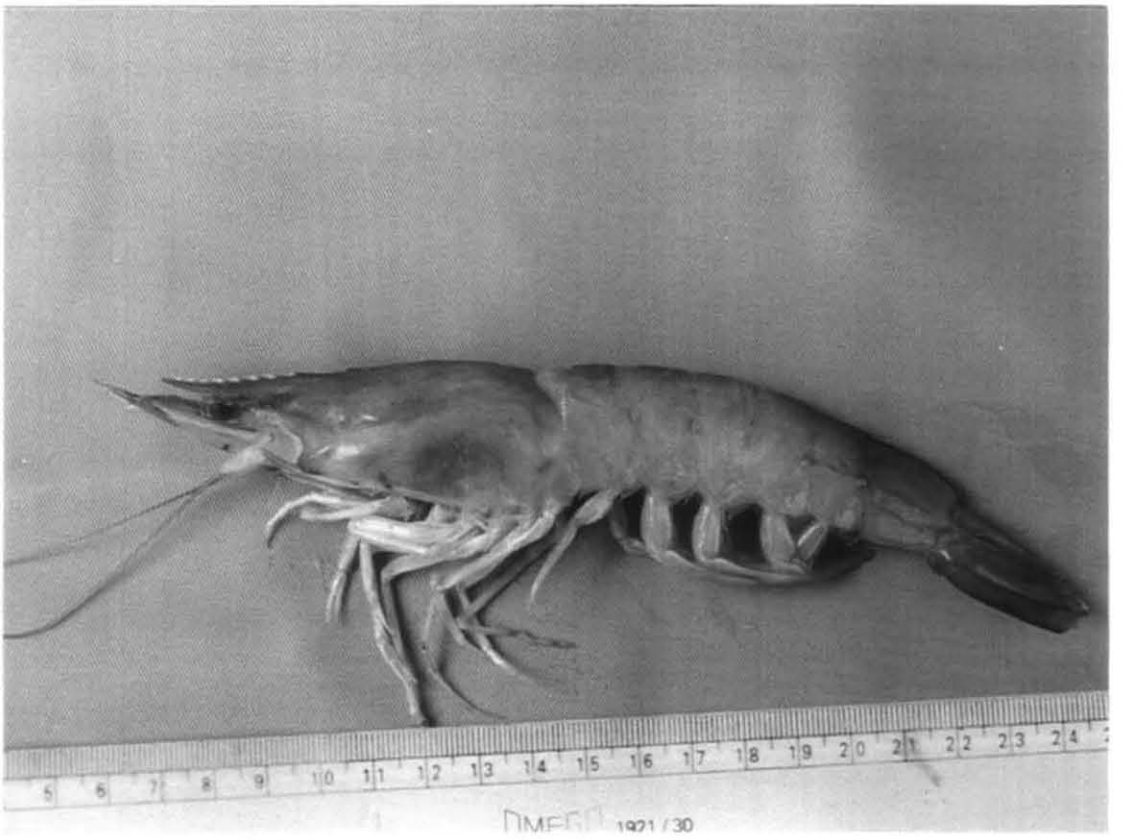

Plate 1. *Metapenaeus monoceros* (Fabricius)

brackish to marine salinities as low as 5 and up to 30 parts per thousand (Holthuis, 1980). *M.monoceros* attains a maximum length of about 190 mm and has high export potential. This species was found to reach a marketable size of fifteen grams in fifty days in culture ponds in Taiwan (Chen, 1976). It forms an important component of the shrimp catch from the Pokkali fields of Kerala and the rice-prawn filtration units in Bangladesh. There is very good scope for this species to be taken up for semi-intensive culture practices in India due to their larger size among the *Metapenaeus* spp and hardy nature.

Development of a viable hatchery technology for controlled production of seed is an essential pre-requisite for attempting commercial culture of any species. The first major contribution towards commercial shrimp culture was made by Hudinaga (Hudinaga, 1942) who was able to achieve maturation and spawning of *Penaeus japonicus* in confinement and rear the larvae till juvenile stage successfully. Since the pioneering work of 1940's (Panouse, 1943) in *Leander serratus*, unilateral eyestalk ablation is used for inducing ovarian maturation and hatchery production of seeds in a number of penaeid species all over the world. But, when this technique is used for rematuration of broodstock, it may result in poor quality eggs, and reduction in fecundity. A thorough understanding of biology of the species, especially reproductive physiology is essential for controlling reproduction in captivity which will in turn help to provide a reliable year round supply of juveniles, help in developing selective breeding programmes and be generally useful for obtaining disease free postlarvae. Studies on reproductive physiology are also helpful in formulating suitable management measures aimed at rejuvenating the over-exploited coastal shrimp stocks.

In penaeid shrimps, ovarian maturation has been classified into a number of stages ranging from four to eight, by different authors. In many studies, morphological features like colour, size, texture and gonadosomatic index (GSI) have formed the basis of classification. Gross morphological description together with cytological changes as studied using light microscope and electron microscope was employed in *Penaeus japonicus* (Hudinaga, 1942; Yano, 1988), *Penaeus duorarum* (Cummings, 1961), *Penaeus merguiensis* (Tuma, 1967), *Penaeus aztecus* and *Penaeus setiferus* (Duronslet *et al.*, 1975), *Sicyonia ingentis* (Anderson *et al.*, 1984), *Parapenaeus longirostris* (Tom *et al.*, 1987), *Penaeus indicus* (Mohamed, 1989), *P.monodon* (Tan-Fermin and Pudadera, 1989), *Metapenaeus dobsoni* (Vasudevappa, 1992) and *Metapenaeopsis dalei* (Sakaji *et al.*, 2000). In *Metapenaeus monoceros*, till date there are no studies dealing with cytological changes during ovarian maturation.

Penaeid male reproductive performance is currently regarded as one of the major drawbacks in the fragile shrimp aquaculture industry (Parnes *et al.*, 2004). In crustaceans, the male reproductive system and its physiology are not as well understood as that of female. Knowledge about the male maturation and semen quality is fragmentary. The failure of many a captive breeding programmes has been attributed to the poor quality of semen stored in spermatophores and its deterioration in captive animals (Aiken and Waddy, 1980). However, reasons for the poor reproductive performance of captive males still remain unclear. Further, an understanding of structural details of the various components of the male reproductive system, the process of spematophore formation and structural details of spermatophore will be helpful for standardization of cryopreservation protocols.

The major gametogenic process in the female reproductive cycle of crustaceans involves the synthesis of nutritive yolk in the ooplasm to meet the basic requirements of embryonic development independent of the maternal organism (Adiyodi and Subramoniam, 1983). Vitellin, the major yolk protein that accumulates within the ovary during vitellogenesis is the nutritive material necessary for the successful development of an embryo independent of its mother. Unlike many decapods, the naupli of penaeid shrimps hatch without any parental care or protection (Cook and Murphy, 1969). During the first two days penaeid naupli subsist entirely on yolk retained from egg. Therefore, the quality and quantity of egg yolk is crucial for early life of penaeid larvae. Vitellogenesis is characterized by vitellogenin synthesis and its subsequent processing and accumulation within the developing oocytes. Vitellogenin, a female specific protein circulating in the haemolymph is the precursor of vitellin/lipovitellin, the nutritive material necessary for the development of embryo. Vitellogenin and vitellin from many penaeid species have been characterized. Since environmental pollutants can affect concentration of vitellogenin in haemolymph, this protein can be of use in pollution studies also (Lee and Noone, 1995).

In crustaceans, the hepatopancreas is considered to function as the storage organ of organic nutrients, which are mobilized during the reproductive cycle to meet specific requirements of maturing gonads (Passano, 1960). Crustacean yolk is a combination of proteins, lipids and sugars complexed with carotenoid pigments. The change in colour from white through cream to green and olive green in penaeid shrimp ovary is due to the accumulation of carotenoids in gonads as chromprotein (Cheeseman *et al.*, 1967). There is very little information on the above aspects in *Metapenaeus monoceros*. Hence the present work " Studies on some aspects of the reproductive physiology of *Metapenaeus monoceros* (Fabricius) " was taken up with the following objectives:

1. To study the morphology of female reproductive system and to draw a classification system for the ovarian maturity stages

2. To study the process of oogenesis with special emphasis on the sequential deposition of yolk protein in ova
3. To study the morphology of male reproductive system and to draw a classification system for the maturity stages
4. To study the process of spermatogenesis and the mechanism of spematophore formation in vas deferens
5. To resolve the expression of yolk protein vitellin in ovary during different maturity stages and to characterize it
6. To study the biochemical changes occurring during gonadal development in ovary, hepatopancreas, muscle and haemolymph.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Metapenaeus monoceros (Fabricius) is an important component of the commercial penaeid shrimp fishery of India, accounting for nearly 7-10% of the total marine shrimp landings (Mohamed, 1967; Sukumaran *et al.*, 1993). With standardization of the hatchery technology, it has got immense potential for diversification of shrimp culture in the country. Some aspects of the fishery and biology of the species in the Cochin region have been dealt with by George (1959) and George *et al.* (1968), and in the Godavari estuarine system by Subrahmanyan (1973). George (1967) has given a synopsis of the available biological data on the species. Based on external appearance and ova diameter studies, five ovarian maturity stages were identified in *M.monoceros* (Nalini, 1976; Rao, 1989 and Nandakumar, 2001). Studies directed towards a better understanding of gonadal development in penaeid shrimps are imperative either to establish guidelines for the management of fisheries (King, 1948; Quintero and Garcia, 1998) or to improve broodstock technology for aquaculture (Tan-Fermin, 1991; Quinitio *et al.*, 1993).

2.1 Female Reproductive System And Oogenesis

Morphology of the female reproductive system of crustaceans is well documented by several research workers (Chandran, 1968; Pillay and Nair, 1970; Komm and Hinsch, 1987). In penaeid shrimps, similar studies have been conducted by Hudinaga (1942), King (1948), Shaikhmahmud and Tembe (1958), Cummings (1961), Subrahmanyam (1965), Oka (1967), Tuma (1967), Rao (1968), Duronslet *et al.* (1975), Anderson *et al.* (1984), Mohamed (1989), Tan-Fermin and Pudadera (1989), Dall *et al.* (1990), Hossain *et al.* (1991), Quinitio *et al.* (1993), Joseph (1996), Quintero and Garcia (1998), Palacios *et al.* (1999a) and Sakaji *et al.* (2000).

Observation of visual traits such as ovarian morphology and colour represent a practical procedure to evaluate female maturation and is routinely applied in commercial shrimp hatcheries. Colour, size and texture of gonads of the female shrimp are closely related to the cellular development. Among the different approaches to study the reproductive cycle of a penaeid shrimp, the external appearance of the ovary is often used for determining the progress of ovarian development. Quintero and Garcia (1998) proposed a chromatic scale to determine the stages of ovarian development in *Penaeus brasiliensis*, which ranges between a translucent appearance to an olive green colour corresponding to the immature and mature stages. Many authors have differently reported the number of maturity stages in penaeid shrimps. Oka and Srihata (1965) classified ovarian maturation in *Penaeus orientalis* into eight stages. Yano (1988) demonstrated ten stages of oocyte development in *P.japonicus*. Oocyte development was classified into four stages in *P.monodon* (Tan-Fermin and Pudadera, 1989), *P.chinensis* (Matsuyama and Matsura, 1983) and in *P.indicus* (Quinitio and Millamena, 1992). Thus, the number of maturity stages reported is not consistent. However, in penaeids the maturity stages generally fall under five groups - immature, early maturing, late maturing, mature and spent (Rao, 1968).

The proportion of gonad weight in total body weight expressed as percentage, called gonado-somatic index (GSI) is an alternative method of assessing gonadal development (Subrahmanyam, 1963; Rahaman, 1967; Pillay and Nair, 1971). Quantitative variables such as oocyte size frequency and oocyte diameter have proved to be good indicators of ovarian development stages. Measuring the ovarian width at first abdominal segment has been reported to be useful for determining maturity in *Penaeus monodon* (Tan-Fermin and Pudadera, 1989). However, both appearance and GSI provide only limited information on actual changes taking place in the ovaries and on the development of oocytes. More detailed information on cellular and sub-cellular level changes during ovarian maturation is obtained through light and electron microscopic studies.

Histological observations on ovarian development process of penaeid shrimps have been made in several species (Dall *et al.*, 1990). In most of the studies, gross developmental changes in size and colouration of the ovary have been related to cytomorphological changes. Such studies have been carried out in *Penaeus japonicus* (Hudinaga, 1942; Yano, 1988); *P.setiferus* (King, 1948; Duronslet *et al.*, 1975; Chow *et al.*, 1993); *Parapenaeopsis stylifera* (Shaikhmahmud and Tembe, 1958; Shaikhmahmud, 1961; Joshi *et al.*, 1982); *P.duorarum* (Cummings, 1961); *P.indicus* (Subrahmanyam, 1965; Rao, 1968; Hossain *et al.*, 1991; Quinitio and Millamena, 1992; Mohamed and Diwan, 1994); *P.orientalis* (Oka and Srihata, 1965); *P.merguiensis* (Tuma, 1967; Ayub and Ahmed, 2002); *Metapenaeus affinis* (Pillay and Nair, 1971; Ayub and Ahmed, 2002); *P.aztecus* (Duronslet *et al.*, 1975); *Sicyonia brevirostris* (Kennedy *et al.*, 1977); *P.monodon* (Motoh, 1978; Tan-Fermin and Pudadera, 1989; Dall *et al.*, 1990; Menasveta *et al.*, 1993; Quinitio *et al.*,1993; Joseph, 1996); *Sicyonia ingentis* (Anderson *et al.*, 1984); *Pandalus kessleri* (Quinitio *et al.*, 1989); *Metapenaeus dobsoni* (Vasudevappa, 1992; Ayub and Ahmed, 2002); *P.kerathurus* (Medina *et al.*, 1996); *Farfentopenaeus paulensis* (Peixoto *et al.*, 2003); *P.brasiliensis* (Quintero and Garcia, 1998); *Metapenaeopsis dalei* (Sakaji *et al.*, 2000) and *P.penicillatus* (Ayub and Ahmed, 2002). Ultrastructural studies on ovarian development were carried out in *P.aztecus* (Duronslet *et al.*, 1975); *P.setiferus* (Duronslet *et al.*, 1975; Chow *et al.*, 1993); *P.indicus* (Mohamed and Diwan, 1994); *P.semisulcatus* (Bose, 1995) and *P.monodon* (Joseph, 1996).

Oogenesis in crustaceans involves two distinct processes: proliferative and differentiative (Adiyodi and Subramoniam, 1983). The proliferative process in the germinal zone increases the number of oogonia cells by mitotic multiplication. The primary oocytes derived from the secondary oogonial cells are transformed into fully ripe ooctyes by sequential deposition of yolk. Vitellogenesis, the process of yolk synthesis and deposition is a very crucial event in female gametogenesis (Charniaux-Cotton, 1985) and vitellin/lipovitellin is the major yolk protein that accumulates in the ovary during this process. The growing oocytes initially

synthesize the yolk materials using their metabolic machinery. This stage is followed by an intensive accumulation of yolk from the hemolymph by endocytosis (Mohamed and Diwan, 1994). These two processes have been designated as primary and secondary vitellogenesis respectively.

The sites of synthesis of yolk proteins in crustaceans have long been a subject of controversy. The sites reported to date include haemocytes in *Callinectes sapidus* (Kerr, 1968); ovarian tissues in *Uca pugilator* (Eastman-Reks and Fingerman, 1985) and *Callinectes sapidus* (Lee and Watson, 1995) and fat bodies in *Armadillidium vulgare* and *Porcellio dilatatus* (Suzuki *et al.*, 1989). In a study employing radio-labelled aminoacids, Rani and Subramoniam (1997) demonstrated high synthetic activity in the sub-epidermal adipose tissue in the initial phase of vitellogenesis, followed by an intense synthesis and storage of vitellogenin in the hepatopancreas of mud crab, *Scylla serrata*.

Vitellogenesis has been well reviewed in Penaeidae (Quackenbush, 1991). The proposed sites for vitellogenin synthesis in penaeid shrimps are ovarian tissue in *Penaeus japonicus* (Yano and Chinzei, 1987), *P.vannamei* (Quackenbush, 1989; Rankin *et al.*, 1989), *P.semisulcatus* (Browdy *et al.*, 1990), *P.monodon* (Chen and Chen, 1994); hepatopancreas in *P.vannamei* (Quackenbush, 1989) and *P.semisulcatus* (Shafir *et al.*, 1992). Subepidermal adipose tissue was also implicated as the site of vitellogenin synthesis during secondary vitellogenesis in *P.japonicus* (Vazquez-Boucard, 1985) and *Parapenaeus longirostris* (Tom *et al.*, 1987). There are a few studies showing that ovary is the main site of vitellogenin synthesis in penaeid shrimps and the contribution of hepatopancreas is negligible. Fainzilber *et al.* (1992) reported that synthesis of vitellogenin immunoreactive protein took place in hepatopancreas of pre-vitellogenic and early vitellogenic females (oocyte diameter<100-200μm) only, and not in late vitellogenic stage when the bulk of yolk protein is synthesized. Vazquez Boucard *et al.* (2002) suggested that ovary is the most important site for vitellogenin synthesis in *Fenneropenaeus indicus* and the

contribution of hepatopancreas is only 0.14%, too meagre to cover the needs of ovary development.

In all the above studies on vitellogenin synthesis, the presence of antigenic substance by immunohistochemical staining methods merely implied the existence or distribution of vitellogenin in a particular tissue examined, but not precisely the sites of synthesis of vitellogenin. Therefore, the site of expression of the yolk precursor gene (represented by mRNA) will certainly be the most reliable criterion for defining the site of vitellogenin synthesis (Tseng *et al.*, 2002). On the basis of *in vivo* vitellogenin gene expression, hepatopancreas was confirmed as the site of extra-ovarian vitellogenin synthesis in *Macrobrachium rosenbergii* (Chen *et al.*, 1999), *Penaeus monodon* (Tseng *et al.*, 2001), *P. japonicus* (Tsutsui *et al.*, 2000) and *P.vannamei* (Tseng *et al.*, 2002).

Cortical crypts are rod shaped bodies encountered in the ripe oocytes of fully mature, ready to spawn ovary of penaeid shrimps (Dall *et al.*, 1990). Previously referred to as jelly-like substance, cortical specializations, rod-shaped bodies or peripheral bodies, they exist in all specimens observed in genera *Penaeus* and *Sicyonia* (Hudinaga, 1942; King, 1948; Ikematsu, 1963; Oka and shrihata, 1965; Oka, 1967; Tuma, 1967; Anderson *et al.*, 1984; Yano, 1988; Tan-Fermin and Pudadera, 1989). According to Tan-Fermin and Pudadera (1989), in *P.monodon*, the cortical bodies are present at the periphery but, as the maturation progresses, these bodies elongate and extend towards the nucleus. Cortical crypts release materials forming a jelly coat when the eggs are exposed to seawater (Clark and Lynn, 1977). As a result of such a cortical reaction, the egg reduces its volume (Clark *et al.*, 1980). Medina *et al.* (1996) reported the absence of cortical crypts in pond reared *P. kerathurus*, while late vitellogenic oocytes in the ovaries of wild specimens contained them. Vasudevappa (1992) and Sakaji *et al.* (2000) reported that cortical rods were not found in oocytes of *Metapenaeus dobsoni* and *Metapenaeopsis dalei* respectively. Contrary to the assumption that cortical rods are a characteristic feature of genus *Penaeus*,

Ayub and Ahmed (2002) detected their presence in mature eggs of *Metapenaeus affinis* and *Parapenaeopsis stylifera*. But these peripheral bodies remain spherical throughout and never take the appearance of rods.

2.2 Characterization Of Vitellin

Vitellogenesis is the process of hormonally regulated synthesis of yolk proteins namely vitellogenin and vitellin. Vitellogenin that circulates in hemolymph is the precursor of vitellin, the yolk protein proper (Gerber-Huber *et al.*, 1987; Kunkel and Nordin, 1985; Van het Schip *et al.*, 1987; Byrene *et al.*, 1989; Chen *et al.*, 1999; Tseng *et al.*, 2002). The term "vitellogenin" was first used instead of "female specific protein" of insects by Pan *et al.* (1969). According to Hagedorn and Kunkel (1979), vitellogenin is found only in females, incorporated into oocytes by micropinocytosis and is the precursor of the intra-oocyte yolk proteins namely lipovitellins or vitellins and contributes to a large part of all the proteinaceous yok in the eggs. The first report of vitellogenin production in decapod males was by Wilder *et al.* (1994), who demonstrated vitellogenin in male hemolymph of *Macrobrachium rosenbergii*, following bilateral eyestalk ablation.

Vitellogenin and vitellins are of low electrophoretic mobility. Moreover, sudan black staining, PAS positivity and pigment analysis have identified them as a lipo-glyco-caroteno protein (Adiyodi, 1968; Kerr, 1969; Chen and Chen, 1994). In *Penaeus monodon*, Chang *et al.* (1994) identified vitellogenin as a lipo-glyco-phospho protein. Association with carotenoid pigments gives a specific colour to vitellogenin and vitellin according to species-violet in *Orchestia gammarella*; red-orange in *Idotea balthica basteri* and *Artemia salina*; orange in several crabs; purple in *Uca pugilator* and green in *Carcinus maenas*, *Ligia oceanica* and in several shrimps, thus making it possible to distinguish these female specific fractions by colour, before staining the electropherograms (Souty-Grosset, 1984). Similarities in electrophoretic patterns and immunological reactions between

vitellogenin and vitellin have been demonstrated in several crustacean species (Suzuki, 1987; Tirumalai and Subramoniam, 1992; Chang *et al.*, 1993; Chang *et al.*, 1994; Han *et al.*, 1994; Chang and Jeng, 1995; Vafopoulou and Steel, 1995; Qiu *et al.*, 1997; Chen *et al.*, 1999 and Vazquez-Boucard *et al.*, 2002).

Employing isopod, *Ligia oceanica* as a model, Picaud *et al.* (1989) have shown that the yolk protein, vitellin consists of a major fraction of extra-ovarian origin in association with the one that is synthesized inside ovary. This investigation has shown that vitellogenin is encountered mainly, but not exclusively in the hemolymph, transported by haemolymph towards the ovary, where it is stored and processed into vitellin. The transformation of vitellogenin into vitellin is progressive and at times simultaneous presence of the two proteins can be seen. Souty-Grosset (1984) has found vitellin in haemolymph when eggs were resorbed owing to non-mating, in which case vitellin band became very intense in hemolymph because of return of material from resorbed oocytes.

In crustaceans, molecular weight of purified vitellogenin is found to range between 200 kDa and 400 kDa. Several workers have dealt with the purification and characterization of vitellogenin in penaeid shrimps. Chang *et al.* (1994) reported a molecular weight of 263 kDa for *Penaeus monodon* vitellogenin with two polypeptide subunits of 170 kDa and 82 kDa. Chen and Chen (1994), reported four subunits of 74, 83,104 and 169 kDa; whereas Logyant *et al.* (1999) reported five sub units of molecular weights 74, 83, 104, 168 and 200 kDa for *P.monodon*,. Chang and Jeng (1995) reported two subunits of 85 and 195 kDa for *P.chinensis* vitellogenin. In *P.semisulcatus*, vitellogenin is found to consist of three subunits of molecular weights 80, 120 and 200 kDa (Lubzens *et al.*, 1997). Thus, crustacean vitellogenin is apparently a composite protein fraction, the production of which is likely to be a multi-gene involved and multi-hormone controlled process (Adiyodi and Subramonian, 1983).

Purification and characterization of ovarian vitellin from several penaeid shrimps have been done. They are *Penaeus japonicus* (Vazquez-Boucard *et al.*, 1986; Yano and Chinzei, 1987), *Parapenaeopsis longirostris* (Tom *et al.*, 1987), *Penaeus vannamei* (Quackenbush, 1989; Tom *et al.*, 1992), *P.monodon* (Quinitio *et al.*, 1990; Chen and Chen, 1993; Chang *et al.*, 1993; Logyant *et al.*, 1999), *P.semisulcatus* (Browdy *et al.*, 1990; Tom *et al.*, 1992; Khayat *et al.*, 1994; Lubzens *et al.*, 1997), *P.chinensis* (Chang *et al.*, 1996) and *Metapenaeus ensis* (Qui *et al.*, 1997). The molecular weight of the isolated vitellin from six crustacean species ranged from 330 to 370 kDa (Wallace *et al.*, 1967). From the above mentioned studies it is evident that the molecular weight of native vitellin in most penaeids ranges from 300 kDa to 500 kDa.

The number of polypeptide subunits in vitellin reported for penaeids is generally between two and five. Browdy *et al.* (1990) reported that vitellin of *Penaeus semisulcatus* had four subunits (50, 63, 80 and 90 kDa), while only two polypeptide subunits (86 and 90 kDa) were reported in the same species by Tom *et al.* (1992). Khayat *et al.* (1994) reported three polypeptides of molecular weights 80, 91 and 158 kDa in *P. semisulcatus*, whereas Lubzens *et al.* (1997) found 80, 120 and 200 kDa polypeptides in the vitellin of the same species. Vazquez-Boucard *et al.* (1986) reported five polypeptide subunits in *P. japonicus*, while Yano and Chinzei (1987) reported only two subunits in the same species. Vitellin of *P. monodon* was found to have four major polypeptide subunits viz., 74, 83,104 and 168 kDa, and a minor one of 90 kDa by Quinitio *et al.* (1990) and Chen and Chen (1993) while Chang *et al.* (1993) reported eight polypeptides (35, 45, 49, 58, 64, 68, 82, 91 kDa). Eight subunits (105, 85, 78, 58 and 40 kDa for Vn1 and 155, 85, 78 kDa for Vn2) were elucidated for *P. chinensis* (Chang *et al.*, 1996). Tom *et al.* (1987) described two subunits for *Parapenaeus longirostris* (45 and 66 kDa). Quackenbush (1989) reported three polypeptides (76, 97 and 158 kDa) in the ovary of *P.vannamei*, while Tom *et al.* (1992) reported only two polypeptides (61, 69 kDa). In *Metapenaeus ensis* two major subunits (76, 102 kDa) were reported by Qiu *et al.* (1997).

2.3 Male Reproductive System And Spermatogenesis

It is a known fact that the reproductive quality of males plays an important role in the productivity of broodstock of captive penaeids (Diaz *et al.*, 2002). When compared to that of females, studies on the male reproductive organs and process of spermatogenesis in penaeid prawns are very few and information on male reproductive physiology is limited (King, 1948; Subrahmanyam, 1965; Lu *et al.*, 1973; Aiken and Waddy, 1980). Penaeid male reproductive performance is currently regarded as one of the major drawbacks in the fragile shrimp aquaculture industry (Parnes *et al.*, 2004).

Unlike females, male shrimps provide no grossly visible clues as to the physiological state of the gonad. Hence very few workers have described well-defined maturity stages in males. Using the small variations in opacity and size of the testes with size of the animal, Subrahmanyam (1965) described five maturity stages in male *Penaeus monodon*. Parnes *et al.* (2004) classified the male reproductive system in *P.vannamei* into four categories based on external appearance of spematophores as observed in the terminal ampoule.

Knowledge of the reproductive tract morphology is one of the most important aspects in any reproductive biology study. Calman (1909) and King (1948) were the first to carry out such studies in penaeids. The male reproductive tract of decapod crustaceans comprises paired testes accompanied by the vasa deferentia, which are divided into several zones: proximal vas deferens, medial vas deferens, distal vas deferens and the terminal ampoule. The testes are paired lobe-like organs located in the cephalothorax ventrally to the heart and dorsally to the hepatopancreas and midgut. In penaeids, testes are comprised of several lobes, each independent of the other, which are connected to the vas deferens through a short and narrow connecting tubule (Ro *et al.*, 1990). The number of testicular lobes in penaeids vary from species to species (Huq, 1980;

Chow *et al.*, 1991b; Mohamed and Diwan, 1993). Vas deferens is a tubular duct, which conveys the spermatozoa from the testis to the gonopore. The vas deferens comprises from five to ten distinct zones depending on the species (Mc Laughlin, 1983). King (1948) distinguished four zones in *Penaeus setiferus*, which were named segments I, II, III and IV. The last segment was also called terminal ampoule. Malek and Bawab (1974a) divided the vas deferns of *P.kerathurus* into three functionally different zones, the medial zone (ascending and descending), the distal and the terminal ampoule. Diaz *et al.* (2002) identified four zones in the vas deferens of *Pleoticus mulleri*.

General accounts on spermatogenesis of decapod crustaceans including penaeids have been provided by many workers like Adiyodi and Subramoniam (1983), Pochon-Masson (1983), Adiyodi (1985), Bauer (1991), Bodekke *et al.* (1990), Felgenhauer and Abele (1990), Hinsch (1990) and Subramoniam (1990, 1993). Detailed histological studies on spermatogenesis have been done in *Penaeus setiferus* (King, 1948); *P.indicus* (Subrahmanyam, 1965 and Mohamed, 1989) ; *Parapenaeopsis stylifera* (Joshi *et al.*, 1982); *Metapenaeus dobsoni* (Vasudevappa, 1992); and *Trachypenaeus similis* (Bauer and Min,1993).

Ultrastructural studies on male reproductive system and the process of spermatogenesis in decapod crustaceans are very few. Hinsch and Walker (1974) probed the role of vas deferens in spermatophore production in spider crab *Libinia emarginata*. Burton (1995) studied the relationship between sperm development and associated structures of the male reproductive system of scyllarid lobster *Thenus orientalis*. Benhalima and Moriyasu (2000) investigated the structure and function of posterior vas deferens of snow crab *Chionoecetes opilio* using scanning and transmission electron microscopy. They reported resorption of spermatophores/spermatozoa in proximal vas deferens, not previously reported in decapods. Ultrastructural studies on penaeid shrimp spermatogenesis are that of Chow *et al.* (1991a, 1991b) in *Penaeus setiferus* and *P.vannamei*; Mohamed and Diwan (1993) in *P.indicus*; Vasudevappa (1992)

in *Metapenaeus dobsoni*; Bose (1995) in *P.semisulactus* and Joseph (1996) in *P.monodon*. Wu *et al.* (2000) employed laser scanning confocal microscopy and electron microscopy in studying vas deferens and spermiogenesis in *P.chinensis*.

In most crustaceans including penaeids, sperm transfer during copulation is accomplished by making use of a specialized sperm packet called spermatophore. Spermatophores are comprised of sperms surrounded by layers of acellular secretions produced by the vas deferens (Aiken and Waddy, 1980). Uma and Subramoniam (1979) studied spermatophore structure and chemical composition in mud crab *Scylla serrata*. Formation of spermatophores was investigated in *Homarus americanus* (Kooda-Cisco and Talbot, 1982); *Albunea symnista* (Subramoniam, 1984) and *Panulirus homarus* (Radha and Subramoniam, 1985). Several studies have dealt with the formation and/or structure of the spermatophore in penaeids. They are Hudinaga (1942) in *Penaeus japonicus*; Subrahmanyam (1965) *in P.indicus*; Malek and Bawab (1974a) in *P.kerathurus*; Farfante (1975) in *P.vannamei*; Champion (1987) in *P.indicus*; Bell and Lightner (1988); Ro *et al.* (1988), Talbot *et al.* (1989) and Ro *et al.* (1990) in *P.setiferus*; Mohamed and Diwan (1993) in *P.indicus*; Vasudevappa (1992) in *Metapenaeus dobsoni*; Bose (1995) in *P.semisulactus* and Joseph (1996) in *P.monodon*. In crustaceans, spermatophores have been studied after teasing out from the distal vas deferens (Radha and Subramoniam, 1985; Ro *et al.*, 1990; Chow *et al.*, 1991a). It was Kooda-Cisco and Talbot (1982) who used electroejaculation technique for understanding the structure of fully formed spermatophore.

Of late, spermatozoal ultrastructural studies of decapod crustaceans have been receiving a lot of attention owing to their use in spermiotaxonomy - application of sperm ultrastructure to phylogeny and taxonomy (Sclezo and Medina, 2003). Sperm morphology has been extensively used for recognizing phylogenic linkages among various taxa (Jamieson, 1989; Tudge and Jamieson, 1991; Medina, 1994a,b; Tudge, 1997; Medina *et al.*, 1998; Jamieson and Tudge,

2000). Hence it appears that knowledge of sperm morphology and components might be very useful for taxonomic studies in penaeid shrimps.

Spermatozoa of mature decapod crustaceans lack a flagellated tail and mitochondrial mid-piece, unlike the sperm of most other animals (Kim *et al.*, 2003). Most decapod sperms are non motile and have only a main body with stellate appendage or spike. In general, decapod sperms are divided into two groups of natantian (shrimps) and reptantian (crayfish, lobster and crabs) morphological types, on the basis of the number and structure of sperm spikes. Natantian sperm possesses a nucleus and a single spike that may contain microfilaments (Brown *et al.*, 1976; Kleve *et al.*, 1980), whereas reptantian sperm possesses a main body and multiple spikes that mostly consist of microtubles (Hinsch, 1969; Talbot and Summers, 1978; Dudenhausen and Talbot, 1982; Tudge and Jamieson, 1991; Lohrmann and Raineri, 1995).

As reviewed by Jamieson and Tudge (2000), sperm ultrastructure has been studied in some caridean shrimps of superfamilies Palaemonidae, *Palaemon serratus* (Papathanasiou and King, 1984), *Palaemonetes paludosus* (Koehler, 1979), *Macrobrachium rosenbergii* (Lynn and Clark, 1983) and *M. australiense* (Butcher and Fielder, 1994); Rhynchocietoidea, *Rhynchocinetes typus* (Dupre and Barros, 1983) and Crangonoidea, *Crangon septemspinosa* (Arsenault *et al.*, 1979; Arsenault *et al.*, 1980). The above studies have shown some morphological differences in the structure of sperm spikes among the caridean superfamilies. The spikes of palaemonoid shrimps are composed of cross-striated fibres, whereas those of crangonoid and rhynchocinetoid are not. Moreover, the spike of the rhynchocinetoid shrimp is a hollow tube-like form, much longer than that of other caridean shrimps. Such differences reflect that spike components of sperm may vary according to superfamilies (Kim et al., 2003).

Within the Dendrobranchiata, the penaeid spermatozoa have been the most extensively studied in terms of the number of species examined (Jamieson, 1991; Medina, 1995a; Jamieson and Tudge, 2000). Such studies have been conducted in *Penaeus aztecus* (Clark *et al.*, 1973); *Sicyonia ingentis* (Clark *et al.*, 1973; Kleve *et al.*, 1980; Clark and Griffin, 1988); *P.japonicus* (Ogawa and Kakuda, 1987; Medina *et al.*, 1994b); *P.vannamei* (Dougherty and Dougherty, 1989); *P.indicus* (Mohamed and Diwan, 1993); *Parapenaeus longirostris* (Medina, 1994); *Penaeopsis serrata* (Medina *et al.*, 1994a) and *Penaeus kerathurus* (Medina *et al.*, 1994b).

In gross morphology, the penaeid spermatozoan basically consists of a rounded or ovoid main body and a spike. The main body comprises the central nuclear region, a peripheral cytoplasmic band and the acrosomal cap, which overlies the nuclear region anteriorly and extends into the spike. Both spike and acrosomal cap make up a membrane bound acrosomal vesicle of heterogeneous content that is directly invested by the plasma membrane. The spike morphology and substructure vary markedly from species to species. According to Scelzo and Medina (2003), an investigation of the sperm ultrastructure of Indian Ocean genus of *Metapenaeus spp* could be of great use to gain a deeper insight into the dendrobranchiate sperm ultrastructure and phylogeny.

2.4 Biochemical Changes During Ovarian Maturation

Crustacean reproduction involves the production of yolk-laden eggs. Yolk is a combination of proteins, lipids, sugars and steroid hormones (Adiyodi, 1968). In shrimps, during maturation, the weight of the ovary increases four to eight folds (Jeckel *et al.*, 1989; Mourente and Rodriguez, 1991; Ravid *et al.*, 1999). During the process, sufficient nutrients must be accumulated in the egg yolk to allow the normal development of the embryos and pre-feeding larvae. In several studies, poor reproductive performance or low offspring quality was observed when sub-optimal broodstock diets were used (Chamberlain, 1988;

Bray *et al.*, 1990; Cahu *et al.*, 1995). Determination of biochemical composition of shrimp ovary and other tissues like hepatopancreas, muscle and hemolymph and the dynamics of various nutrients like lipids, proteins, carbohydrates and carotenoid pigments during the process of ovarian maturation is an effective approach towards increasing our knowledge regarding shrimp broodstock nutrition (Harrison, 1990), which will in turn pave the way for production of high quality nauplii.

Studies on biochemical changes in relation to ovarian maturation in invertebrates have been pioneered by Giese and co-workers (Giese and Pearse, 1974). Several such studies have dealt with changes in metabolites like protein, lipid, carbohydrates and carotenoid pigments in tissues such as ovary, hepatopancreas, muscle and haemolymph during reproductive maturation in *Callinectes sapidus* (Dean and Vernberg, 1965); *Cancer borealis* (Telford, 1968); *Portunus pelagicus* (Rahaman, 1967); *Charybdis variegata* (Chandran, 1968); *Paratelphusa hydrodromous* (Adiyodi, 1968); *Uca annulipes* and *Portunus pelagicus* (Pillay and Nair, 1973); *Barytelphusa cunicularis* (Diwan and Nagabhushanam, 1975); *Orconectes nais* (Rice and Armitage, 1974); *Clibanarius clibanarius* (Varadarajan and Subramoniam, 1982); *Paratelphusa masoniana* (Kumar and Agarwal, 1983); *Panulirus homarus* (Vijayakumaran and Radhakrishnan, 2002) and crayfish, *Cherax quadricarinatus* (Garcia-Guerrero *et al.*, 2003).

In penaeid shrimps, such studies were initiated by Pillay and Nair (1973). Lawrence *et al.* (1979) made a comparison of the percentage protein, carbohydrate and lipid in the ovary and hepatopancreas of *Penaeus vannamei*, *P. stylirostris* and *P.setiferus* using both wild and pond reared specimens. Other studies dealing with biochemical changes during ovarian maturation are in *Parapenaeopsis hardwickii* (Kulkarni and Nagabhushanam, 1979; Nagabhushanam and Kulkarni, 1980); *Penaeus japonicus* (Teshima and Kanazawa, 1978; Teshima and Kanazawa, 1983; Teshima *et al.*, 1988; Teshima

et al., 1989); *Penaeus setiferus* (Middleditch *et al.*, 1980; Castille and Lawrence, 1989), *P.indicus* (Reid and Caulton, 1980; Galois, 1984; Mohamed and Diwan, 1992); *P.notialis* (Trujillo and Luna, 1981) *Metapnaeus affinis* (Sarojini *et al.*, 1986); *P.aztecus* (Castille and Lawrence, 1989); *P.monodon* (Dy-Penaflorida and Millamena, 1990; Millamena and Pascual,1990); *M. dobsoni* (Vasudevappa, 1992); *P.semisulcatus* (Bose, 1995); solenocerid shrimp, *Pleoticus muelleri* (*Jeckel et al.*, 1989) and caridean prawn *Crangon crangon* (Haefner and Spaargaren, 1993; Spaargaren and Haefner, 1994). Clarke *et al.* (1990) studied the biochemical composition in *Macrobrachium rosenbergii* in relation to embryonic development. The role of hepatopancreas in polypeptide synthesis during vitellogenesis has been probed in *Litopenaeus vannamei* (Rankin *et al.*, 1989), *Penaeus semisulcatus* (Shafir *et al.*, 1992 and Fainzilber *et al.*, 1992). A sequential increase in the haemolymph concentration of female specific protein vitellogenin was noticed in *Penaeus vannamei* (Quackenbush, 1989), *Pandalus kessleri* (Quinitio *et al.*, 1989) and *P. indicus* (Quinitio and Millamena, 1992). Arcos *et al.* (2003) studied the variations in haemolymph metabolic variables in relation to gonad development in *P. vannamei*.

Palacios *et al.* (2000) investigated the tissue biochemical composition in relation to multiple spawning in wild and pond reared *Penaeus vannamei* broodstock. They did not detect any depletion of hepatopancreatic reserves after successive rematurations. They also noticed that females with multiple spawnings had a higher GSI, increased levels of protein in the ovary and increased levels of acylglycerides and total protein in the hepatopancreas. Contrary to this, Vazquez-Boucard *et al.* (2004) observed a depletion of total protein and lipid levels in hepatopancreas and ovary of *Fenneropenaeus indicus* after three successive spawnings. They observed that GSI fell by about 20% between first and fourth spawnings.

In shrimps, lipid metabolism is subject to the greatest modification during the three stages of reproduction: vitellogenesis, embryogenesis and larval

development (Vazquez-Boucard *et al.*, 2002). Lipids play an essential role as energy reserve (triacylglycerols), as the basic component of membranes (phospholipids), and for hormone synthesis. Lipids stored in the hepatopancreas of spawners are necessary for the synthesis of vitellins, the development of oocytes, embryonic development and feeding the young larvae. Vitellin is a lipo-glyco-caroteno-protein, a complex macromolecule of which the most important component is lipids (Galois, 1983). Lipids stored in hepatopancreas are transported to the ovaries during vitellogenesis (Castille and Lawrence, 1989). Wouters *et al.* (2001b) noticed that during ovarian development of *Litopenaeus vannamei*, lipid accumulation in the ovaries occurred prior to the depletion of lipid in the hepatopancreas, thus contradicting the argument that midgut gland is at the origin of lipids accumulated in the ovaries. While the midgut gland is accurately identified as major lipid storage and processing organ (Clarke, 1982; Teshima *et al.*, 1989; Vogt *et al.*, 1985), it seems that some of the ovarian lipids must be derived either from other body reserves, *de novo* synthesis or diet.

Phospholipids are structural lipids considered to form the basic matrix of cell membranes, and not used for other purposes. However, Galois (1983) demonstrated that a large portion of these structural lipids is mobilized when energy requirements increase significantly during embryonic development in *Palaemon serratus* and during starvation in *Penaeus japonicus*. Phospholipids are predominant in shrimp ovaries, mainly phosphatidyl choline and phosphatidyl ethanolamine (Gehring, 1974; Teshima and Kanazawa, 1983; Mourente and Rodriguez, 1991; Ravid *et al.*, 1999). Cholesterol is also an important lipid class in mature shrimp ovaries (Middleditch *et al.*, 1980; Teshima and Kanazawa, 1983; Mourente and Rodriguez, 1991; Ravid *et al.*, 1999). Cholesterol is known to fulfill several endocrinological functions, and its mobilization during maturation was reviewed by Harrison (1990).

It is assumed that protein requirements are higher during maturation and reproduction of animals as compared to the non-reproductive stages, given the

intense biosynthesis that takes place during these processes (Harrison, 1990). An increase of protein content in the ovaries of *Penaeus indicus* (Read and Caulton, 1980), *P. aztecus* and *P. setiferus* (Castille and Lawrence, 1989) during maturation has been documented. Palacios and Racotta (1999b) and Palacios *et al.* (2000) observed a different hepatic and ovarian protein content between females with excellent repeat performance and females with moderate repeat performance of both wild and domesticated *P. vannamei*. The protein contents of the hepatopancreas and the ovaries of the best performing animals were significantly higher. Females that did not spawn had the lowest protein levels in their tissues. Lubzens *et al.* (1995) and Ravid *et al.* (1999) focused on the structure of the haemolymph lipoprotein, vitellogenin and ovarian lipoprotein, vitellin and their role in lipid metabolism. Being insoluble in water, lipids would be transported in the haemolymph as lipoproteins after binding to vitellogenin.

Carotenoids are a group of pigments that cannot be biosynthesized by animals. Castillo *et al.* (1982) have carried out a survey of carotenoids in crustaceans. They are taken up from the diet, and can be transformed afterwards from one carotenoid form into another. In juvenile shrimp, they are important for pigmentation. According to recent research, they do also play vital roles in shrimp larvae and broodstock, among which the role of natural anti-oxidant is the most important (Dall *et al.*, 1995; Merchie *et al.*, 1998). During early maturation, free and esterified carotenoids accumulate in the hepatopancreas and during secondary vitellogenesis they are mobilized from there via the haemolymph to the ovaries (Vincent *et al.*, 1988; Harrison, 1990). This accumulation of carotenoids in the ovaries during maturation results in their darkening, on which the staging of females into different maturation classes is based. According to Jeckel *et al.* (1989), the form of the carotenoids that accumulate depends largely on the diet. Dall *et al.* (1995) conducted biochemical studies on wild *Penaeus esculentus*, determining astaxanthin and its esters as principal carotenoids. Free astaxanthin was predominant in maturing ovaries forming up to 80% of the total carotenoids and increasing from 2 to 34 ppm. In the digestive gland, free

astaxanthin and esters increased from 20 to 120 ppm. In the integument, carotenoid levels remained relatively constant (90 ppm) throughout the maturation cycle. A comparison with carotenoids of the natural diet of *P. esculentus* indicates that after ingestion dietary carotenoids are converted to astaxanthin. Dall *et al.* (1995) also emphasize that carotenoids play a crucial role as vitamin A precursors. Wyban *et al.* (1997) studied the effect of dietary carotenoid on reproductive exhaustion of *Litopenaeus vannamei*. At the start of their experiment, broodstock was exhibiting exhaustion symptoms such as color loss in the ovaries and decreased larval survival. When, paprika, a low-cost carotenoid source was added to the maturation diet, it resulted in an increased larval survival. This effect on larval quality can probably be attributed to the anti-oxidant properties of carotenoids. Free radicals, initiated by various factors including active oxygen, attack lipids and proteins in biomembranes, leading to a deterioration of egg quality (Bromage and Roberts, 1995). Carotenoids, particularly astaxanthin, are strong scavengers of free radicals and protect eggs from oxidative deterioration. They also prevent peroxidation of poly-unsaturated fatty acids (PUFA) in the diet. Finally, carotenoids also play an important role in providing the necessary reserves in embryos and pre-feeding larvae for the development of chromatophores and eyes (Dall *et al.*, 1995).

Materials and Methods

3. MATERIALS AND METHODS

Live specimens of *Metapenaeus monoceros* were collected during November 2001 to June 2003 from trawlers operating from Kalamukku and Murikkumpadam fish landing centers of Vypeen islands (Latitude 10.08° N, Longitude 76.21° E). Live adult shrimps of size ranging from 90 mm to 160 mm were used. Aerators and 25 litre plastic bins were used for live transport of shrimp from the fishing ground to the landing centers. Shrimps were then transported live to the CMFRI laboratory where they were kept in 1 ton fibre glass tanks with aeration till the various tissues were collected and fixed for histological preparations and electron microscopy and preserved for biochemical and electrophoretic studies. The following studies were carried out:

1. Gross morphology of male and female reproductive structures
2. Histology
3. Ultrastructure
4. Electrophoresis to characterize vitellin
5. Biochemical changes during ovarian development

3.1 Gross Morphology

At the laboratory, specimens were segregated sexwise and maintained in 1-ton fiberglass tanks. For both the sexes, total length (TL) and carapace length (CL) were measured to the nearest mm and total weight (TW) to the nearest milligram. Based on colour and size of the ovary observed through the exoskeleton, female shrimps were classified into 5 maturity stages according to Rao (1968). The stages were later verified by gonadosomatic index (GSI) and histology. Impregnated and non-impregnated condition of thelycum of females was noted. In males, presence or absence of terminal ampoule and external appearance of petasma were observed.

Ovaries of shrimps were dissected out carefully and observed under stereoscopic dissection microscope to study their morphology. All dissections were done in normal saline (0.85% NaCl). The exposed ovary was then carefully excised out and weighed to the nearest milligram. The gonadosomatic index (GSI) was calculated as per the method described by Giese and Pearse (1974).

$$GSI = \frac{\text{Wet weight of ovary} \times 100}{\text{Wet weight of shrimp}}$$

Testes of male shrimps were carefully dissected out by proceeding from the terminal ampoule. The various parts of the male reproductive system, viz. the testicular lobes, proximal vas deferens, medial vas deferens and distal vas deferens were observed under light microscope.

3.2 Histology

The process of oogenesis was studied employing histological preparations of ovaries from females belonging to different maturity stages, with normal haematoxylin and eosin staining. For studying spermatogenesis and spermatophore formation, histological sections of testicular lobes, proximal vas deferens, medial vas deferens, terminal ampoule and spermatophores were prepared.

3.2.1 Fixation

Preliminary examinations showed no difference in the state of ovarian development between anterior, middle and posterior sections of the ovary. For conducting routine histological studies, one of the middle lobes of the ovary was cut and fixed in Bouin's fluid for 24-48 hours. Spermatophores were extruded by subjecting the male specimens to 12 V electric current at the base of the fifth

walking leg, using the electrodes of an electrocautery. From male specimens, parts of testicular lobes, proximal vas deferens, medial vas deferens, terminal ampoule and spermatophores were fixed in Bouin's fluid in the same way.

3.2.2 Processing and sectioning

Tissue preparation for light microscopy was performed according to Bell and Lightner (1988). All tissues were washed overnight in running tap water to remove the excess picric acid. The tissues were then dehydrated using propanol series (30% to 100%) and cleared in chloroform. The tissues were further cold - impregnated overnight with wax using a mixture of chloroform and wax shavings in a ratio of 1:1. Subsequently, the solvent was evaporated by placing the tissues in an oven at 58°C. The tissues were then passed through two changes of fresh molten wax. Tissue blocks were prepared and serial sections were cut at approximately 5-6 µm thickness using a rotary microtome. Sections were then affixed to clean glass slides using fresh Meyer's egg albumin and flattened by placing them on a slide warmer with a drop of distilled water. Subsequently, the water was drained off and the slides were allowed to air dry. These slides were then used for histological staining.

3.2.3 Staining

Representative 5-6 µm paraffin cross sections were subjected to routine staining in haematoxylin, with 1% alcoholic eosin as the counter stain, following standard procedures for general histology. Sections to be stained were deparaffinized in two changes of xylene and then rehydrated through a descending series of propanol grades. Sections were made blue using tap water or ammonia solution. Eosin stained sections were repeatedly washed in an ascending series of propanol grades to remove excess eosin and cleared in xylene. Sections were then mounted with DPX.

3.2.4 Photomicrography

Mounted sections were examined under a LEICA MPS 60 binocular microscope. Microphotographs were taken using a LEICA camera attached to the microscope.

3.2.5 Micrometric Measurements

Since the size increase of the oocytes is a function of oogenensis, the micrometric measurements of the oocytes in different stages of maturation were taken using an ocular micrometer calibrated with a stage micrometer. As oocytes strongly deviate from a spherical shape, the largest and smallest axes of the oocyte diameter were taken and the average used as the actual oocyte diameter. Spermatogonia and sperm cell diameters were also recorded. A minimum of 100 cells was measured from a single slide.

3.3 Transmission Electron Microscopy

The ovarian lobes from females and testicular lobes, proximal vas deferens, medial vas deferens, distal vas deferens, terminal ampoule and spermatophores from male specimens were washed with saline and cut into small pieces of 1 mm^3. These were fixed in 3% buffered glutaraldehyde solution for two hours at 4°C, following immersion fixation technique. Decanted tissues were washed with the buffer (0.1 M sodium cacodylate) for 15 minutes giving three changes. These were stored in the buffer till the same were post fixed in 1% osmium tetroxide for two hours at 4°C. These tissues were then dehydrated in acetone at different concentrations of 30%, 50%, 70% and 90% for ten minutes each and finally in 100% acetone, with two changes of twenty minutes each, at room temperature. The infiltration was done in Spurr's resin (Spurr, 1969) combined with acetone in the ratio of 1:3, 1:1 and 3:1 for one hour each. After infiltration, the tissues were made into blocks in plastic vials and kept in

'cinex' incubator, setting the temperature at 70°C, for nine hours. From the polymerized blocks, ultra thin sections (60-90 nm) were taken in a LKB Nova Ultramicrotome (LKB – Producter AB, Sweden) using glass knives. The sections were double-stained in Uranyl actetate and Lead citrate to enhance the contrast. The ultrathin sections were then mounted on grids and the images were observed and photographed in the Hitachi H 600 Transmission Electron Microscope.

3.4 Electrophoresis

3.4.1 Sample preparation

Lipovitellin for the present study was isolated from vitellogenic ovary (stage III and stage IV). Female shrimp of different maturity stages were dissected to collect the ovaries. Ovaries were washed in ice-cold 100 mM phosphate buffer (0.2M solution of NaH_2PO_4 + 0.2M solution of $Na_2HPO_4 .7H_2O$, pH 7.2) containing 0.001% phenyl methyl sulfonyl fluoride (PMSF) as a protease inhibitor. Ovarian samples were frozen and stored at -20°C till further use. The frozen ovary was thawed and homogenized in three volumes of the same buffer, using a hand homogeniser, and centrifuged at 10,000 rpm for 15 minutes, at 4°C. The floating fatty layer and the precipitate from the ovaries, were removed. The centrifugation and removal of the fat cap at the top were done three times and the supernatent containing lipovitellin was frozen and stored at -80°C. Ovarian samples of all the five stages of maturity and testicular lobe samples were prepared likewise and stored.

3.4.2 Native Polyacrylamide Gel Electrophoresis (Native-PAGE)

For separation and characterization of vitellin from crude ovarian homogenates, vertical native PAGE (Davis, 1964) was employed.

3.4.2.1 Standardization of PAGE

Discontinuous electrophoresis was carried out with a stacking gel of 5%. Resolving gels of 6.5%, 7%, 7.5% and 8% were tried to choose an ideal percentage. Finally 7.5% concentration gave a better separation and this concentration was selected for further studies (Table 1 and 2).

3.4.2.2 Casting of gel

The resolving gel components were mixed gently and poured into the prepared cassette. Few drops of butanol were over-layered to prevent meniscus formation and the gel was left undisturbed to set. After polymerization of the resolving gel, the overlying butanol was removed and the cassette was washed with double distilled water and dried. The prepared stacking gel mixture was then poured over the separating gel. The comb was placed in the stacking gel and allowed to set. After the gel got polymerized, the comb was removed without distorting the shape of the well.

3.4.2.3 Sample application and electrophoresis

Sample was prepared by mixing 10 µl of the ovarian supernatent with 10 µl of the loading buffer. The gel was carefully removed from the gel casting unit and set on the electrophoretic apparatus, with the notched plate facing the upper tank, using screws. Electrode buffer was poured in the tanks. Care was taken to avoid entrapment of air bubbles at the bottom of the gel. The prepared samples were applied to the wells in the stacking gel and were layered with running buffer in order to avoid disturbance of the sample. The electrodes were then connected to the power pack. Electrophoresis was conducted at a constant voltage of 50 V, until the dye front crossed the stacking gel when it was increased to 100V. The entire run was carried out at 4°C till the dye front reached the bottom of the gel.

Table 1. Reagents for electrophoresis

	PAGE		
1.	Stock acrylamide solution		
	Acrylamide	29.100 g	100 ml double
	Bisacrylamide	0.900 g	distilled water (DDW)
2.	Resolving gel buffer (pH 8.8) 1.8 M Tris base	18.2 g	100 ml DDW
3.	Stacking gel buffer (pH 6.8)	6.1 g	100 ml DDW
4.	10% Ammonium persulphate prepared freshly		
5.	TEMED		

Table 2. Composition of native PAGE

7.5% Resolving gel		5% Stacking gel	
Acrylamide stock solution	5.0ml	Acrylamide stock solution	0.83ml
Separating gel buffer	5.0ml	Separating gel buffer	1.25ml
APS	0.2ml	APS	50µl
TEMED	14µl	TEMED	3.5µl
DDW	9.58ml	DDW	2.81ml

Table 3. Details of staining technique adopted in electrophoresis

Tests	Fixation time	Staining time	Destaining solution and duration	Colour of band
Protein: Coomassie Brilliant Blue	10% TCA for 30 minutes	30 minutes in dark	Methanol, water and acetic acid (5:5:1) for 30 minutes	Blue
Polysaccharide: Periodic acid Schiff's reagent	12.5% TCA for 30 minutes	1hour in 1% periodic acid in 3% acetic acid; 1hour wash in double distilled water; 3 hour in Schiff's reagent in dark under refrigeration	1% aqueous metabisulphite followed by repeated wash in 7% acetic acid	Magenta
Lipoprotein: Sudan Black B		2 hours in saturated solution of sudan black B in ethanol	Ethanol for 30 minutes	Brownish black
Calcium: Alizarin red S		20 minutes	Repeated washing in double distilled water; rinse in acid ethanol until background is clear	Deep reddish orange

3.4.2.4 Staining the gels

Native PAGE gels were removed from the plates and subjected to an overnight fixation. This was followed by staining, for 2 hours, in Coomassie Brilliant Blue R-250, to study the general protein profile. The excess stain was washed off and the gels were finally immersed in destainer (Table 3). For characterization of lipovitellin band, the gels were stained for carbohydrate, lipid and calcium moieties, with periodic acid Schiff's reagent, Sudan Black B and Alizarin Red 'S', respectively, as shown in Table 3.

3.4.2.5 Determination of molecular weight

For determination of molecular weight of lipovitellin, native PAGE molecular weight marker (Urease trimer - 272 kDa) was run along with the samples (Bryan, 1977). Relative mobility (Rf) values of the standard markers were calculated by using the formula

Rf = Solute front / Dye front

Using the Rf values calculated for standard markers, a graph was drawn between Rf and $\log_{10}$ of the molecular weights of the standard proteins on a semi-log graph. The Rf values of vitellin band were calculated and extrapolated on the standard graph to determine the molecular weight of vitellin.

3.5 Biochemical Studies

3.5.1 Haemolymph collection and tissue sampling

Samples of haemolymph from prawns of all reproductive stages were analysed for total proteins, lipids, carbohydrates, and carotenoid contents. Gonad, hepatopancreas and muscle tissues from prawns belonging to different

maturity stages were analysed for moisture, protein, lipid, carbohydrate and carotenoid contents. Carotenoids were not estimated in muscle tissues because of the very low levels present in them. Dried and powdered tissue samples were used for the estimation of protein, lipid and carbohydrate, while fresh tissues were used for the estimation of moisture and carotenoid content.

Live females were collected from the tank and blotted dry using a filter paper. Haemolymph samples from individual prawns were drawn by direct cardiac puncture using a hypodermic syringe fitted with a No.22 needle. The syringe used for haemolymph collection was rinsed in modified citrate EDTA buffer (Soderhall and Smith, 1983) prior to each collection. The collected haemolymph samples were frozen and stored in plastic vials at -20°C until analysis. Shrimps were dissected for tissue samples and the ovary, hepatopancreas and muscle tissues were quickly excised out. Tissues were dried to a constant weight at 60°C and then powdered using an agate mortar and pestle. They were then stored in a dessicator with silica gel until analysis. Five replicates were carried out for each estimation.

3.5.2 Biochemical analysis

3.5.2.1 Estimation of moisture content

The moisture content of ovary, hepatopancreas and muscle tissues was determined by keeping pre-weighed wet samples at 60°C in a hot air oven till constant weights were obtained. The loss in weight was taken as the water content and expressed as percentage.

3.5.2.2 Estimation of total proteins

Folin – Ciocalteu phenol method of Lowry *et al.* (1951) was used for total protein estimation using crystalline bovine serum albumin as standard. Pre-

weighed dried tissue or a known aliquot of haemolymph was taken and deproteinized with 10% trichloroacetic acid (TCA). The supernatant obtained was used for estimation of total carbohydrates. The protein precipitate in each tube was dissolved in 5ml of IN NaOH. From this solution, 0.1 ml was taken in triplicate and made upto 0.5 ml with double distilled water. To this, 5 ml of freshly prepared alkaline mixture (50 ml of 2 % Na_2CO_3 in 0.1 N NaOH + 1 ml of 0.5 % $CuSO_4.5H_2O$ in 1% Sodium potassium tartarate) was added and the tube was kept at room temperature for 10 minutes. After 10 minutes, 0.5 ml of Folin - Ciocalteu's reagent (2 N stock solution diluted 1:1) was added and mixed. After 30 minutes, the optical density of the blue colour developed was measured against the reagent blank at 660 nm using a UV-VIS spectrophotometer (Model: GENESYS-10 Thermospectronic, USA).

3.5.2.3 Estimation of total carbohydrates

Total simple sugars; oligosaccharides and polysaccharides were estimated using the phenol - sulphuric acid method of Dubois *et al.* (1956). The supernatant obtained during the protein estimation procedure was used for analysis. From this supernatent, 0.1 ml was made upto 1 ml with saturated solution of benzoic acid in double distilled water and to this solution, 1 ml of 5 % phenol was added. Then, 5 ml of concentrated sulphuric acid was added directly against the liquid surface, to obtain good mixing. After 30 minutes, the stable orange-yellow colour developed was read at 490 nm in UV-VIS spectrophotometer (Model: GENESYS-10 Thermospectronic, USA) along with D - glucose standard and reagent blanks.

3.5.2.4 Estimation of total lipids

The total lipids were quantitatively determined by sulphophosphovanillin method of Barnes and Blackstock (1973). Pre - weighed dried tissue or a known aliquot of haemolymph was homogenized in chloroform: methanol mixture (2:1

V/V) and kept overnight at 4°C for complete extraction. The mixture taken in glass stoppered centrifuge tubes were then centrifuged for 15 minutes at 3000 rpm and the clear supernatent containing all the lipids was transferred to clean, dry glass tubes. 0.5 ml of this lipid extract taken in glass tubes was dried in vacuum over silica gel in a dessicator. To the dried samples, 0.5 ml of concentrated sulphuric acid was added and shaken well. The tubes were then plugged with non - absorbent cotton wool and heated at 100°C in a boiling water bath for 10 minutes. The tubes were collected and cooled rapidly to room temperature under running tap water. To 0.1 ml of this acid digest, 2.5 ml of phosphovanillin reagent was added and mixed well in a cyclomixer. After 30 minutes, the pinkish red colour developed was read at 520 nm in UV-VIS spectrophotometer (Model: GENESYS-10 Thermospectronic, USA) along with cholesterol standard and reagent blanks.

3.5.2.5 Estimation of total carotenoids

The total carotenoid content was estimated by following the extraction procedure of Olson (1979). Pre-weighed fresh tissue or a known aliquot of haemolymph was taken in a clean 10 ml screw cap glass vial and 2.5 g anhydrous sodium sulphate was added. The sample was gently mashed with a glass rod until it was well mixed with sodium sulphate. The caked residue was covered with 5 ml of chloroform and placed at 4°C for 24 hours. An aliquot of the chloroform extract was diluted with ethanol and the absorption was read in UV-VIS spectrophotometer (Model: GENESYS-10 Thermospectronic, USA) at 450 nm.

3.5.2.6 Statistical analysis of data

The data were presented as mean ± standard deviation and analysed by one way ANOVA using the SPSS 7.5 version of WINDOWS and the significance was tested at 5 % and 1 % levels.

RESULTS

4. RESULTS

4.1 Female Reproductive System

Female reproductive system of *Metapenaeus monoceros* was studied based on gross morphological observations of the ovary as viewed through the dorsal exoskeleton, gonadosomatic index and observation of thelycum. Oogenesis, the process of formation of yolky oocytes from oogonial cells through sequential deposition of yolk was studied employing histological preparations and electron microscopy sections. The results are presented below.

4.1.1 Gross morphology

The female reproductive system in *Metapenaeus monoceros* consists of the internal organs - the paired ovaries and oviducts and the external organ, thelycum. The ovaries are bilaterally symmetrical and partly fused along the inner margin and extend the entire length of the animal from the base of the rostrum to the telson (Fig. 1) Ovaries are located dorsal to the hepatopancreas and ventral to the heart in the cephalothoracic region and dorsal to the gut in the abdominal region. Each half of the ovary consists of three main lobes - anterior, middle and posterior. The anterior lobes constitute a pair of elongated structures in the cephalic region and are located close to the esophagus in the cardiac region. The middle lobe, located in the cardiac and thoracic regions consists of seven finger like lobules surrounding the hepatopancreas. A pair of lobes, one from each half of the ovary extending over the entire length of the abdomen constitutes the posterior lobes. Oviducts are a pair of short, narrow tubes originating from the sixth or penultimate lateral lobule of the middle lobe of each ovary and opening through the genital pore at the coxa of the third pair of pereopods.

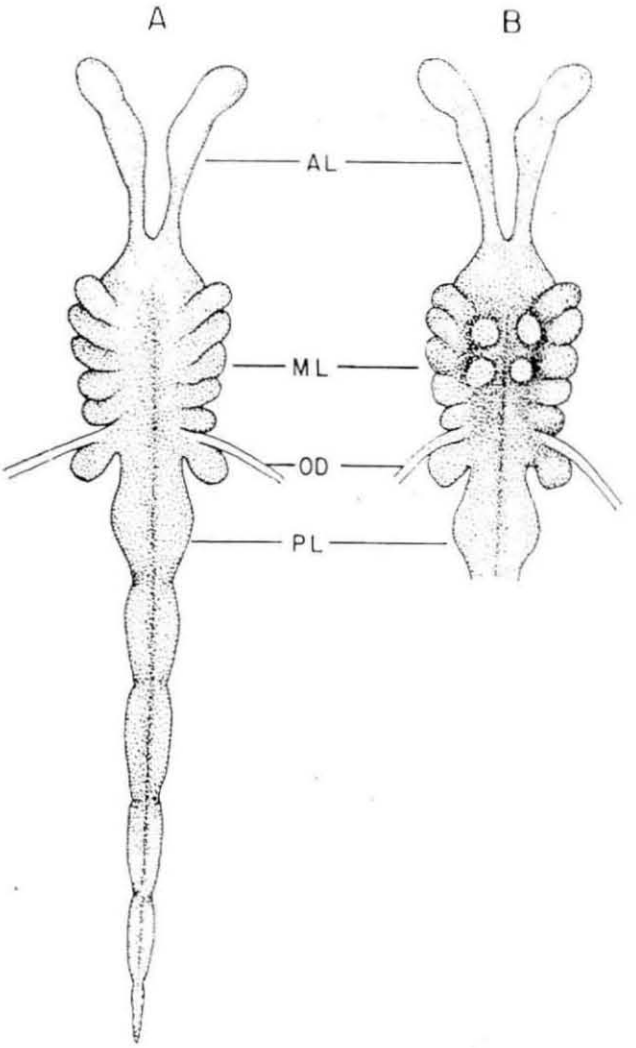

Fig. 1 Structure of female reproductive system of *Metapenaeus monoceros*.
AL - anterior lobe, ML - middle lobe, OD - oviduct, PL - posterior lobe
A. Dorsal view B. Ventral view

The thelycum of *M.monoceros* is closed type. It is located on the ventral side between the fourth and fifth pereopods and serves as the receptacle for spermatophores. Thelycum consists of an anterior and two lateral plates. A cavity in the thelycum behind the lateral plates is for the storage of spermatophores.

4.1.2 Visual assessment of ovarian development

Based on the colour and size of the ovary as observed through the dorsal exoskeleton and further observation upon dissection, the ovary in *M.monoceros* was divided into five stages.

4.1.2.1 Stage I or Immature

Ovary was not visible through dorsal exoskeleton. Upon dissection, it was thinner than gut, strand like, translucent and smooth in texture. Ovary was formed of developing anterior lobes, which were confined to the posterior half of the cephalothorax, and posterior lobes, which were situated on the dorsal aspect of the abdomen, extending up to the middle of the sixth abdominal segment.

4.1.2.2 Stage II or Early maturing

Ovary was visible through the dorsal exoskeleton as a thin strand. Upon dissection, it was observed to be light yellow in colour and granular. The anterior lobes had further extended forward into the cephalothorax, the middle lobes and the rudiments of their lobules developing. The posterior lobes increased in girth and was larger than the gut and minute brown pigments appeared on the dorsal surface of the ovary.

4.1.2.3 Stage III or Late maturing

Ovaries were clearly visible through the dorsal exoskeleton as a dark thick band. Dissected out ovaries appeared light green with branched brownish

chromatophores distributed over the entire dorsal surface with a firm and granular texture. The anterior, middle and posterior lobes were fully formed, but did not fill the cephalothorax completely.

4.1.2.4 Stage IV or Mature

Ovaries were clearly visible through the exoskeleton as a thick band on the entire dorsal side of the animal, with the characteristic diamond shaped expansion of the first abdominal segment. Upon dissection, some fully mature female specimens had dark green ovaries, while a few others had dark brown ovaries. The texture was firm and granular. The ovarian lobes were considerably larger and filled up all the available space in the body cavity, both in the cephalothoracic and abdominal region.

4.1.2.5 Stage V or Spent

Ovaries were not visible through the exoskeleton as in the case of the immature stage. Hence spent stages were differentiated from immature stages based on the size of the specimens. Upon dissection, the ovaries appeared whitish or creamy in colour with a flaccid appearance. Incompletely spawned specimens were also encountered with anterior and middle lobes whitish/creamy and posterior lobes green in colour.

4.1.3 Gonadosomatic index (GSI)

GSI values during the different maturity stages are presented in Table 4. There was an increase in GSI values with the progression of maturity, except from stage IV to stage V when there was a sharp decrease. The GSI values ranged from 0.353 in immature females (Stage I) to 6.98 in mature specimens (Stage IV) showing a 20-fold increase. From the immature (Stage I) to the early maturing stage (Stage II) the GSI increased from 0.353 to 2.15 (a six fold

Table 4. Classification of maturity stages in female *Metapenaeus monoceros* based on colour of the ovary, GSI and OD

Ovary Stage	Colour and appearance of ovary	GSI ± S.D (n=25)	Oocyte diameter µm ±S.D (n = 100)	Nucleus diameter µm ±S.D (n = 100)
Stage I (Immature) Primary oogonial cells Secondary oogonial cells Pre vitellogenic oocytes	Translucent Smooth	0.353±0.21	6.43±1.92 17.64±3.84 35.68±12.36	4.8±1.83 14.23±2.84 21.3±5.28
Stage II (Early maturing)	Light yellow Smooth-granular	2.15±0.83	129.46±18.34	42.64±9.56
Stage III (Late maturing)	Light green Granular	3.98±1.20	174.38±16.8	55.68±9.38
Stage IV (Mature)	Dark green/ Dark brown Granular	6.98±1.52	230.56±15.89	58.39±6.39
Stage V (Spent)	Pale cream Flaccid-Granular	0.894±0.29	21.87±3.86	14.32±2.18

increase), whereas from the second to the third stage the increase was less than two fold, i.e., from 2.15 to 3.98. From Stage III to Stage IV the GSI increased from 3.98 to 6.98 (around 1.75 times). Mean GSI value recorded for the spent stage was 0.894.

4.1.4 Histological and ultrastructural studies on oogenesis

Even though staging of ovarian development based on gross morphology alone can be quite handy for routine hatchery use, it is not very accurate. For the correct staging of ovarian development, a proper understanding of the changes taking place in oocyte cytoplasm and nucleus during the process of ovarian maturation is imperative. Light microscopic examination of the ovaries of at different stages of maturity revealed the chain of nuclear and cytoplasmic changes that occurred inside the developing oocytes. Transmission electron microscopic studies helped to understand the subcellular level changes. Based on the histological and ultrastructural studies, the process of oogenesis was classified into five different phases – pre-vitellogenic, early vitellogenic, late vitellogenic, vitellogenic and spent. These phases correspond to the stages I to V described earlier based on gross morphology.

From histological sections it was evident that the ovarian tissue of *Metapenaeus monoceros* was encompassed by a thin ovarian wall which was composed of three layers – a thin outermost pavement epithelium, an inner layer of germinal epithelium and a relatively thick layer of connective tissue in between (Plate 3). The middle layer of connective tissue was eosinophilic while the two epithelial layers were basophilic in nature. Blood capillaries were observed in the ovarian wall. The germinal zone or the germarium was observed in all sections as a thin band along the innermost layer of the ventral ovarian wall (Plates 2 & 3). It was observed that young oocytes moved farther from the germinal zone upon maturation. Smaller primary oogonial cells were found at the periphery of

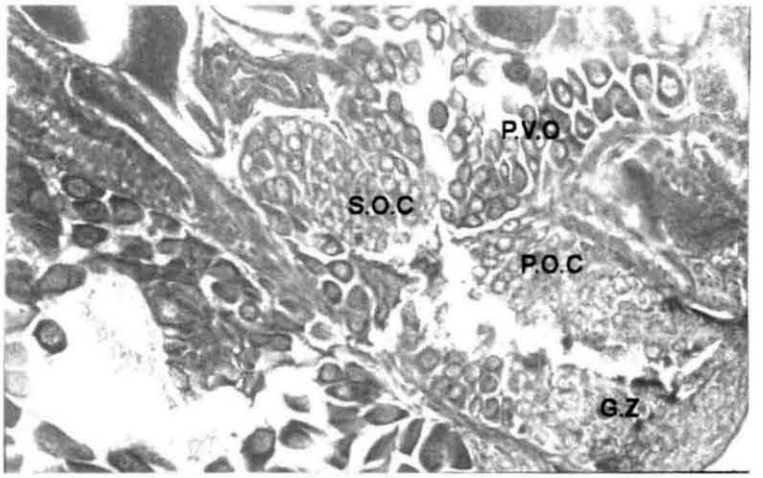

Plate 2. Light micrograph of stage I ovary showing germinal zone (GZ), primary oogonial cells (POC), secondary oogonial cells (SOC) and pre-vitellogenic oocytes (PVO) x 200

Plate 3. Light micrograph of stage II ovary with perinucleolar oocytes (PNO) (GZ-germinal zone, FC-follicle cells and O.W-ovarian wall) x 200

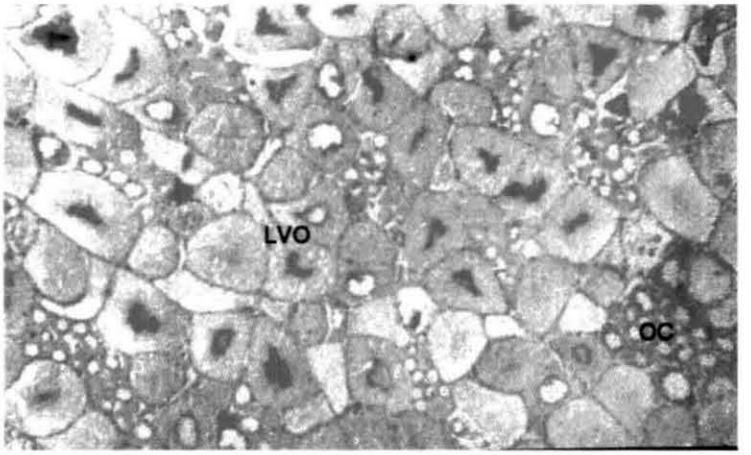

Plate 4. Light micrograph of stage III ovary showing late vitellogenic oocytes (LVO) (OC-oogonial cells) x 100

the germinal zone, while the secondary oogonial cells and pre vitellogenic oocytes towards the centre in a graded manner.

4.1.4.1 Pre-vitellogenic stage (Stage I ovary)

Ovary at the pre-vitellogenic stage was observed to possess three types of cells – primary oogonial cells and secondary oogonial cells arising from the germarium and moving towards the periphery in a graded manner and deeply basophilic pre-vitellogenic oocytes further away from the germinal zone (Plate 2). The primary oogonial cells had a mean diameter of 6.43 µm and a nucleus of 4.8 µm (Table 4). They had pale eosinophilic cytoplasm but the nucleus was basophilic with the nucleoli arranged along the periphery of nuclear membrane (Plates 2 & 3). Secondary oogonial cells were formed by the mitotic division of the primary oogonial cells and had a mean diameter of 17.64 µm with a nucleus of 14.23 µm.

Pre-vitellogenic oocytes are formed by meiotic division of the secondary oogonial cells. These oocytes had a deeply basophilic cytoplasm with a mean diameter of 35.68 µm. The nucleus with a mean diameter of 21.3 µm was lightly stained with haematoxylin. The oocytes at this stage were not surrounded by follicle cells.

4.1.4.2 Early vitellogenic stage (Stage II ovary)

Oocytes in the early vitellogenic stage were characterized by the arrangement of 12-15 basophilic nucleoli as a circular ring along the periphery of the nuclear membrane and was called the perinucleolar oocytes (Plate 3). Perinucleolar oocytes had a mean diameter of 129.46 µm and nuclear diameter of 42.64 µm (Table 4). Cytoplasm of perinucleolar oocytes was eosinophilic and appeared granular due to the presence of vesicular primary yolk. A germinal zone with oogonial cells was apparent at this stage. Folliculogenesis, the

investment of follicle cells around developing oocytes, was also completed during this stage. Basophilic follicle cells were found moving along the periphery of the germinal zone towards the centre of the ovary and encircling individual oocytes (Plate 3).

Electron microscopic observations of the early vitellogenic oocytes also revealed the ring shaped arrangement of nucleolar material along the periphery of nuclear membrane (Plate 10). Nucleus of early vitellogenic oocytes also possessed euchromatic and heterochromatic regions. Cytoplasm of early vitellogenic oocytes was abundant in mitochondria, rough endoplasmic reticulum and free ribosomes (Plate 11).

4.1.4.3 Late vitellogenic stage (Stage III ovary)

Late vitellogenic oocytes were characterized by a rough granular cytoplasm, which was fully eosinophilic (Plates 4 & 5). The mean oocyte diameter at this stage was 174.38 µm with a nuclear diameter of 55.68 µm. The nucleus was basophilic and nuclear material was found to diffuse into the cytoplasm (Plate 5). Owing to the increased diameter of growing oocytes due to accumulation of densely packed yolk platelets, the follicle cells were stretched and flattened.

Ultrastructurally, the late vitellogenic oocytes had the cytoplasm filled with yolk bodies, lipid vacuoles, electron dense bodies and organelles like mitochondria (Plate 12). Cytoplasm was rich in golgi bodies, rough endoplasmic reticulum and free ribosomes (Plate 15). Concentric layers of rough endoplasmic reticulum were abundant at the junction of oolemma and follicle cells. Follicle cells at this stage were flattened with hypertrophied nucleus (Plate 14). Nucleolemma developed pores through which nuclear material diffused freely into cytoplasm (Plate 13).

4.1.4.4 Vitellogenic stage (Stage IV ovary)

Ovary in the vitellogenic stage was filled with vitellogenic oocytes with a mean diameter of 230.56 µm and nuclear diameter of 58.39 µm (Table 4). Fully mature oocytes appeared more elongate than circular with a thin rim of basophilic follicle cells around it (Plates 6 and 7). Follicular detachment from the oolemma, which occurs just prior to ovulation, was observed (Plate 6). Vitellogenic oocytes had a granular and eosinophilic cytoplasm filled with yolk. Nucleus was basophilic and nucleoli were not apparent. Cortical bodies, which are characteristic features of fully mature oocytes of most penaeid species, were absent in the vitellogenic oocytes of *Metapenaeus monoceros* (Plate 7).

Ultrastructurally the cytoplasm was similar to late vitellogenic stage, filled with yolk bodies, yolk platelets and lipid vacuoles (Plate 16). Follicle cells were flattened and the follicle cell nucleus showed hypertrophy with 8-9 nucleoli arranged along the periphery of nucleolemma (Plate 17).

4.1.4.5 Spent stage (Stage V ovary)

Oocytes in spent ovary were similar to those present in pre-vitellogenic stage except for the larger number of resorbing oocytes. Strongly basophilic oogonial cells and pre-vitellogenic oocytes were present (Plate 8). Vacuolated follicle cells were found to encircle the resorbing oocytes (Plate 9).

Electron microscopic observation revealed nuclear condensation. The cytoplasm was mostly devoid of organelles with plenty of vacuoles (Plates 18 & 19).

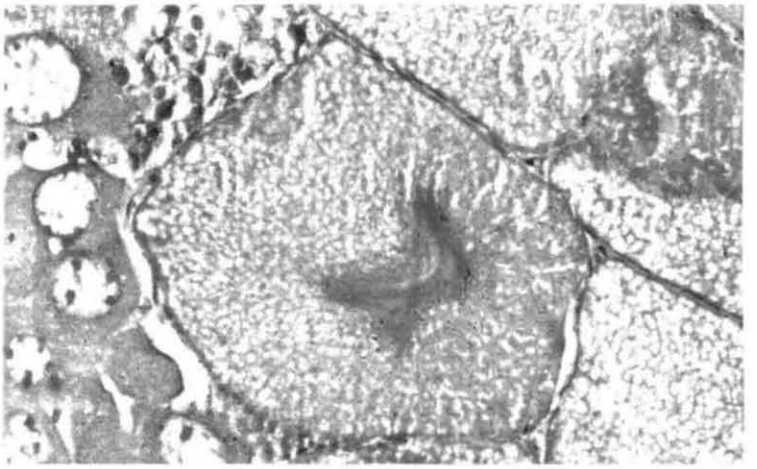

Plate 5. Higher magnification of a late vitellogenic oocyte
Note the diffustion of nuclear material x 400

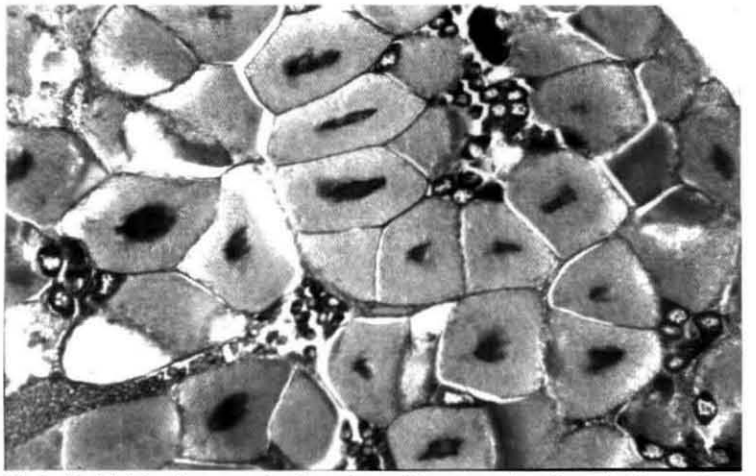

Plate 6. Light micrograph of a stage IV ovary showing vitellogenic oocytes x 100

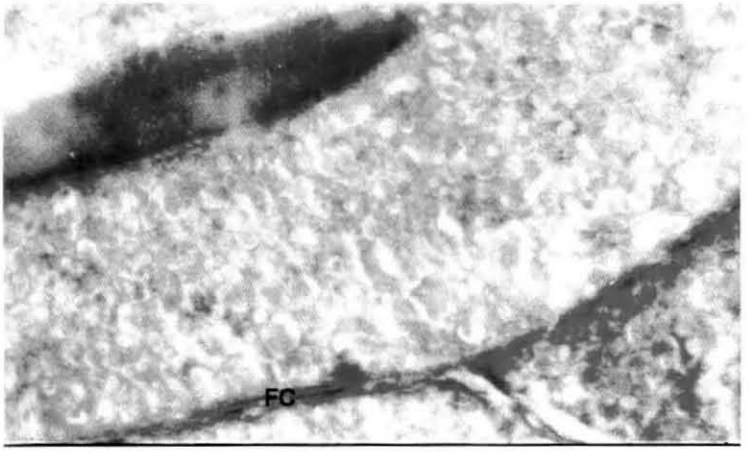

Plate 7. Higher magnification of a vitellogenic oocyte surrounded by flattened follicle cells (FC). Note the absence of cortical rods x 1000

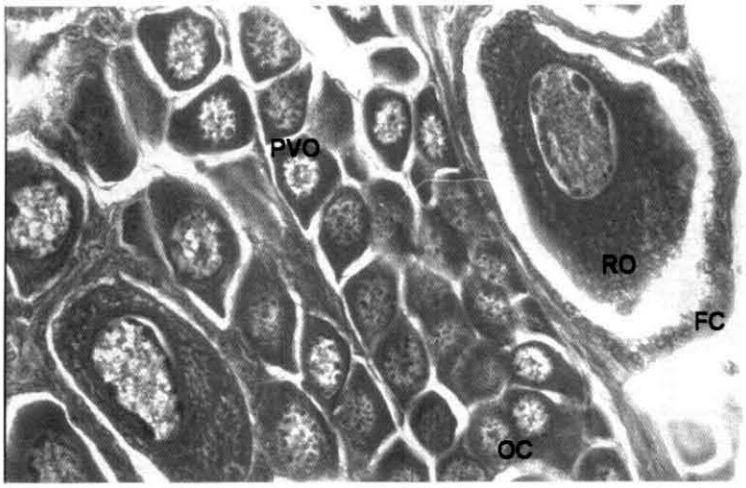

Plate 8. Light micrograph of a stage V ovary showing resorbing oocytes (RO), oogonial cells and pre-vitellogenic oocytes (PVO) (FC-follicle cells) x 400

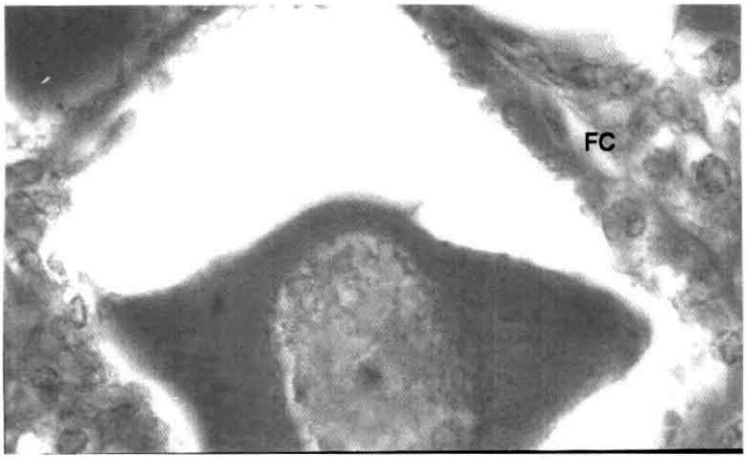

Plate 9. Higher magnification of a resorbing oocyte (RO) surrounded by vacuolated follicle cells (FC) x 1000

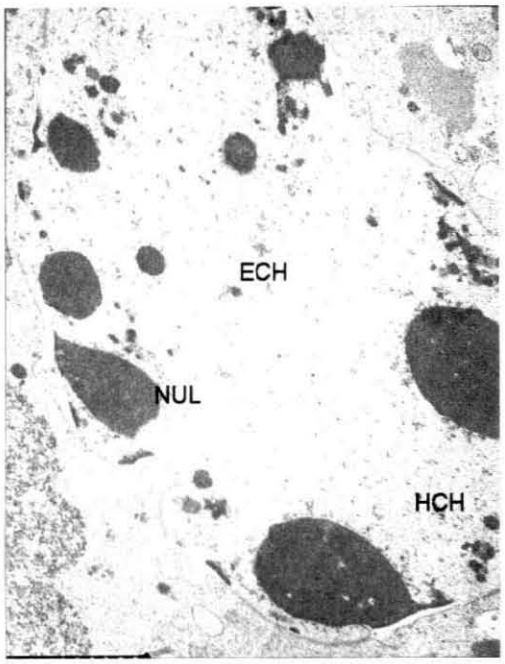

Plate 10. Electron micrograph of nucleoplasm in pre-vitellogenic oocyte showing the presence of nucleoli (NUL) along the peripheral nucleoplasm as well as euchromatic (ECH) and heterochromatic (HCH) regions x 3500

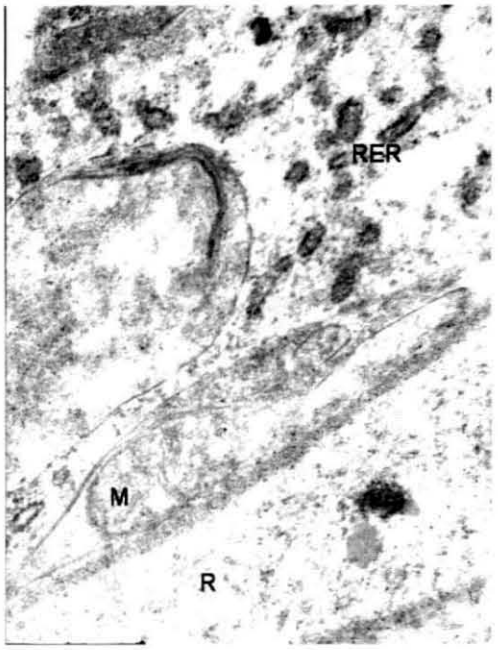

Plate 11. Electron micrograph of the cytoplasm of pre-vitellogenic oocyte with mitochondria (M), rough endoplasmic reticulum (RER) and free ribosomes (R) x 15000

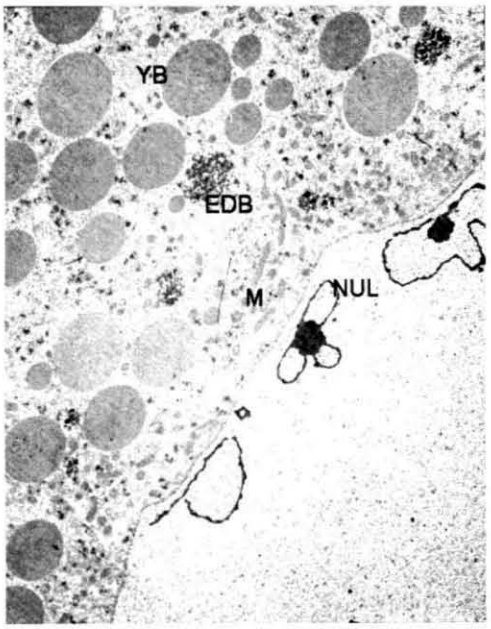

Plate 12. Electron micrograph showing a late vitellogenic oocyte with nucleoli (NUL) arranged along the peripheral nucleoplasm and cytoplasm with yolk bodies (YB), mitochondria (M) and electron dense bodies (EDB) x 4000

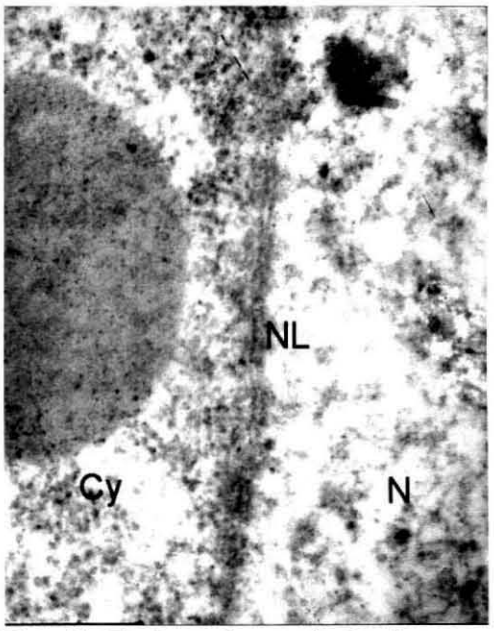

Plate 13. Electron micrograph showing pores (arrows) in the nucleolemma (NL) of a late vitellogenic oocyte with nuclear material diffusing into the cytoplasm (Cy) (N-nucleus) x 25000

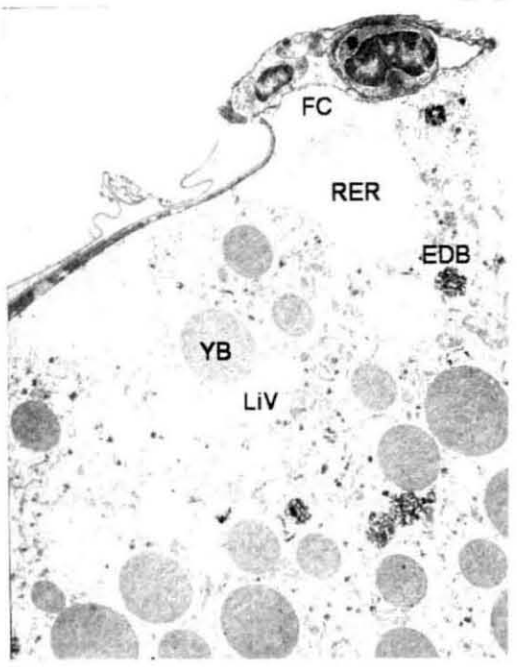

Plate 14. Electron micrograph of late vitellogenic oocyte showing the adjacent follicle cell(FC), cytoplasm filled with yolk bodies (YB), lipid vacuoles (LiV), electron dense bodies (EDB) and concentric layers of rough endoplasmic reticulum (RER) in the peripheral cytoplasm x 4000

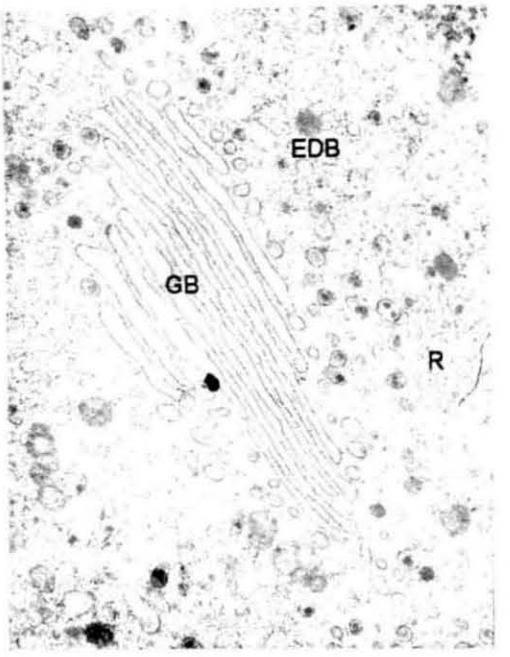

Plate 15. Electron micrograph of a late vitellogenic oocyte having cytoplasm with densly packed free ribosomes (R) and golgi bodies (GB). Also seen are electron dense bodies (EDB) x 6000

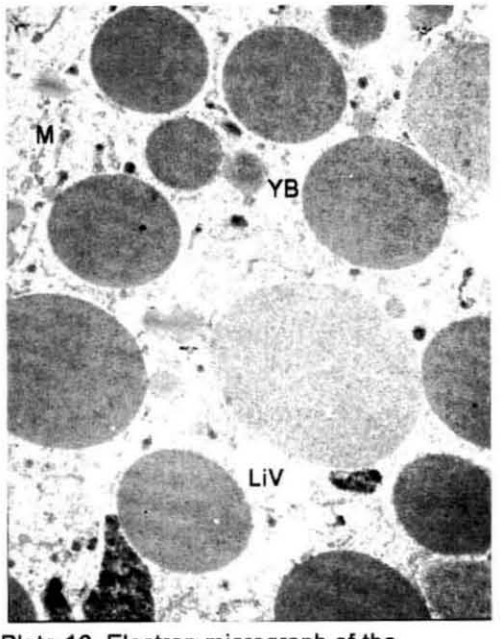

Plate 16. Electron micrograph of the cytoplasm of a vitellogenic oocyte filled with yolk bodies (YB). Also seen are lipid vacuoles (LiV) and mitochondria (M) x 4000

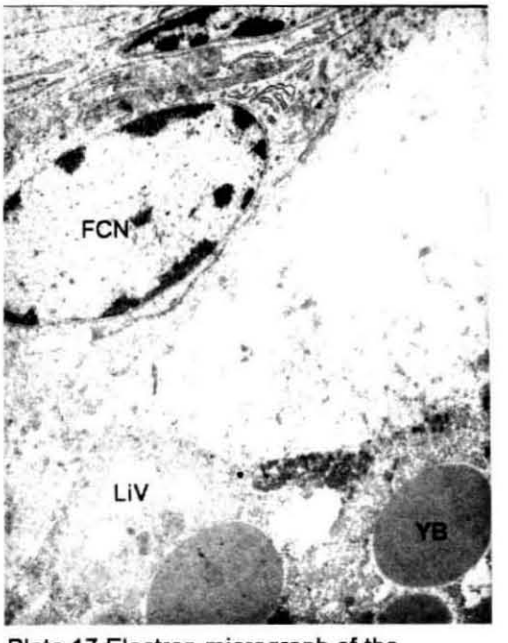

Plate 17 Electron micrograph of the peripheral cytoplasm of a vitellogenic oocyte with adjacent follicle cell (FCN-follicle cell nucleus, LiV-lipid vacuoles, YB-yolk bodies) x 4000

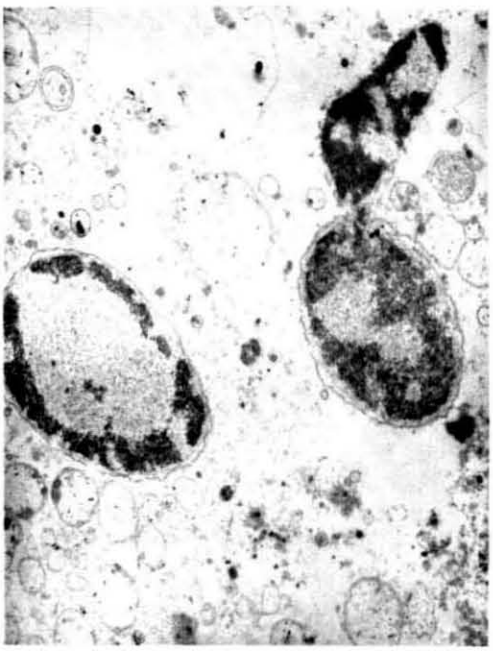

Plate 18. Electron micrograph of a section of spent ovary showing nuclear condensation. Note the cytoplasm devoid of organelles x 3500

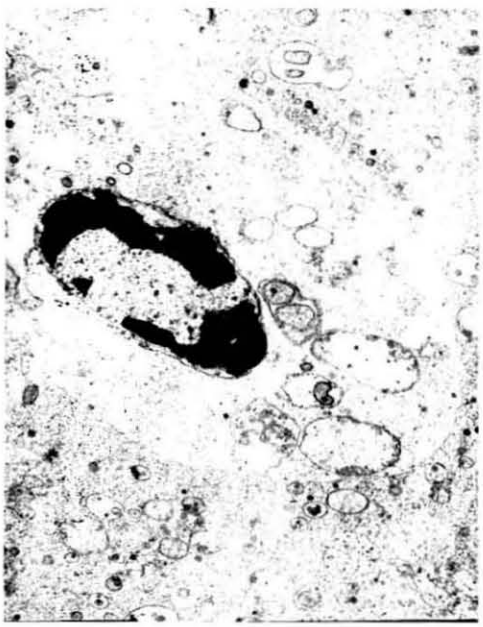

Plate 19.Electron micrograph of a section of spent ovary showing nuclear condensation x 8000

4.2 Male Reproductive System

Male reproductive system of *Metapenaeus monoceros* was studied based on morphological observations, histological preparations and electron microscopy. Gross morphological observations were conducted on the external organs like petasma and appendix masculina. Internal organs like testes, vas deferens and terminal ampoules were dissected out and subjected to detailed examination. The structural organization of testes and vas deferens was studied employing histology and electron microscopy. The process of spermatophore formation was also studied in detail using histology and electron microscopy. The results of the above studies are presented below.

4.2.1 Gross morphology

The male reproductive system of *Metapenaeus monoceros* consisted of both internal and external organs. A pair of testes, paired vas deferens and paired terminal ampoules constituted the internal organs (Fig. 2) while a petasma and a pair of appendix masculina constituted the external organs.

The testis was an unpigmented, translucent organ composed of six or seven lateral lobes located in the cardiac region, dorsal to the hepatopancreas. The testicular lobes were connected to each other at their inner ends and led to the vas deferens.

The paired vas deferens of *Metapenaeus monoceros* originated from the posterior margin of the main axis of the testicular lobes and consisted of four portions. The short and narrow proximal vas deferens (PVD) was located nearer to the testis, the thickened and long medial vas deferens (MVD) at the middle portion and the long and narrow distal vas deferens (DVD) at the farther end of the testicular duct just prior to the terminal ampoule which was a greatly dilated muscular organ found embedded in the coxal muscles of the fifth pereopod. The

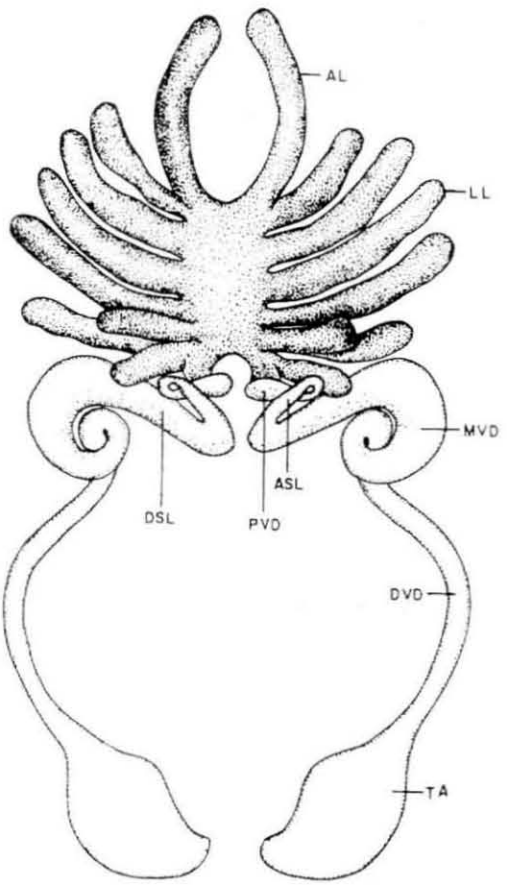

Fig. 2 Structure of male reproductive system of *Metapenaeus monoceros*. AL - anterior lobe, LL - lateral lobe, MVD - median vas deferens, ASL - ascending limb of MVD, DSL - descending limb of MVD, DVD - distal vas deferens, TA - terminal ampoule

MVD in turn consisted of a blind pouch at its junction with PVD, an ascending limb and a descending limb. The fully developed spermatophores or sperm packets were found inside the terminal ampoules, which opened to the outside through the gonopores situated on the coxae of the fifth pereopod.

4.2.2 Maturity stages

Based on gross external morphology, male specimens of *Metapenaeus monoceros* were classified into three stages of maturity, viz. immature, maturing and mature.

4.2.2.1 Immature stage

Testis was a thin, translucent and extremely delicate organ in the cardiac region. Testicular lobes could not be differentiated. Vas deferens appeared as a thin translucent thread like structure and the different components like proximal, medial and distal vas deferens were not differentiated. The terminal ampoule appeared as a thin, delicate membranous bag. The secondary sexual characteristics like the petasma and appendix masculina were not developed.

4.2.2.2 Maturing stage

Testis was much more developed with larger lobes, which could now be differentiated. Vas deferens was thicker and tubular. Eventhough different parts of the vas deferens could be distinguished, they were not well defined. The terminal ampoule increased in thickness. The entire vas deferens including the terminal ampoule was translucent without any spermatophore. The endopods of the first pair of pleopods were modified and partially united to form the petasma. Appendix masculina appeared as small buds at the base of the second pair of pleopods.

4.2.2.3 Mature stage

All the internal and external reproductive organs were well developed. Fully developed testes appeared milky white in colour. Testicular lobes were well separated and distinguishable. Components of vas deferens could be well differentiated. Terminal ampoule was a thick, muscular, membranous bag and appeared white due to the presence of fully developed spermatophores inside. The endopods of the first pair of pleopods were fully linked by a series of minute hook-like structures, forming the petasma. Appendix masculina was fully developed.

4.2.3 Anatomy of the reproductive system

The structural organization of the various male reproductive organs - testes, proximal vas deferens, medial vas deferens (ascending and descending limbs), distal vas deferens and terminal ampoule was studied using histological and electron microscopic sections.

4.2.3.1 Testis

The entire testis was covered by a thin wall of outer epithelium. Histological studies of testis at different maturity stages revealed that each testicular lobe was composed of innumerable testicular acinii held together by connective tissue (Plates 20 & 21). In immature animals the testicular acinii were completely empty. The acinar wall was found thicker in immature animals and contained only a small germinal zone with non-differentiated germ cells. In cross sections of testicular lobes of maturing animals, the germinal zone was shifted to the periphery and contained spermatogonial cells (Plates 20 & 21). Spermatocytes were encountered towards the centre of the acinii and dividing stages of spermatocytes were noticed. Later development stages of spermatogonia were found towards the centre of acinii whereas lesser stages

along periphery. In fully mature males, the germinal zone was very much restricted. Different acinii were fully occupied with cells of a particular type like spermatids and spermatozoa (Plates 22 & 23).

4.2.3.2 Vas deferens

From the histological sections of vas deferens of *Metapenaeus monoceros* it was evident that in immature animals it was a simple tube throughout its entire length. But in maturing and mature animals there was a lot of structural modification. Hence histological investigations were made along different regions of the sperm duct from animals at different stages of maturity. Since different morphological divisions of the tract were clearly observed in maturing and mature animals, sections from such animals were used for identification of different parts. The different regions of the vas deferens like proximal vas deferens (PVD), medial vas deferens (MVD, both ascending and descending limbs) and distal vas deferens (DVD) were subjected to detailed studies to understand their structure.

4.2.3.3 Proximal vas deferens (PVD)

The proximal vas deferens was a short, slender tube leaving the testis. In immature animals it was a simple tube consisting of an outermost connective tissue sheath and inner circular muscle layer with a few blood vessels in between. However in mature animals this duct showed structural modifications. PVD was identified as the site of completion of spermatogenesis, where spermatozoa mix with seminal fluid and are packed as spermatophores. In immature and maturing animals the tube was shorter than in mature ones and the lumen was found empty.

In fully mature animals, PVD was made up of an outer connective tissue sheath, a layer of circular muscle fibres and an innermost layer of basophilic low columnar epithelial cells (Plates 24 & 25). Mature spermatozoa were found

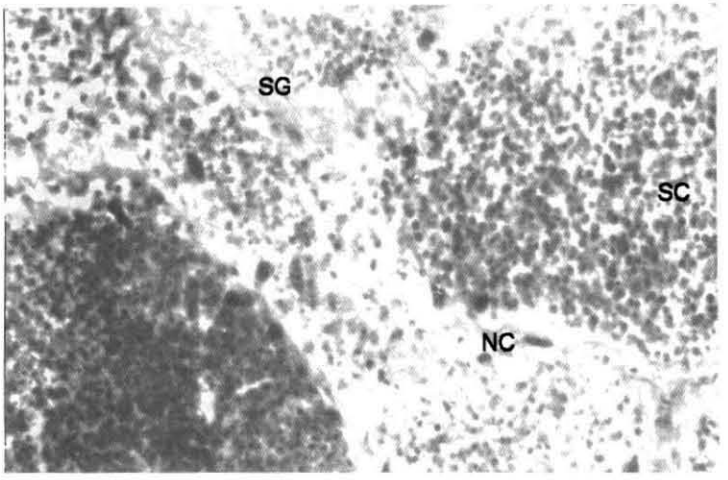

Plate 20. Light micrograph of a maturing testis with spermatogonia (SG) in germinal zone, spermatocytes (SC) and nurse cells (NC) x 400

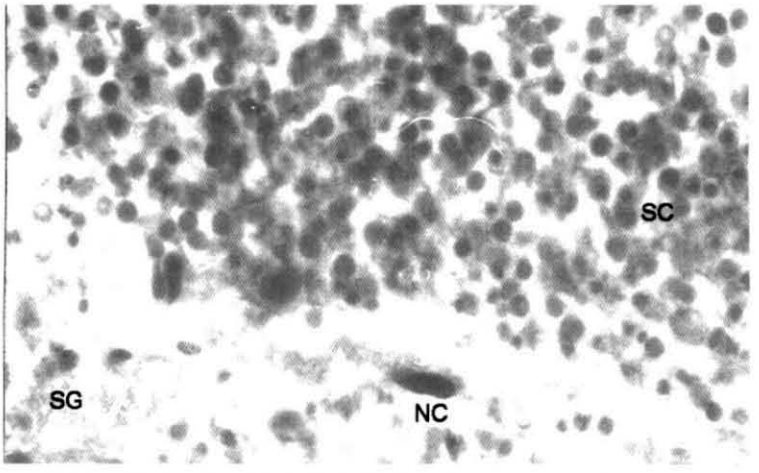

Plate 21. Higher magnification of a maturing testis showing diakinetic stages of spermatocytes (SC) (SG-spermatogonia, NC-nurse cells) x 1000

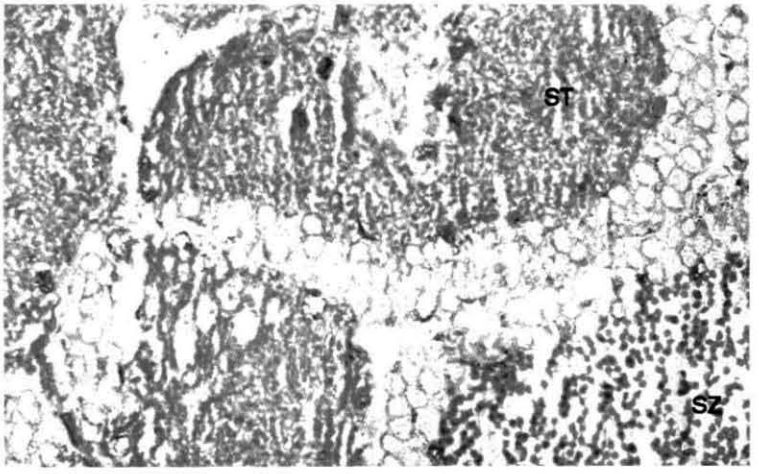

Plate 22. Light micrograph of a mature testis showing spermatids (ST) and spermatozoa (SZ) x 400

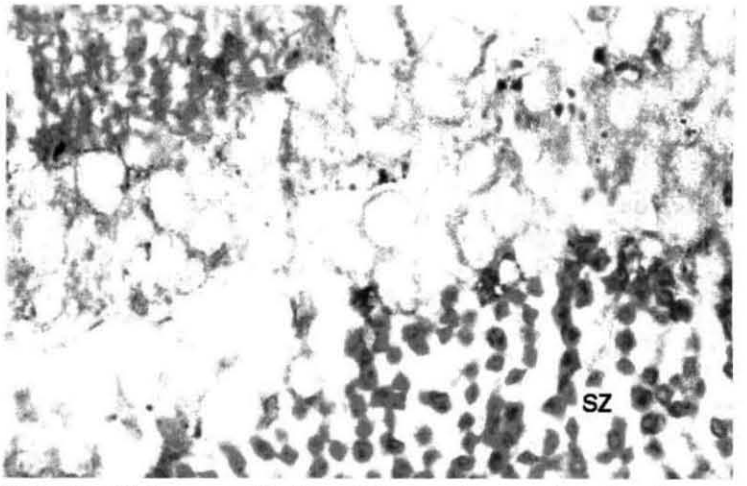

Plate 23. Higher magnification of the previous section showing spermatozoa (SZ) with main body and spike x 1000

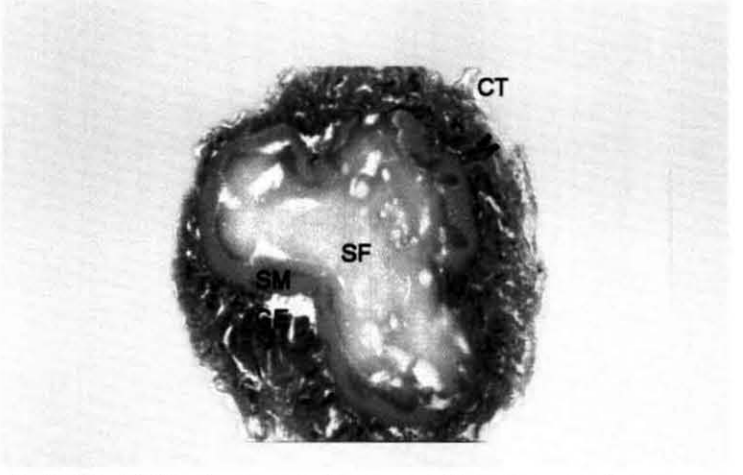

Plate 24. Light micrograph of cross section of proximal vas deferens (CT-connective tissue, M-musIce layer, GE-glandular epithelial cells, SM-sperm mass, SF-seminal fluid) x 200

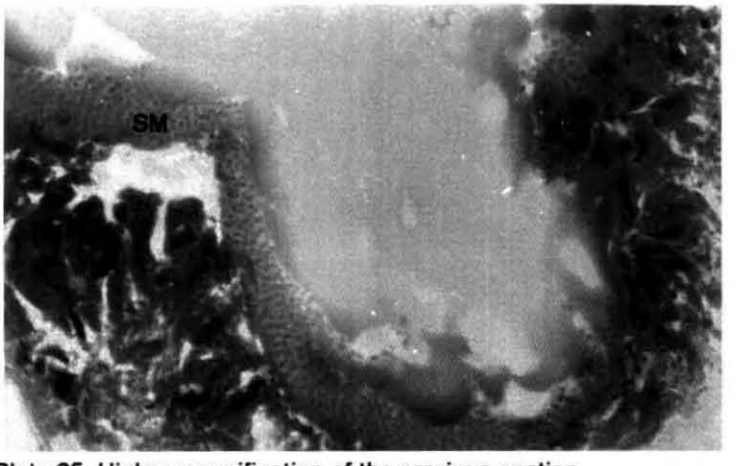

Plate 25. Higher magnification of the previous section (GE-Glandular epithelial cells, SM-sperm mass) x 400

packed in an amorphous matrix. Spermatozoa were basophilic and the supporting matrix eosinophilic (Plate 25). Spermatozoa in the supporting matrix were found bathed in seminal fluid.

4.2.3.4 Medial vas deferens (MVD)

The proximal vas deferens is dilated towards the posterior to form the medial vas deferens. In immature animals the sperm duct was a straight tube and MVD formed as the middle portion of this tube. The MVD at this stage was made up of an outer connective tissue sheath and an inner layer of circular muscle fibres. In immature animals the MVD had only one lumen, which was empty. In maturing and mature animals MVD was comprised of three distinct parts, viz. blind pouch, ascending limb and descending limb. The blind pouch was the junction of PVD and MVD and it dilated into the wider ascending limb of MVD.

The ascending limb of MVD in maturing and mature animals was structurally and functionally different from immature ones. It was made up of an outermost connective tissue sheath and an inner layer of muscle fibres (Plates 26 & 27). The innermost layers, which lined the lumen, were made up of columnar epithelial cells. The ascending limb was divided into two unequal compartments – the larger spermatophoric duct and the much smaller accessory duct. The two ducts were separated by an intermittent septum, which was made up of a narrow layer of connective tissue with muscle fibres and blood vessels (Fig. 26, 28 and 30) and lined on both sides by epithelial cells. The spermatophoric duct was in continuation with the blind pouch and the sperm mass passes through it. Spermatophoric duct deposited the different spermatophoric layers around the sperm mass during its forward movement towards the terminal ampoule whereas accessory duct secreted the accessory structures (wing) of spermatophore. The compartmentalization of MVD into spermatophoric and accessory ducts was often found incomplete in maturing specimens

Walls of the wider spermatophoric duct and the much smaller accessory duct were lined with glandular columnar epithelial cells in a state of supreme activity. The spermatophoric duct was found to have two large typhlosoles (Plates 26, 27 & 29), which were nothing but massive growth of glandular epithelial cells projecting into the lumen to increase the surface area to compensate for the greater secretory demand. These typhlosoles were supported by connective tissue, strengthened by muscle fibres and nourished by blood vessels. They were found in a state of intense secretory activity discharging amorphous secretions into the lumen, which formed discrete layers around the spermatozoa. The narrow accessory duct also had two typhlosoles which were considerably smaller in size compared with their counterparts in the larger duct (Plates 28 & 30). They were involved in secretion of materials, which constituted the accessory substances associated with spermatophore. The sperm mass covered with the discrete layers formed of cellular secretions, moved towards the descending limb of MVD.

The descending limb of MVD also had the same internal anatomy as the ascending limb. The presence of the septum which divided the lumen into spermatophoric and wing ducts after remaining for some distance gradually disappeared uniting the sperm mass and accessory structures (Plate 31), which entered together into the lumen of distal vas deferens. Entire lumen was found packed with amorphous secretions of the epithelial cells. Sperm mass was found covered with various layers.

Ultrastructural studies of MVD showed that the plasma membranes of adjacent cells showed extensive interdigitations, a mechanism to increase the cell surface area to compensate for the greater secretary activity. Mitochondria and vesicles were present. Apex of the epithelial cells had numerous microvilli (Plate 39).

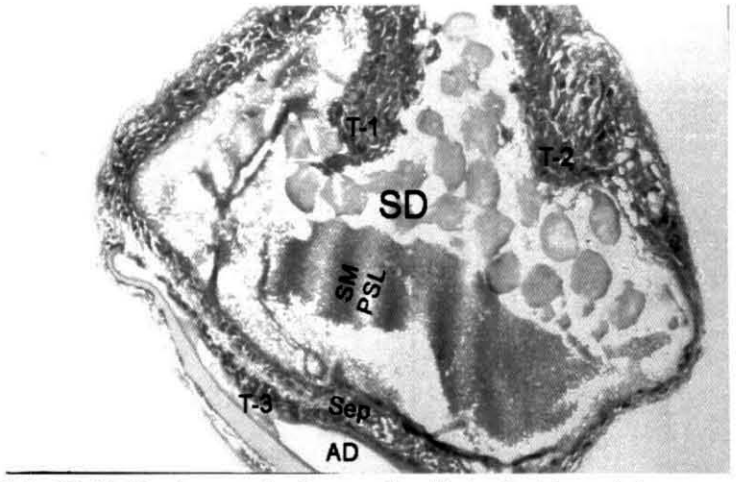

Plate 26. Light micrograph of ascending limb of mid vas deferens showing sperm duct (SD) and accessory duct (AD) with septum (Sep) in between. Typhlosoles in sperm duct (T-1 and T-2) are in active secretion. Primary spermatophore layer (PSL) around sperm mass (SM) (T-3 - ryphlosole in wing duct) x 50

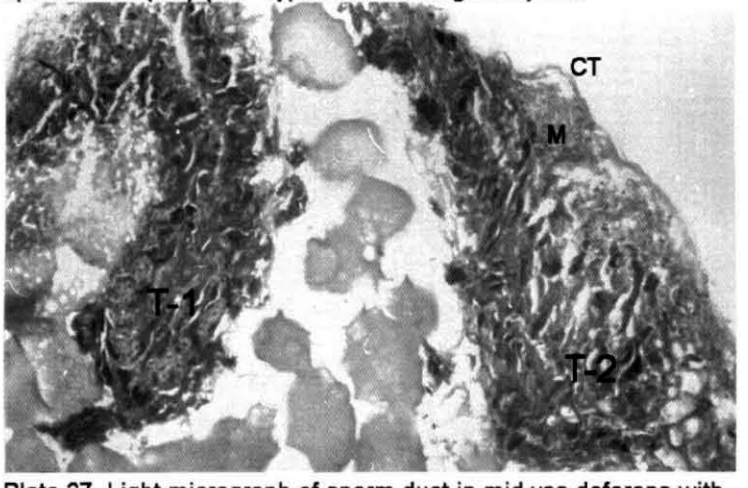

Plate 27. Light micrograph of sperm duct in mid vas deferens with typhlosoles, T-1 and T-2 in active secretion (CT-connective tissue, M-muscle layer) x 200

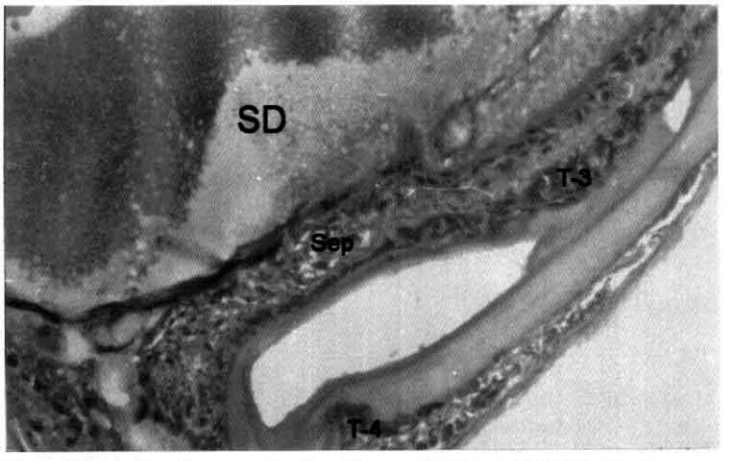

Plate 28. Light micrograph of mid vas deferens showing two typhlosoles (T-3 and T-4) in wing duct and septum (Sep) separating sperm duct (SD) and accessory duct (AD) x 200

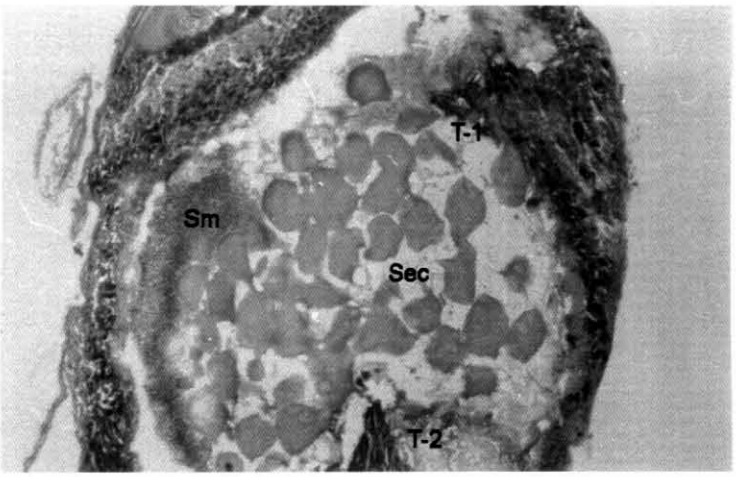

Plate 29. Light micrograph of distal part of ascending MVD showing sperm duct with Typhlosoles (T-1 and T-2) in active secretion (Sec-secretions of T-1 and T-2, Sm-sperm mass) x 100

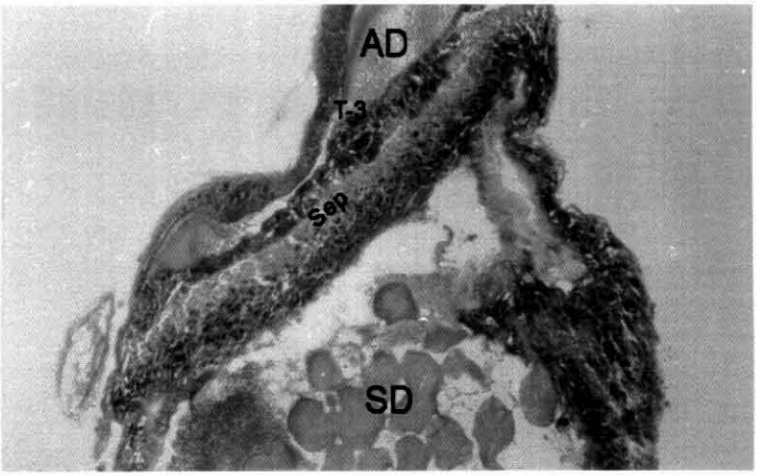

Plate 30. Light micrograph of distal part of ascending MVD showing sperm duct (SD) and acessory duct (AD) with intermediate septum (Sep) (T-3 -Typhlosole in WD) x 100.

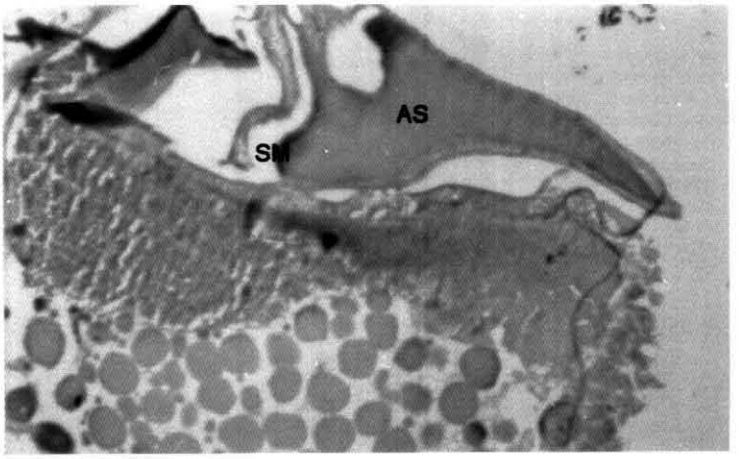

Plate 31. Light micrograph of descending limb of MVD showing the fully formed acessory substance (AS). Note the disappearance of the septum between SD and WD (SM- sperm mass) x 100

4.2.3.5 Distal vas deferens (DVD)

Distal vas deferens was a long slender tube, which connected the MVD with the terminal ampoule. Transverse sections showed that it had the same basic structure of the PVD and MVD with an outer connective tissue layer, and inner layer of muscle fibres. Tall basophilic columnar epithelial cells lined the inner lumen of the duct, which was engaged in active secretion of amorphous secretions (Plate 32). These secretions were found deposited over the sperm mass. Unlike the MVD, no typhlosoles were found in the DVD of *Metapenaeus monoceros*. The distal vas deferens ended in the pear shaped muscular organ, viz. the terminal ampoule.

4.2.3.6 Terminal ampoule

The terminal ampoule or ejaculatory duct as the name indicated was a muscular organ forming the terminal portion of the sperm duct. It was found embedded in the coxal muscles of the fifth pereopod. In immature animals it was not well developed. Cross sections of the terminal ampoule showed that it was composed of an outer thin layer of connective tissue and an inner thick layer of circular muscle fibres. Glandular epithelial cells were not found lining the lumen of the terminal ampoule of immature animals and the lumen was found empty. In maturing animals the size increased considerably due to increased size of the muscle layers but the epithelial lining was not well developed and the lumen was found empty.

Terminal ampoule of mature animals had an outer connective tissue layer (Plates 34 & 35). A layer of circular and longitudinal muscle fibres was seen inner to the outermost layer. The lumen of the terminal ampoule was not continuous but was divided into different chambers (Plates 34 & 35). Each chamber was lined by basophilic glandular epithelial cells, which showed intense secretory

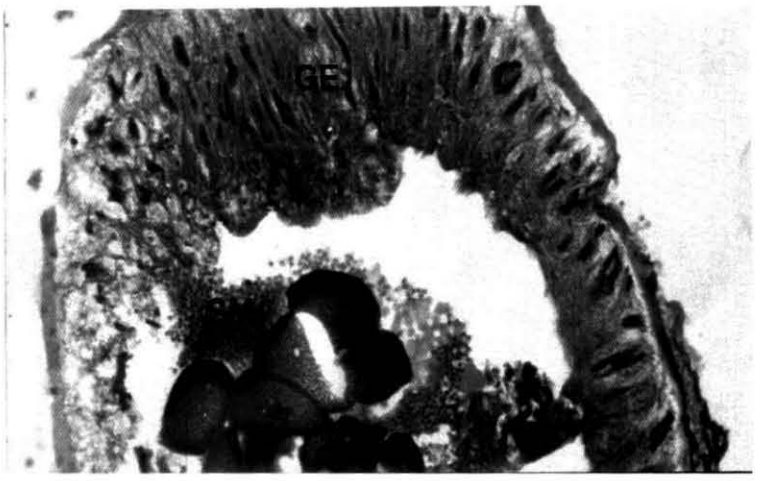

Plate 32. Light micrograph of cross section of the distal vas deferens with lumen containing sperm mass (SM) (GE-Glandular epithelial cells) x 200

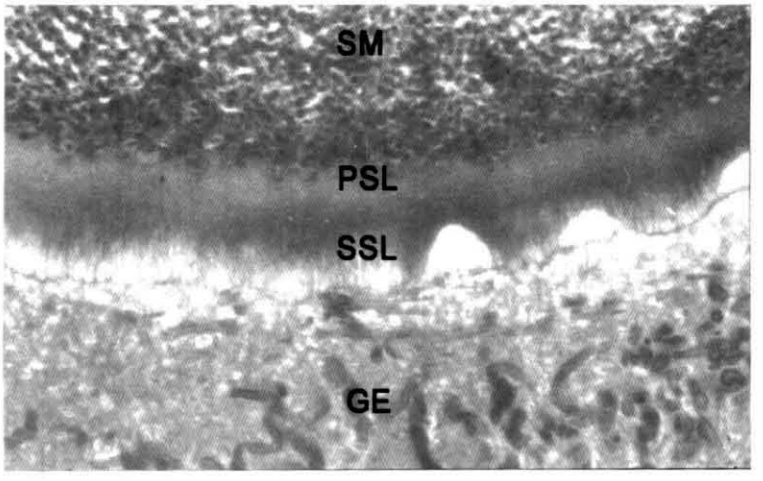

Plate 33. Light micrograph of higher magnification of DVD showing sperm mass (SM) in the lumen with primary spermatophore layer (PSL) and secondary spermatophore layer (SSL) (GE-glandular epithelial cells) x 400

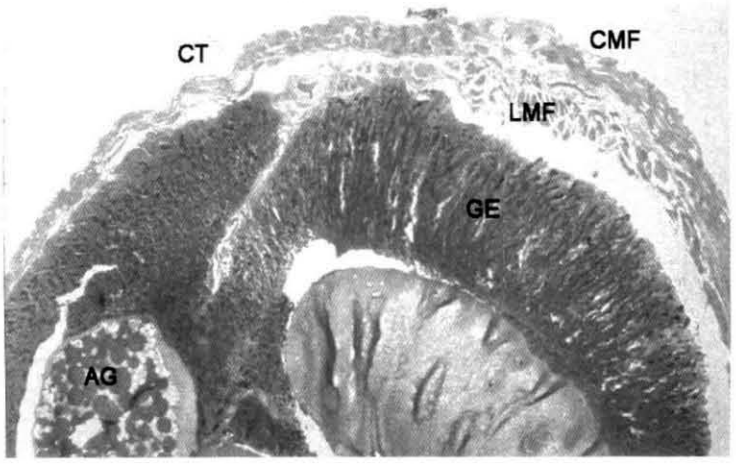

Plate 34. Light micrograph of terminal ampoule showing connective tissue (CT), longitudinal and circular muscle fibres (LMF &CMF), glandular epithelial cells (GE) and adhesive globules (AG) x 50

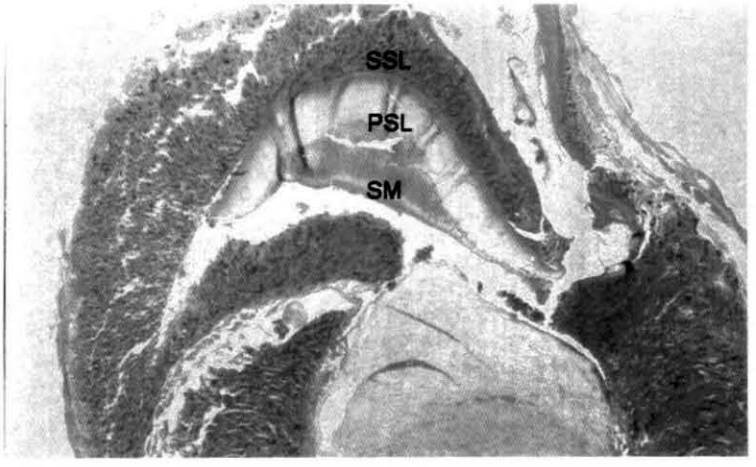

Plate 35. Light micrograph of terminal ampoule showing sperm mass (SM) surrounded by primary and secondary spermatophore layers (PSL & SSL) x 50

activity. Muscle fibers were often seen in between the layers of glandular epithelial cells (Plate 35).

4.2.4 Spermatogenesis

Formation of mature spermatozoa from germinal cells in the lumen of testicular acinii is called spermatogenesis. In histological sections of testicular lobes of *Metapenaeus monoceros* a germinal zone was apparent at one side of the acinar wall (Plates 20 & 21). Germinal zone contained spermatogonial cells and nurse cells. Spermatogenesis always progressed from the periphery of acinii to the centre and therefore subsequent developmental stages were found towards the centre in a graded manner. Spermatogonia passed through a period of quick growth to become primary spermatocytes, which underwent reduction division to form two secondary spermatocytes. The two spermatocytes divided mitotically to form four spermatids from each primary spermatocyte. Spermatid modified to spermatozoa without further division (Plates 22 & 23).

Spermatogonial cells had a round vesicular nucleus with diffused chromatin. Cell boundaries were not clearly demarcated. Nurse cells were found dispersed in between spermatogonia (Plates 20 & 21). These were elongate cells measuring 9-10 µm in length and 3-4 µm in width. By virtue of their close association with gonial cells, they are assumed to have a nutritive and supportive role. The cell boundaries of spermatocytes, spermatids and spermatozoa were distinct. Spermatocytes had a prominent basophilic nucleus and a thin rim of eosinophilic cytoplasm around it. These spermatocytes were usually the dividing cells in the testis and diakinetic stages characteristic of dividing cells were frequently observed among them (Plate 21). Electronmicrographs of testicular acinii showed actively dividing cells (Plates 36 & 37). Spermatids were smaller than spermatocytes with condensation of chromatin matter. The spermatozoa developed from these cells through cellular differentiation (Plate 22). In histological sections spermatozoa appeared almost circular in outline with

basophilic condensed chromatin matter. A 'Y' shaped acrosome vesicle was apparent at the apical region but the spike was not very clear (Plate 23).

4.2.5 Ultrastructure of spermatozoa

Fully mature spermatozoan of *Metapenaeus monoceros* consisted of a spherical main body and a spike (unistellate spermatozoan) (Plate 44). The main body was made up of the central nuclear region, a peripheral cytoplasmic band and an acrosomal cap, which overlaid the nuclear region anteriorly. From the acrosomal cap projected the extended spike towards the anterior of the spermatozoan. The spike and the acrosomal cap are together called the acrosomal vesicle. The average length of the spermatozoa was 6 µm. The main body had a mean diameter of 3.4 µm with the spike measuring 2.6 µm in length.

The acrosomal structure was complex, consisting of the membrane bound acrosomal vesicle and the subacrosomal substance (Plates 44 & 45). The acrosomal vesicle was made up of two distinct elements surrounded by a continuous membrane: the acrosomal cap, which overlaid the anterior surface of the sperm cell and the spike, which projected anteriorly. Over the outer surface of the acrosomal vesicle, the sperm plasma membrane and the acrosomal membrane were closely joined, so that both the spike and convex side of the acrosomal cap were surrounded by a double membrane. The spike was composed of a limiting membrane and the internal spike material. The spike material was found more electron dense than the constituents of the acrosomal cap (Plates 44 & 45). Beneath the inner surface of the acrosomal vesicle was a homogeneous, electron-lucent subacrosomal substance separating the acrosome from the nuclear region.

The central region of the sperm body was occupied by the nucleoplasm, which was not surrounded by a membrane (Plate 46). The nuclear material was in direct contact with the cell membrane as a discrete nuclear membrane was not

present. Inside the nucleus, chromatin formed a network of fibrillar material. The nucleus was outlined postero-laerally by the cytoplasmic band, which extended up to the edge of the acrosomal cap.

4.2.6 Mechanism of spermatophore formation

The mechanism of spermatophore formation in *Metapenenaeus monoceros* was studied in detail employing light and electron microscopic sections of vas deferens, which revealed that each part of the highly modified testicular duct had specific roles in the process. Serial sections of the testicular duct taken at different parts showed that spermatozoa produced by the testicular lobes were modified by secretions of columnar epithelial cells lining the duct converting them into fully mature spermatophore by the time they reached the distal part of the duct, the terminal ampoule.

Histological sections of the proximal vas deferens showed that the basophilic epithelial cells lining the lumen of this duct was in active secretion, producing seminal fluid in which the spermatozoa were bathed (Plates 24 & 25). Spermatozoa were found packed in an amorphous sperm matrix, which was stained with eosin (Plate 25). Inside the proximal vas deferens spermatozoa were converted into a coherent mass before they were transported to the medial vas deferens. Ultrastructural studies showed that PVD is the site of completion of spermatogenesis as it received the sperm cells, which were individually released from the testes. The glandular epithelial cells lining the lumen of the duct had numerous microvilli. The epithelial cells showed intense secretary activity with numerous mitochondria and free ribosomes in the cytoplasm (Plate 38).

Proximal vas deferens joined the medial vas deferens through the blind pouch where the sperm mass was packed more densely. Immediately next to the blind pouch was the ascending limb of MVD followed by the descending limb. In the ascending limb of MVD the lumen was divided into a spermatophoric duct

and an accessory duct by a septum of connective tissue lined on either side by a single layer of epithelial cells (Plates 26 & 28). The glandular epithelial cells in the spermatophoric duct and the two large typhlosoles were in active secretion (Plates 26 & 29). These secretions in the sperm duct formed the primary spermatophore layer (PSL) (Plate 26).

Accessory duct, which was the smaller of the two ducts, also had epithelial cells lining them. The two typhlosoles in the accessory duct were considerably smaller but were in active secretion (Plate 28). These secretions formed two discrete layers in this duct. Towards the distal part of the ascending limb, the accessory duct was found to contain two layers – a predominant layer made up of granular material, which occupied most of the duct and a homogenous layer (Plate 30). In the distal portion of the ascending limb the typhlosoles in the sperm duct were in active secretion of an amorphous material that surrounded the sperm mass (Plate 29).

Serial sections of the descending of MVD showed that the septum that separated the lumen into wing duct had regressed aiding the joining of the contents of the two ducts (Plate 31). A fully formed accessory structure was joined to the sperm mass and was surrounded by common layers around them. Epithelial cells still continued secretion of amorphous material around the sperm mass.

Electron microscopic studies of the medial vas deferens showed that the columnar epithelial cells were supported on a basal lamina made of connective tissue supported on muscle layers (Plate 39). Plasma membranes of adjacent cells showed extensive interdigitations, mitochondria and vesicles. Moderately large inclusions were also seen. Apex of epithelial cells showed microvilli. The lumen of accessory duct was found to contain the granular secretary products that were observed in histological sections (Plate 40).

Histological sections of the distal vas deferens revealed that its lumen was lined with columnar epithelial cells but typhlosoles were absent in this part of the duct. The partitioning of the lumen into two compartments was not to be seen (Plate 32). Epithelial cells continued their secretions around the sperm mass. The sperm mass in the DVD was covered by a primary spermatophore layer (PSL) and for the first time a secondary spermatophore layer (SSL) was seen over the former (Plate 33).

Terminal ampoule in *Metapenaeus monoceros* was a highly muscular chamber, which was found at the distal end of the sperm duct. It had only a single lumen that was divided into four chambers. Highly secretory glandular epithelial cells lined all the chambers (Plates 34 & 35). One of the chambers had sperm mass surrounded by the primary and secondary spermatophore layers. In another chamber an eosinophilic matrix secreted by the glandular epithelial cells was present. The secretion of the glandular epithelial cells of the terminal ampoule also aided in the formation of adhesive globules required to cement and join the parts of the spermatophores ejaculated from the terminal ampoule on either side. Terminal ampoule was a highly muscular organ having a thick layer of outer circular and inner longitudinal muscle fibres whose contraction resulted in the ejaculation of spermatophores. The acellular matrix helped in the final molding of the spermatophores. No spermatophoric layers were found secreted by the terminal ampoule. Ultrastructural investigations showed that epithelial cells of the terminal ampoule were highly secretory in nature. Cytoplasm of the epithelial cells had numerous free ribosomes and rough endoplasmic reticulum (Plates 41 & 42). Vesicles were found discharged into the lumen. Concentric arrangement of rough endoplasmic reticulum in the cytoplasm was seen and many electron dense bodies were found in the cytoplasm (Plate 43).

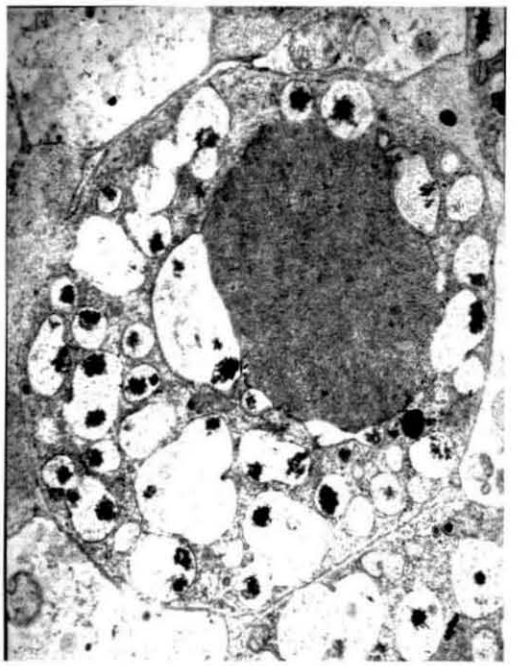

Plate 36. Electron micrograph of testicular acinus in a maturing testis showing cells in active division x 3500

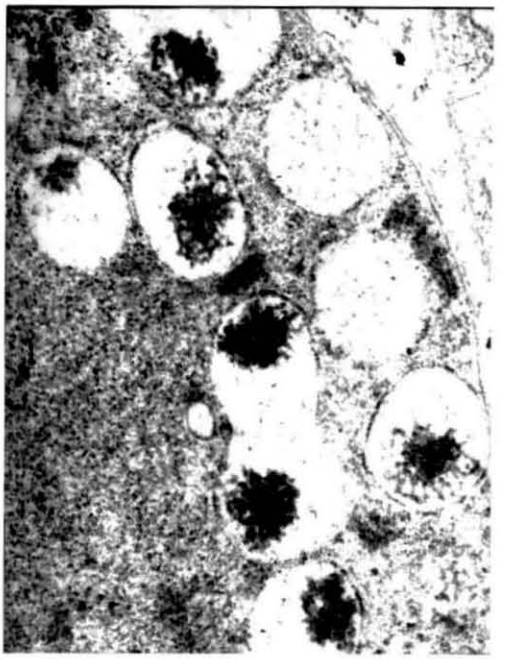

Plate 37. Higher magnification of the previous section x 25000

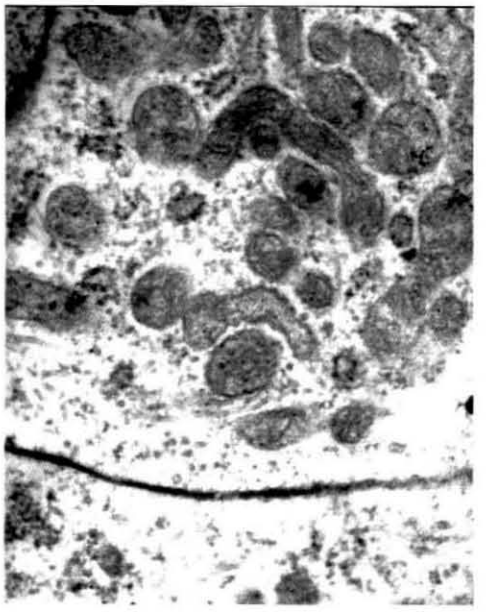

Plate 38. Electron micrograph of epithelial cells lining the proximal vas deferens showing secretory activity with numerous mitochondria and free ribosomes x 40000

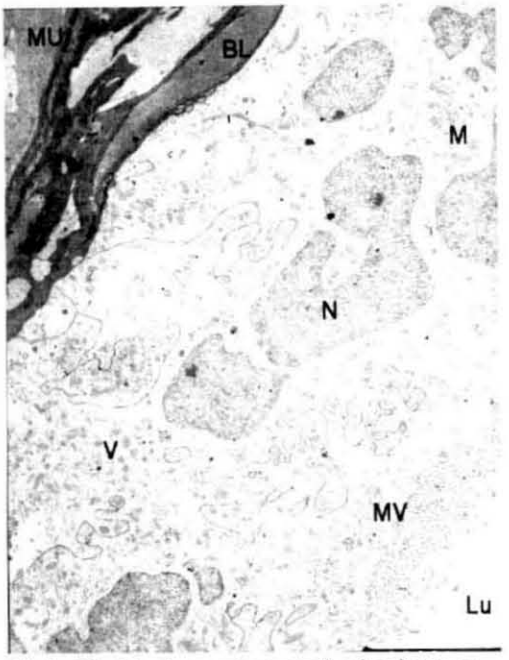

Plate 39. Electron micrograph of columnar epithelial cells of mid vas deferens. Plasma membranes of adjacent cells show extensive interdigitations, mitochondria(M) and vesicles(V). Apex of epithelial cells show microvilli(MV), (N-nucleus, BL-basal lamina MU-muscle unit, Lu-lumen) x 3500

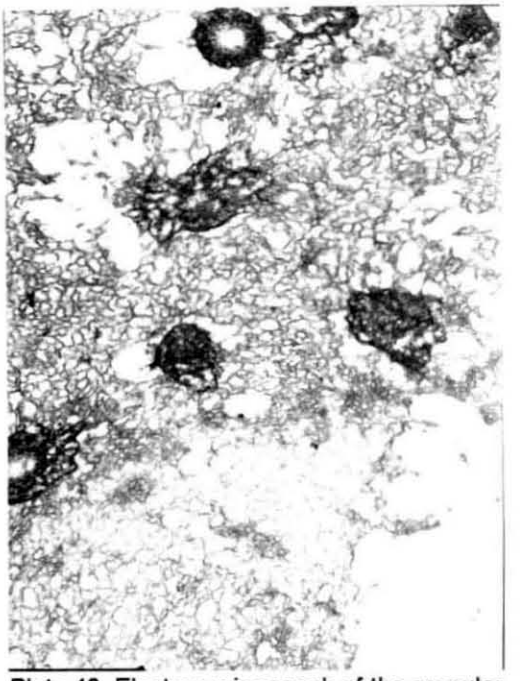

Plate 40. Electron micrograph of the granular secretory product in the lumen of accessory duct x 8000

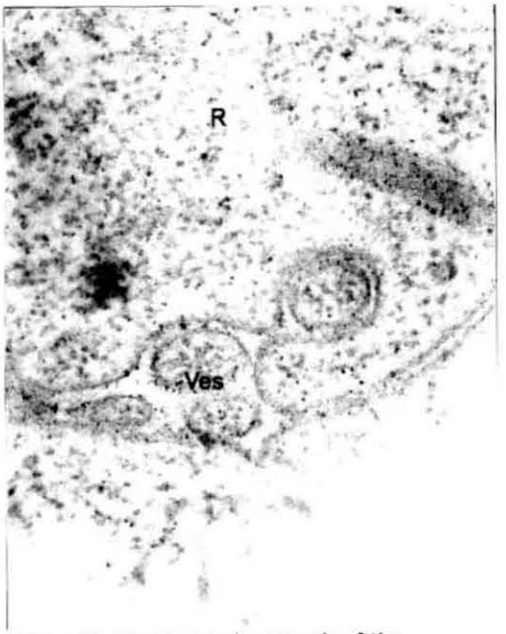

Plate 41. Electron micrograph of the epithelial cells of terminal ampoule showing intense secretory activity with free ribosomes (R) and vesicles(Ves)x 35000

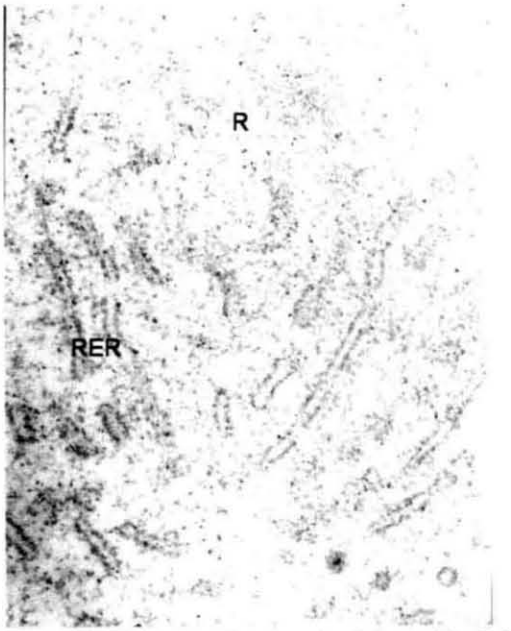

Plate 42. Electron micrograph of the epithelial cells of terminal ampoule with rough endoplasmic reticulum (RER) and free ribosomes (R) x 35000

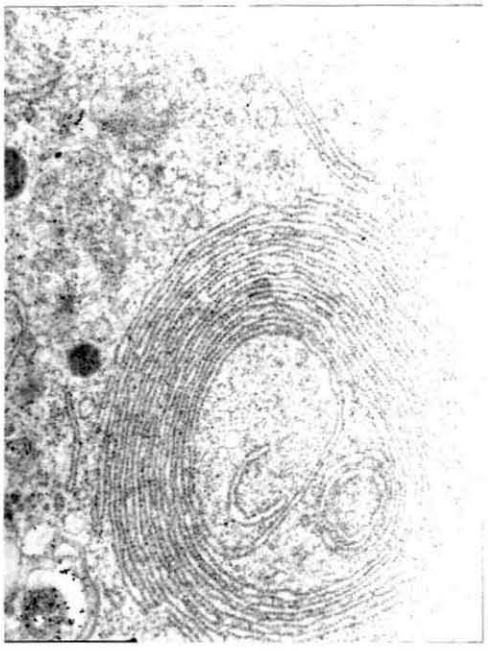

Plate 43. Electron micrograph of the epithelial cells of terminal ampoule showing concentric arrangement of R.E.R x 17000

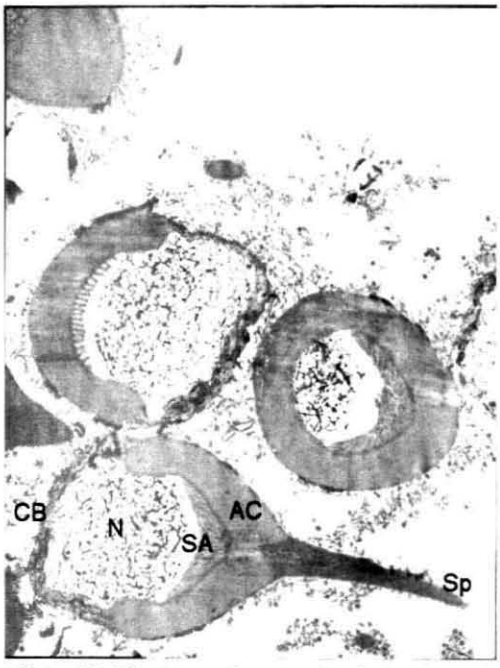

Plate 44. Electron micrograph of sperm-atozoa from a fully formed spermatophore showing main body with nucleus (N) and cytoplasmic band (CB) and acrosomal complex with acrosomal cap (AC), sub-acrosomal material (SA) and elongated spike (Sp) x 10000

Plate 45. Electron micrograph of a mature spermatozoan (N-nucleus, Sp-spike, AC-acrosomal cap, SA-sub-acrosomal substance) x 25000

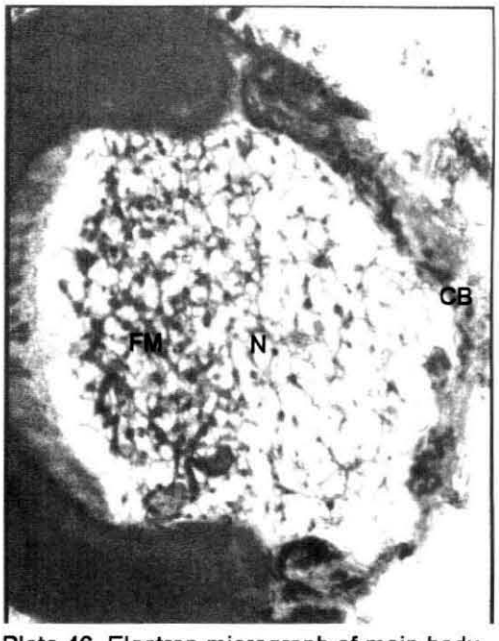

Plate 46. Electron micrograph of main body of mature spermatozoan showing Nucleus(N) with Fibrillar material (FM), cytoplasmic band (CB) x 30000

4.3 Biochemical Changes During Ovarian Maturation

Changes in biochemical constituents viz. proteins, lipids, carbohydrates, carotenoids and moisture, in relation to ovarian development in *Metapenaeus monoceros* were analyzed in tissues like ovary, hepatopancreas, haemolymph and muscle. The results showed that there were significant differences in these metabolites during the process of ovarian maturation. The results of the study are presented in tables 5 to 9 and graphically shown in figures 3 to 6.

4.3.1 Proteins

The average values of total proteins recorded in tissues like ovary, hepatopancreas, muscle and haemolymph during the different stages of ovarian maturation are presented in table 6.

In the ovary there was significant difference at $p < 0.01$ in protein content during the maturity stages I to V. The results showed a gradually increasing trend from stage I to stage IV followed by a steep fall in stage V (Fig. 3). In stage I ovary, protein constituted 52.01 % of dry weight, which increased to 59.36 % in stage IV but decreased sharply to 53.18 % in stage V.

The difference in protein contents of hepatopancreas with the maturity stages was not significant. No particular trend was seen in the variation of protein content in hepatopancreas (Fig. 4). In stage I, proteins constituted 53.53 % of the dry weight of hepatopancreas. The highest value of 54.12 % was recorded in stage III.

Significant ($p<0.01$) difference was noticed in the protein concentration of haemolymph. The haemolymph recorded 68.82 mg/ml during stage I and the maximum value of 72.8 mg/ml in stage II (Fig. 6). The lowest recorded value was for stage V (66.66 mg/ml).

There was no significant difference in the protein content in muscle tissue with maturity stages even at $p < 0.05$. The maximum value of 76.23 % was recorded in stage IV (Fig. 5).

4.3.2 Lipids

The average values for total lipids in the various tissues estimated during the present study are given in table 7.

The difference in total lipids in the ovary with different maturity stages was significant ($p<0.01$). There was a gradual increase from stage I, reaching maximum values during stages III and IV and followed by a steep decrease in stage V (Fig. 3). In stage I ovary, lipids constituted 27.68 % of the total dry weight and it increased to 30.50 % in stage III and 31.23 % in stage IV.

Lipid concentration in hepatopancreas showed significant ($p<0.01$) difference between maturity stages. From a low of 30.26 % on dry weight basis in stage I, it increased to 35.48 % in stage III. There was a marginal decrease in stage IV (33.01 %) (Fig. 4). The lowest value of 29.28 % was recorded for stage V.

There was significant ($p<0.01$) difference in lipid concentration of haemolymph with maturity stages. From 3.22 mg/ml in stage I, it reached a peak value of 10.22 mg/ml in stage IV and fell to 3.50 mg/ml in stage V (Fig. 6).

Lipid concentration in muscle tissue did not show any significant difference with maturity stages even at $p<0.05$. No clear trend was noticeable (Fig. 5). However the lowest value of 7.31 % on dry weight basis was observed in stage V and the highest of 8.32 % in stage III.

Table 6. Variations in the concentration of total proteins during the different maturity stages of *Metapenaeus monoceros*

Tissues	Maturity Stages				
	I	II	III	IV	V
Ovary ** (mg/100mg) dry weight	52.01±0.83	56.18±2.58	58.02±1.18	59.36±1.18	53.18±2.53
Hepatopancreas (mg/100mg) dry weight	53.53±0.97	53.19±0.55	54.12±0.23	53.23±1.15	53.81±0.47
Muscle (mg/100mg) dry weight	76.18±1.83	75.61±1.34	75.12±1.60	76.23±1.82	75.50±1.16
Haemolymph** (mg/ml)	68.82±1.03	72.8±1.69	69.8±0.97	68.46±3.98	66.66±2.52

** $P<0.01$

Table 7. Variations in the concentration of total lipids during the different maturity stages of *Metapenaeus monoceros*

Tissues	Maturity Stages				
	I	II	III	IV	V
Ovary ** (mg/100mg) dry weight	27.68±1.67	29.13±2.58	30.5±2.70	31.23±2.98	27.93±2.49
Hepatopancreas** (mg/100mg) dry weight	30.26±1.63	32.45±1.70	35.48±3.21	33.01±3.68	29.28±2.56
Muscle (mg/100mg) dry weight	7.86±1.18	7.92±0.74	8.32±1.18	7.32±0.47	7.31±1.08
Haemolymph** (mg/ml)	3.22±1.63	7.67±2.12	7.81±2.60	10.22±2.34	3.50±1.54

** $P<0.01$

4.3.3 Carbohydrates

The average values of total carbohydrates estimated in the present study are given in table 8.

Ovary of *Metapenaeus monoceros* showed significant ($p<0.01$) difference in carbohydrate concentration with the different maturity stages. The trend in variation of ovarian carbohydrates is shown in Figure 3. Ovarian carbohydrates constituted 1.6 % of dry weight of ovary during stage I. A maximum concentration of 2.97 % was recorded for stage IV that decreased to 1.68 % for stage V.

The difference in carbohydrate content of hepatopancreas was not significant ($p<0.05$) during stages I to V. No clear trend was perceivable (Fig. 4). The lowest value of 3 % was for stage II and the highest value of 4.53 % for stage IV.

The haemolymph carbohydrate concentration showed significant ($p<0.01$) difference with the various maturity stages. From the lowest value of 0.26 mg/ml in stage I, there was a steady increase (Fig. 6) through stages II and III which reached a peak of 0.996 mg/ml in stage IV. The value estimated for stage V was 0.61 mg/ml.

Carbohydrate variations in muscle did not show significant difference with maturity stages even at $p<0.05$. The carbohydrate levels remained almost constant during different stages (Fig. 5).

4.3.4 Carotenoids

Mean values of total carotenoids in various tissues estimated during the present study are given in table 9.

There was significant difference ($p<0.01$) in carotenoid concentration in ovary during different maturity stages. Carotenoids showed a slow build up in the ovary with the progression of maturity stages till stage IV (Fig. 3). From a concentration of 1.49 µg/mg in stage I, there was not much increase in stage II where it was only 1.54 µg/mg. However, there was a steady increase to 4.35 µg/mg in stage III and 7.03 µg/mg in stage IV, followed by a decrease to 2.28 µg/mg in stage V.

There was significant difference ($p<0.01$) in carotenoid levels in hepatopancreas with maturity. A clearly increasing trend in carotenoid levels was observed till stage III followed by a decrease during stages IV and V (Fig. 4). The lowest value of 14.71 µg/mg was recorded in stage I and the highest of 49.94 µg/mg in stage III.

Haemolymph carotenoid levels showed significant ($p<0.01$) difference with maturity stages. The trend showed a gradual increase from stage I to stage III, and stabilized during stages III and IV, followed by a decrease during stage V (Fig. 6). The lowest value recorded was 9.55 µg/ml in stage I and the highest, 25.80 µg/ml in stage III.

4.3.5 Moisture

Average values of moisture content recorded in the present study are given in table 5.

In the ovarian tissue there was significant ($p<0.01$) difference in moisture content with maturity stages. The moisture content showed a decreasing trend till stage IV followed by a sharp increase during stage V (Fig. 3). From a moisture content of 71.95 % in stage I, there was a decrease to 68.3 % in stage IV. The highest value recorded was 74.18 % for stage V ovary.

Table 5. Variations in moisture content (%) during the different maturity stages of *Metapenaeus monoceros*

Tissues	Maturity Stages				
	I	II	III	IV	V
Ovary **	71.95±1.53	70.20±1.34	69.68±2.38	68.30±1.40	74.18±2.10
Hepatopancreas**	67.64±1.59	62.28±1.38	57.3±2.19	58.4±1.07	70.54±1.58
Muscle **	73.18±0.72	73.04±0.39	73.12±0.53	72.2±0.30	73.54±0.68

** $P<0.01$

Table 8. Variations in the concentration of total carbohydrates during the different maturity stages of *Metapenaeus monoceros*

Tissues	Maturity Stages				
	I	II	III	IV	V
Ovary * (mg/100mg) dry weight	1.596±0.59	1.852±0.46	1.899±1.52	2.968±0.81	1.683±0.56
Hepatopancreas (mg/100mg) dry weight	3.679±1.73	3.002±1.36	3.513±1.53	4.537±1.44	3.627±1.63
Muscle (mg/100mg) dry weight	0.950±0.023	0.972±0.020	0.973±0.016	0.962±0.022	0.951±0.036
Haemolymph** (mg/ml)	0.260±0.096	0.620±0.23	0.830±0.15	0.996±0.17	0.610±0.14

* $P<0.05$

** $P<0.01$

Table 9. Variations in the concentration of total carotenoids during the different maturity stages of *Metapenaeus monoceros*

Tissues	Maturity Stages				
	I	II	III	IV	V
Ovary ** (µg / mg)	1.49±0.59	1.54±0.46	4.35±1.52	7.03±0.81	2.28±0.56
Hepatopancreas** (µg / mg)	14.71±3.11	22.79±5.60	49.94±16.21	27.28±6.57	18.31±6.87
Haemolymph** (µg / ml)	9.55±4.16	13.50±2.97	25.80±5.89	25.14±5.21	16.99±4.13

** $P<0.01$

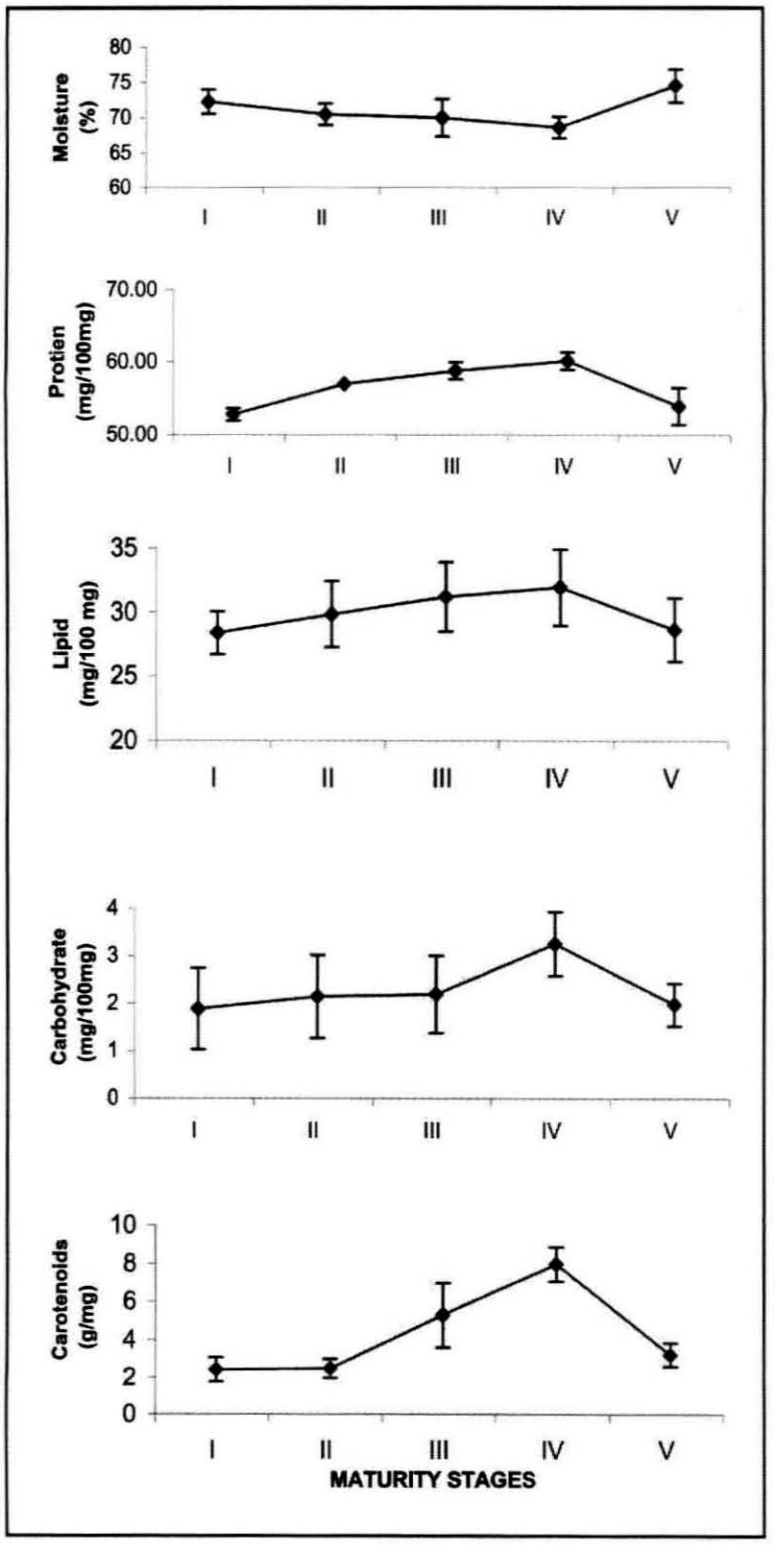

Figure 3. Trends in biochemical composition of ovary at different stages of maturity

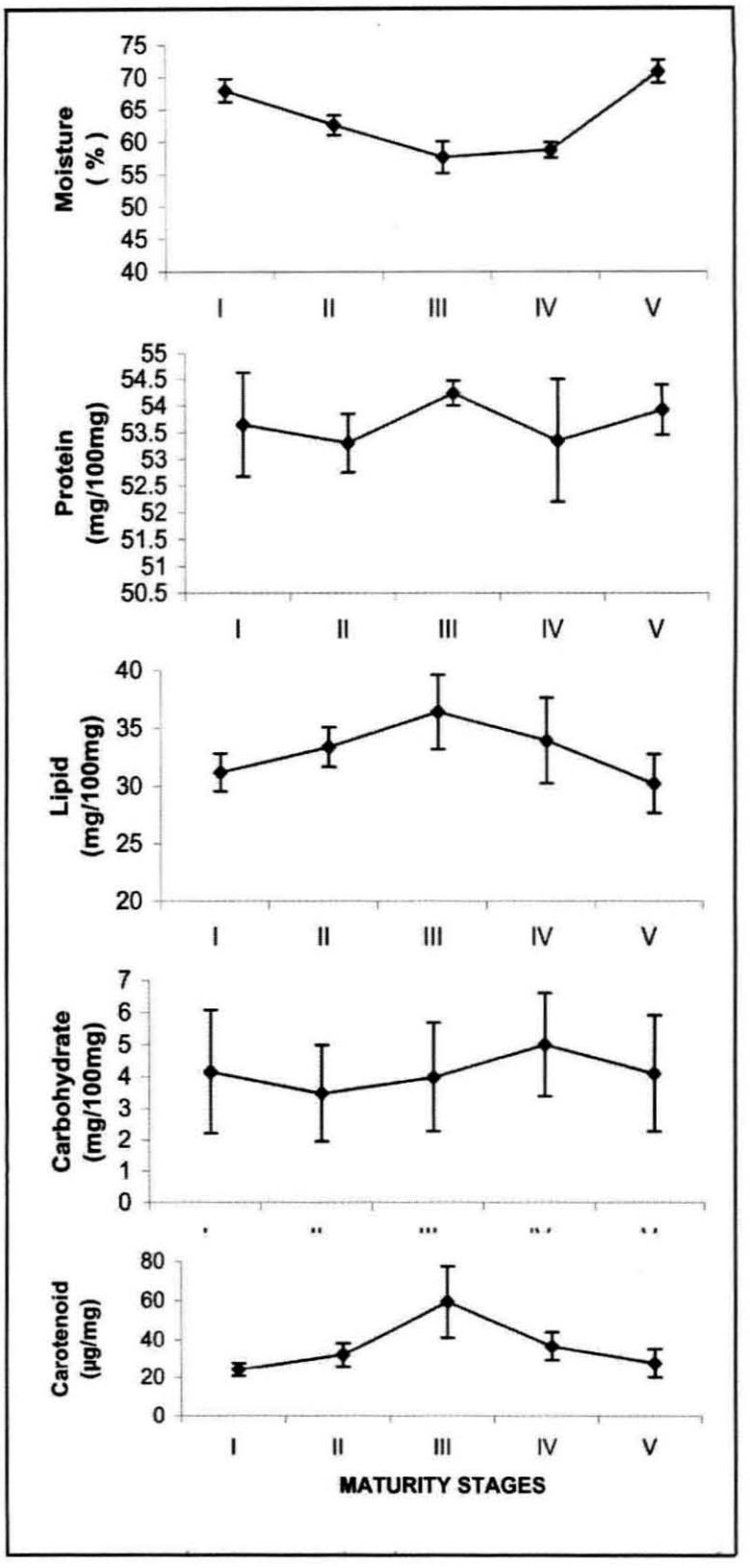

Figure 4. Trends in biochemical composition of hepatopancreas at different stages of maturity

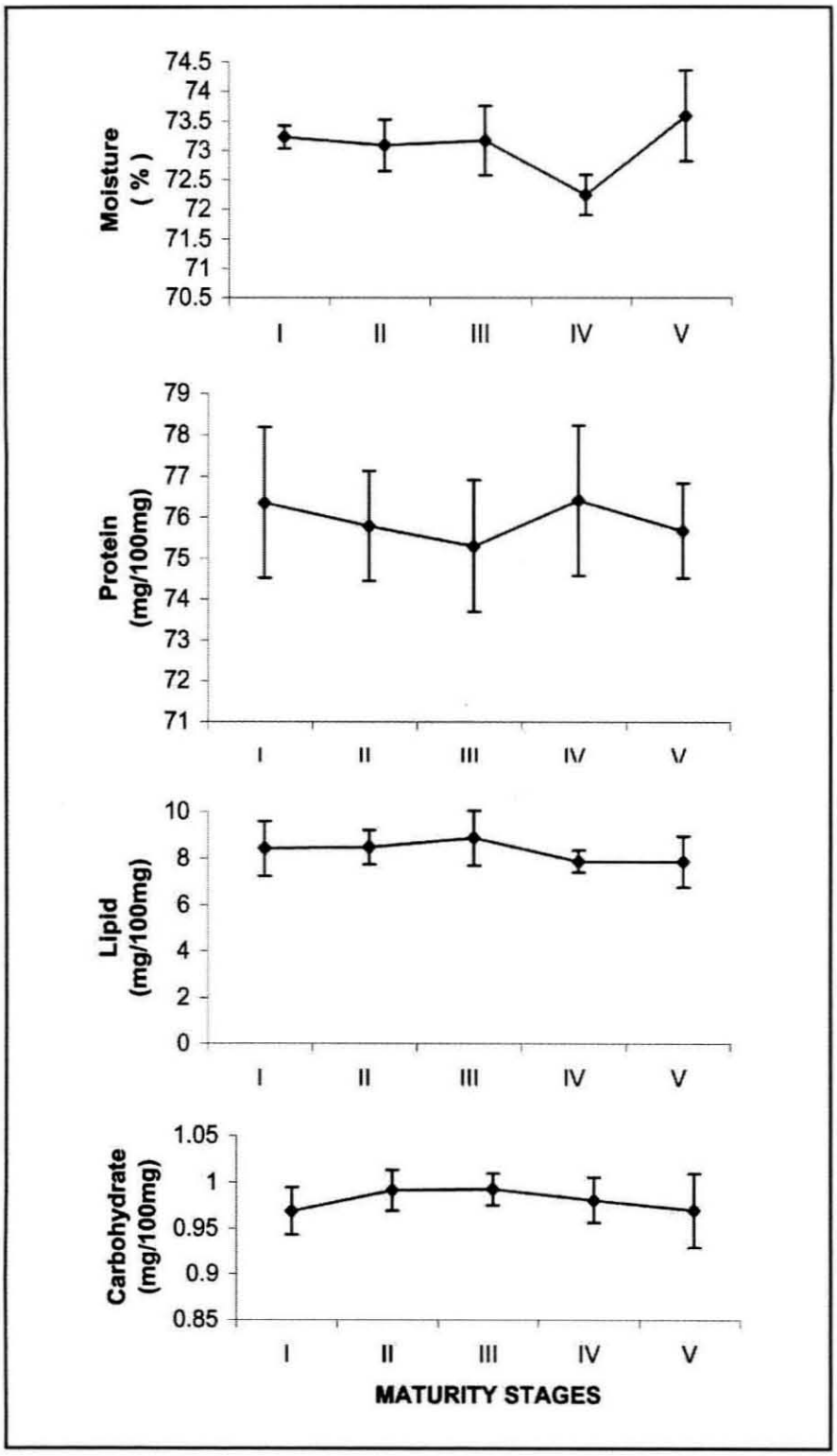

Figure 5. Trends in biochemical composition of muscle at different stages of maturity

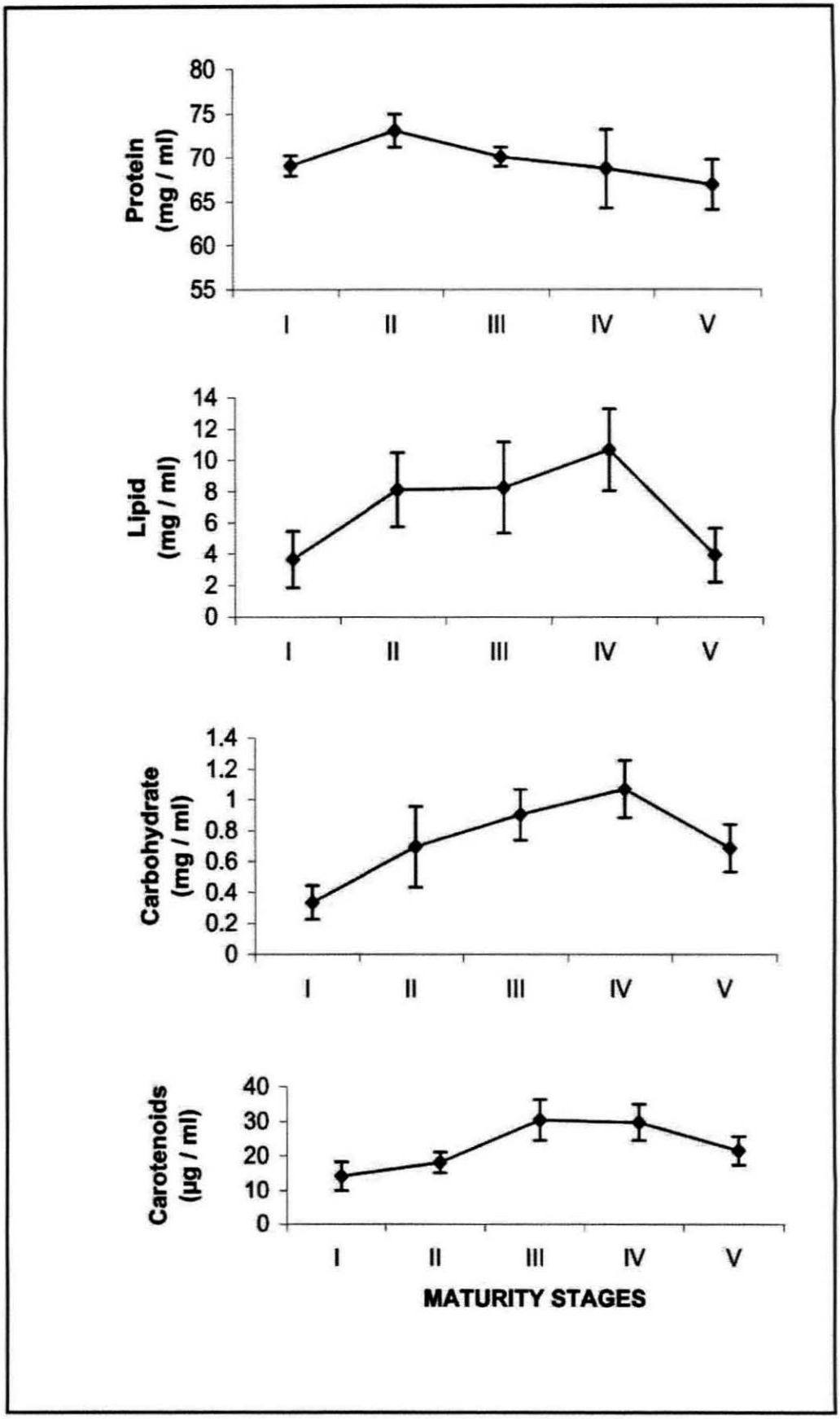

Figure 6. Trends in biochemical composition of haemolymph at different stages of maturity

Moisture content of hepatopancreas also showed significant ($p<0.01$) difference with the progression of maturity. The values showed a sharply decreasing trend from stage I to III, followed by an increase (Fig. 4). The value recorded was 67.64 % for stage I, which decreased to 57.3 % during stage III. The moisture content of hepatopancreas increased marginally to 58.4 % in stage IV followed by a sharp increase to the maximum value of 70.54 % in stage V.

The moisture content of muscle ranged from 72.2 % to 73.54 % during the different maturity stages. The values remained almost constant with the progression of maturity (Fig. 5).

4.3.6 Composition of mature oocytes

Based on the analysis conducted, the biochemical composition of mature oocytes of *Metapenaeus monoceros* was determined. The values are expressed in percentage, as given below.

Metabolite	**Percentage**
Moisture	68.30 % wet weight
Protein	59.36 % dry weight
Lipid	31.23 % dry weight
Carbohydrate	2.97 % dry weight
Carotenoids	0.70 % wet weight

4.4 Characterization Of Vitellin

Native Polyacrylamide gel electrophoresis (Native PAGE) of ovarian tissue was conducted to resolve the level of expression of yolk protein vitellin during the different maturity stages of *Metapenaeus monoceros*. The protein profiles of ovarian homogenates of stages I to V and testicular homogenate resolved through the native PAGE are presented in plate 47. From an array of many ovarian proteins, a high molecular weight protein fraction was identified as the yolk protein or vitellin. It was often possible to identify the vitellin fraction from unstained gels because of their characteristic green colouration. The expression of yolk protein vitellin was observed to be dependent on maturity stage. This protein fraction showed a steady increase in the level of expression with the maturity stages (stage II to IV). The vitellin fraction was not expressed in immature ovaries. Vitellin was absent in spent overies also. It was absent in testicular homogenates (Plate 47). Molecular weight of vitellin was found to be 326 kDa (Plate 51).

Confirmation of this protein as vitellin was made through selective staining of proteins in the gel. Premise of the staining is the knowledge that yolk protein is a lipo-glyco-carotenoprotein possessing a calcium moiety. Therefore the native PAGE gels were stained with Sudan black B, periodic acid schiff (PAS) reagent and alizarin red for detecting the presence of lipid, carbohydrate and calcium respectively. The results of the selective staining are shown in plates 48, 49 and 50. The 326 kDa band was stained with Sudan black B, periodic acid schiff and alizarin red indicating that it is a lipo-glycoprotein with calcium.

Another interesting observation from PAGE was the presence of a protein fraction with molecular weight 270 kDa that was expressed in ovaries of all maturity stages (Plate 47). Surprisingly this protein fraction was observed in the testis samples also. This protein fraction was stained with Sudan black and

alizarin red but not with periodic acid schiff. Hence the 270 kDa protein fraction was a lipoprotein bound to calcium.

Plate 47. Native PAGE illustrating the appearance of vitellin in ovary of females at different maturity stages.
Lane 1: Stage I ovary; Lane 2: Testis; Lane 3: Stage II ovary; Lane 4: Stage III ovary
Lane 5: Stage IV ovary; Lane 6: Stage V ovary

← vitellin

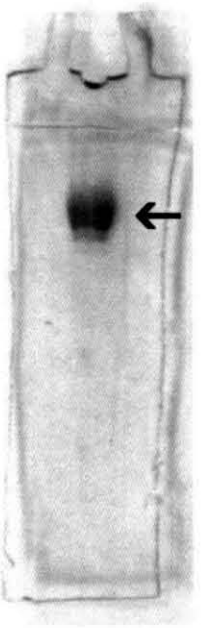

Plate 48. Native PAGE of vitellin stained

with Sudan Black

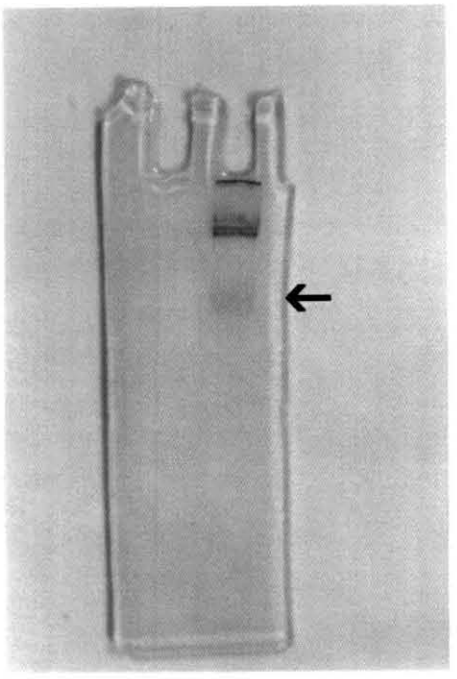

Plate 49. Native PAGE of vitellin

stained with PAS

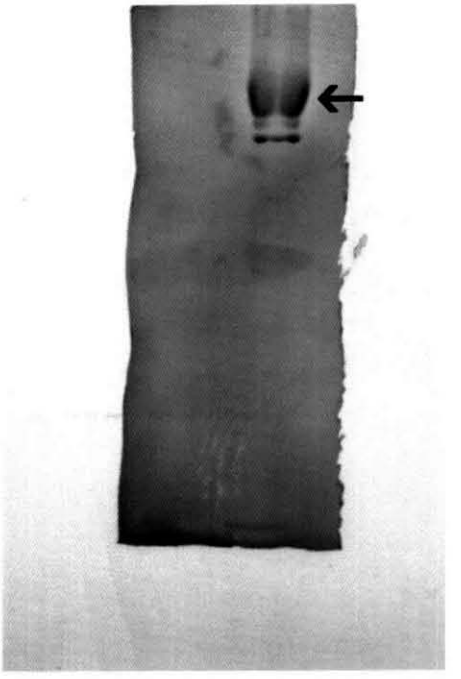

Plate 50. Native PAGE of vitellin

stained with Alizarin red S

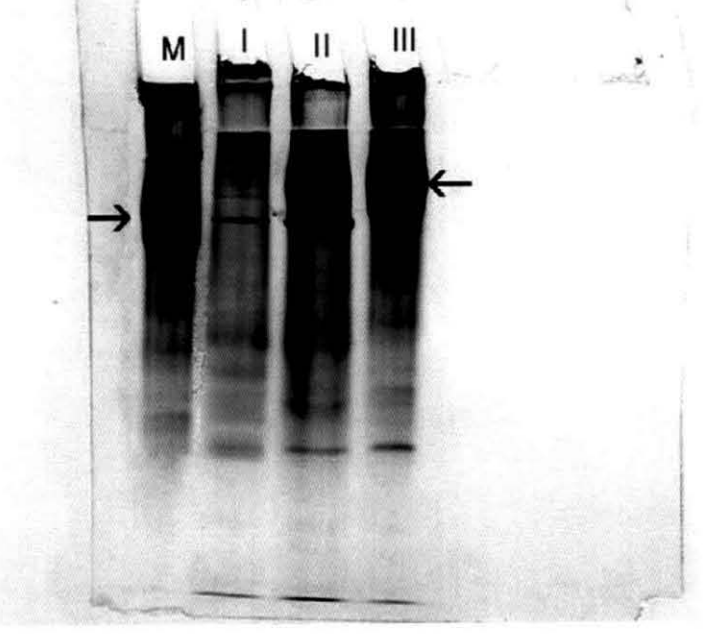

Plate 51. Native PAGE for molecular weight determination of vitellin
Lane 1: Marker; Lane 2: Stage I ovary; Lane 3: Stage II ovary
Lane 3: Stage III ovary

← Vitellin; → Urease (272 KD)

DISCUSSION

5. DISCUSSION

5.1 Female Reproductive System And Oogenesis

In *Metapenaeus monoceros* the female reproductive system consists of the internal organs – paired ovaries and oviducts and the external organ, thelycum. The structure of the ovary in this species conforms to the general penaeid pattern (King, 1948; Rao, 1968), having a pair of anterior lobes, a pair of middle lobes consisting of seven lobules on each side and posterior lobes. Nalini (1975) reported that the middle lobe consisted of six or seven lateral lobules. However, in the present study all the specimens observed had invariably seven lobules with the oviduct originating from the sixth lobule. But for the number of lobules in the middle lobe the external morphology of *M.monoceros* is similar to that in other penaeids like *Penaeus setiferus* (King, 1948), *P.duorarum* (Cummings, 1961), *P.merguiensis* (Tuma, 1967) *P.indicus* (Subrahmanyam, 1965; Mohamed and Diwan, 1994); and *M.dobsoni* (Vasudevappa, 1992). Therefore it is evident that the general structure of the female reproductive system in penaeids is homologous with little inter–specific variation.

In *Metapenaeus monoceros* the ovarian maturation is accompanied by distinct changes in colour, size and appearance and the process is almost similar to that described by Rao (1968) for four species of penaeids. Colour change in the ovary during maturation is well known for decapod crustaceans, particularly for penaeids (Dall, 1990). In the present study, for fully mature ovaries of *M. monoceros* two distinct colours were noticed viz. dark green and dark brown. Other workers have reported the ripe ovaries of this species to be dark green or brownish green (Nalini, 1976; Rao, 1989 and Nandakumar, 2001). Colour, size and texture of the ovaries are closely related to their cellular development (Quintero and Garcia, 1998). Ayub and Ahmed (2002) found that in fully mature / ripe specimens of *Penaeus penicillatus*, *P.merguiensis*, *Parapenaeopsis stylifera* and *M.affinis* the ovaries were dark green in colour. The green ovaries of shrimps

as seen from the dorsal exoskeleton help in the identification of ripe individuals for aquaculture purposes (Tan – Fermin and Pudadera, 1989).

The proportion of gonad weight in total body weight, expressed as percentage, called gonadosomatic index (GSI) is an alternative method for assessing gonadal development (Subrahmanyam, 1963; Rahaman, 1967; Pillay and Nair, 1971). In the present study the Gonadosomatic index (GSI) ranged from 0.353 in immature females to 6.98 in mature ones. Vadudevappa (1992) reported a GSI of 6.17 for fully mature females of *Metapenaeus dobsoni* whereas Mohamed and Diwan (1989) reported a value of 7.312 for fully mature females of *Penaeus indicus*. In the present study the mean ova diameter (OD) of mature oocytes of *M.monoceros* was 230.56 µm. The various OD values for fully mature oocytes of *M.monoceros* reported hitherto are 260 µm (Mohamed *et al.*, 1978), 110 – 270 µm (Rao, 1989) and 174 – 232 µm (Nandakumar, 2001). According to Gurney (1942) the size of eggs of the same penaeid species occurring in different habitats and localities of India varied considerably. Rao (1974) observed that the spawned eggs of *M.dobsoni*, *P.indicus* and *Parapenaeopsis stylifera* collected from the Cochin waters were larger than the eggs of penaeid prawns of the other parts of India.

Based on the colour, size and appearance of the ovary as observed through the dorsal exoskeleton and further observation upon dissection, the ovarian maturation in *Metapenaeus monoceros* was divided into five stages – immature (Stage I), early maturing (Stage II), late maturing (Stage III), mature (Stage IV) and spent (Stage V). Earlier workers like Nalini (1976), Rao (1989) and Nandakumar (2001) had also recognized five stages of ovarian maturation for the species based on the external appearance. However, in the present study an attempt was made to correlate the gross morphology with cytological changes using histological sections. On the basis of this the ovary was classified into the following stages – pre-vitellogenic, early vitellogenic, late vitellogenic, vitellogenic and spent which correspond to the stages I to V based on gross morphology.

Many authors have differently reported the number of maturity stages in penaeid shrimps. Vasudevappa (1992) proposed five stages of maturity for *M.dobsoni* whereas Sakaji *et al.* (2000) grouped the ovarian maturation in *Metapenaeopsis dalei* into seven stages. Oka and Srihata (1965) classified ovarian maturation in *Penaeus orientalis* into eight stages. Yano (1988) demonstrated ten stages of oocyte development in *P.japonicus*. Oocyte development was classified into four stages in *P.monodon* (Tan-Fermin and Pudadera, 1989), *P.chinensis* (Matsuyama and Matsura, 1983) and in *P.indicus* (Quinitio and Millamena, 1992). Thus, the number of maturity stages reported is not consistent. However, in penaeids the maturity stages generally fall under five groups - immature, early maturing, late maturing, mature and spent (Rao, 1968).

In agreement with the observations of King (1948) in *Penaeus setiferus* and Subrahmanyam (1965) in *P.indicus*, it was seen that the thin ovarian wall of *Metapenaeus monoceros* is composed of three layers viz. a thin outermost layer of pavement epithelium, an inner layer of germinal epithelium or germarium and a layer of connective tissue in between. In *M.monoceros* a germinal zone was observed as a thin band along the innermost layer of the ventral ovarian wall. Adiyodi and Subramoniam (1983) reported a wide variation in the placement of germinal zone in the ovary of crustaceans. In the present study, it has been observed that from the germinal zone a continuous crop of oogonial cells are produced, from an area called the zone of proliferation. Similar observations have been made by Shaikhmahmud and Tembe (1958) in *Parapenaeopsis stylifera* and Vasudevappa (1992) in *M.dobsoni*. The gradual movement of oogonia towards the centre of the lumen of ovary during their transformation into primary and secondary ooctyes is clearly demonstrated in the present study. In *M.monoceros* and in the above-mentioned studies the immature ova present in the centre of lumen have been observed to move towards the periphery as they grow and become mature.

The process of oogenesis in *Metapenaeus monoceros* is completed in two phases like that in other crustaceans. First is the proliferative phase in which the primary oogonial cells undergo mitotic division forming secondary oogonial cells which after meiotic division give rise to primary oocytes. The second phase is the differentiative phase wherein the immature ova accumulate yolk and develop into mature oocytes. More or less similar observations have been reported in many other decapod crustaceans (Yano, 1988). The stages of mitotic division of primary oogonial cells were difficult to observe and record as they occurred in rapid succession (Adiyodi and Subramoniam, 1983). Oogonial cells had pale eosinophilic cytoplasm whereas in pre-vitellogenic oocytes the cytoplasm was deeply basophilic. Mohamed and Diwan (1994) reported that the oogonial cells of *Penaeus inidcus* possessed a large, conspicuous nucleus and weakly eosinophilic cytoplasm. The pre-vitellogenic oocytes in *M.dobsoni* had deeply basophilic cytoplasm, which was homogeneous and granular (Vasudevappa, 1992).

In *Metapenaeus monoceros* early vitellogenic oocytes were characterized by the perinucleolar arrangement of 12–15 nucleoli. Vasudevappa (1992) made similar observations in the early vitellogenic ovaries of *M.dobsoni*. He also noticed abundance of atretic cells in the early vitellogenic oocytes. In *Metapenaeopsis dalei*, Sakaji et al. (2000) noticed perinucleolar oocytes in stage III ovaries. Ultrastructurally, the nucleoli of the pre-vitellogenic oocytes of *M.monoceros* appeared as electron–dense bodies along the inner periphery of the nuclear wall and the ooplasm was rich in rough endoplasmic reticulum, free ribosomes and mitochondria. Duronslet *et al.* (1975) in *Penaeus setiferus* and *P.aztecus* and Mohamed and Diwan (1994) in *P.indicus* reported that during early vitellogenesis emphasis is on the intra–oocytic synthesis aided by extrusion of RNA–rich nucleolar material into the cytoplasm through nuclear pores and the presence of abundant free ribosomes, rough endoplasmic reticulum and mitochondria. In the shrimp *Aristaeomorpha foliacea*, an increase in size and number of nucleoli occurred in pre-vitellogenic oocytes (Desantis *et al.*, 2001).

Similar features with transfer of nuclear materials to the ooplasm have been reported in *Paratelphusa hydrodromous* (Adiyodi, 1969) and *Libinia emarginata* (Hinsch, 1970). Nuclear emission from young oocytes is common in autosynthetic oocytes and the transfer of nuclear material to the ooplasm is a prelude to protein synthesis in various crustaceans (Adiyodi and Subramoniam, 1983).

The late vitellogenic ovary of *Metapenaeus monoceros* was characterized by the presence of rough granular cytoplasm which was fully eosinophilic. In *M.dobsoni* also Vasudevappa (1992) reported that a clear shift from the basophilic to eosinophilic nature of cytoplasm occurred at the late vitellogenic stage. Mohamed and Diwan (1994) reported that the granular nature of the oocyte cytoplasm of *Penaeus indicus* at this stage was mainly due to the formation of dense yolk platelets and accumulation of lipid globules. The basophilic reaction of cytoplasm of oocytes of pre-vitellogenic and early vitellogenic stages and the gradual shift to acidophilic nature in late vitellogenic and vitellogenic stages noticed in *M.monoceros* are similar to that noticed in *P.setiferus* (King, 1948) and *P.monodon* (Tan – Fermin and Pudadera, 1989). Electron micrographs of late vitellogenic stage in the present study clearly demonstrated the arrangement of nucleoli along the peripheral nucleoplasm. Cytoplasm was abundant in mitochondria, free ribosomes, rough endoplasmic reticulum, golgi bodies, yolk spheres and yolk platelets. Komm and Hinsch (1987) reported that the rough endoplasmic reticulum is commonly associated with active golgi complexes which give rise to numerous vesicles and these vesicles containing intra-cisternal granules fuse and the granules begin to coalesce and increase in electron density. Almost the same sequence of events was seen in *M.monoceros* in the present study. Initial yolk vesicles were electron lucent and filled with tiny elctron dense flocculent granules in *P.indicus*. Through progressive differentiation these granules aggregated to form a moderately electron dense yolk sphere which finally devolved into yolk platelet (Mohamed and Diwan, 1994).

The vitellogenic ovary in *Metapenaeus monoceros* was filled with mature oocytes which were more elongate than circular with patches of oogonial cells in between. The eosinophilic cytoplasm was filled with yolk granules and conspicuous was the absence of cortical bodies. It is of a general consensus that full ovarian maturation of penaeids is indicated by the presence of large acidophilic oocytes with cortical bodies (King, 1948). Previously referred to as jelly-like substance, cortical specializations, rod-shaped bodies or peripheral bodies, they exist in all specimens observed in genera *Penaeus* and *Sicyonia* (Hudinaga, 1942; King, 1948; Ikematsu, 1963; Oka and shrihata, 1965; Oka, 1967; Tuma, 1967; Anderson *et al.*, 1984; Yano, 1988; Tan-Fermin and Pudadera, 1989). The appearance of rod–like bodies in the ripe ova of penaeid shrimps has been reported by several workers including Hudinaga (1942) in *Penaeus japonicus*, King (1948) and Duronslet *et al.* (1975) in *P.setiferus*, Cumming (1961) in *P.duorarum*, Tuma (1967) in *P.merguiensis*, Duronslet *et al.* (1975) in *P.aztecus* Mohamed and Diwan (1994) in *P.indicus* and Joseph (1996) in *P.monodon*. According to Tan–Fermin and Pudadera (1989), in *P.monodon* the cortical bodies are present at the periphery but as maturation progresses, these bodies elongate and extend towards the nucleus. However, the absence of cortical bodies in fully ripe ova has been reported by Shaikhmahmud and Tembe (1958) in *Parapenaeopsis stylifera*; Rao (1968) in *M.affinis*, *M.dobsoni* and *P.stylifera*; Vasudevappa (1992) in *M.dobsoni* and Sakaji *et al.* (2000) in *Metapenaeopsis dalei*. Contrary to the assumption that cortical rods are a characteristic feature of the genus *Penaeus*, Ayub and Ahmed (2002) detected their presence in mature eggs of *Metapenaeus affinis* and *Parapenaeopsis stylifera*. But they reported that these peripheral bodies remain spherical throughout and never take the appearance of rods. Cortical crypts release materials forming a jelly coat when the eggs are exposed to seawater (Clark and Lynn, 1977). As a result of such a cortical reaction, the egg reduces its volume (Clark *et al.*, 1980). Because of the absence of cortical rods in the oocytes of *M.monoceros*, it is expected that there is little or no jelly coat around the eggs

exposed to seawater and that reduction in volume after the cortical reaction is lesser than that in species, which have them.

Ultrastructural investigations of the ova of *Metapenaeus monoceros* at different maturity stages in the present study show that the mode of synthesis of yolk is mostly autosynthetic. The oolemma did not develop microvilli or micropinocytotic vesicles to incorporate yolk proteins from an outer source. Duronslet *et al.* (1975) and Mohamed and Diwan (1994) reported that yolk formation apparently takes place by the selective deposition and sequestration of organic material from within (autosynthesis) and without (heterosynthesis). According to them during the early vitellogenic phase the emphasis is on intra–oocytic synthesis of proteins aided by the extrusion of RNA–rich nucleolar material into the cytoplasm through the nuclear pores and the presence of abundant free ribosomes, rough endoplasmic reticulum and mitochondria (as seen in the present study). From the late vitellogenic phase onwards extra – oocytic sequestration of organic reserves is prevalent through the formation of micropinocytotic vesicles (not seen in the present study) on the oolemma. In *M.monoceros* nuclear pores were noticed during the late vitellogenic stage also, through which nuclear material diffused into the ooplasm. Nuclear emission from young oocytes is common in autosynthetic oocytes and the transfer of nuclear material to the ooplasm is a prelude to protein synthesis in various crustaceans (Adiyodi and Subramoniam, 1983). In *M.*dobsoni, Vasudevappa (1992) reported the dispersal of the nuclear material into the cytoplasm during the late vitellogenic stage from histological sections. In shrimp *Aristaeomorpha foliacea*, Desantis *et al.* (2001) reported that the production of yolk components seems to have intra–oocytic origin, since oolemma is lacking in microvilli and micropinocytotic vesicles. An exclusively endogenous synthesis of yolk by rough endoplasmic reticulum in association with golgi apparatus has been found in a few species of crustaceans (Kessel, 1968; Ganion and Kessel, 1972).

In the present study, the histological sections of spent ovaries of *Metapenaeus monoceros* showed that bulk of the area was occupied by pre-vitellogenic oocytes and oogonial cells with stray occurrence of resorbing oocytes. Vacuolated follicle cells were seen surrounding the atretic oocytes. Mohamed and Diwan (1994) observed that in *Penaeus indicus* spent ovaries resembled those in pre-vitellogenic and early vitellogenic stages. The presence of atretic or resorbing oocytes is considered to be a major trait in distinguishing immature from spent ovaries of penaeids (Medina *et al.*, 1996; Quintero and Garcia, 1998; Peixoto *et al.*, 2003). Ultrastructure of the spent ovaries of *M.monoceros* indicated that the ooplasm was devoid of organelles, filled with vacuoles and the nuclear condensation was conspicuous. Several workers like Joseph (1996) in *P.monodon* and Sakaji *et al.* (2000) in *Metapenaeopsis dalei* have observed the co–existence of more than three or four developmental stages of oocytes in a single section of the ovary, leading to the conclusion that they are multiple spawners. In *Metapenaeus monoceros*, however not more than two stages of oocytes were encountered in a single ovarian section. Hence it can be inferred that this species spawns during a short period in the spawning season. Nalini (1976) reported that the maturing and mature stages of *M.monoceros* from Cochin waters contained only two widely separated groups of ova, one representing the immature stock and the other mature stock, indicating that the spawning is restricted to a short and definite period.

Folliculogenesis in *Metapenaeus monoceros* was found to start in pre–vitellogenic stage and complete in early vitellogenic stage. In pre–vitellogenic stage, the round follicle cells surround a large number of pre–vitellogenic oocytes. During the late vitellogenic stage, the follicle cells are flattened as the ova diameter increases and they form a thin ribbon like covering around the ova in the vitellogenic stage. Similar observations were made by Vasudevappa (1992) in *M.dobsoni* and Mohamed and Diwan (1994) in *Penaeus indicus*. Sakaji *et al.* (2000) reported that the follicle cells, which were found encircling the ova till then, were not observed in the final stage of maturation in *Metapenaeopsis dalei*.

Ultrastructure of ripe ovaries in the present study demonstrated the presence of flattened follicle cells with the follicle cell cytoplasm having rough endoplasmic reticulum and free ribosomes, pointing towards their ability for protein synthesis. In *P.japonicus*, follicle cells are implicated as the possible cell type responsible for ovarian vitellogenin synthesis (Yano and Chinzei, 1987). Concentric layers of rough endoplasmic reticula were seen at the periphery of oocyte cytoplasm adjacent to the follicle cells in *M.monoceros*. According to Charniaux–Cotton (1975), follicle cells facilitate vitellogenic activity by aiding in uptake of yolk protein from external sources. Mohamed and Diwan (1994) opined that in *P.indicus*, follicle cells might serve and facilitate the exogenous supply of yolk material.

5.2 Male Reproductive System

5.2.1 Gross morphology

The morphology of male reproductive system has been studied in penaeid shrimps, *Penaeus setiferus* (King, 1948); *Parapenaeopsis stylifera* (Shaikhmahmud and Tambe, 1958; Rao, 1968); *Penaeus indicus* (Subrahmanyam, 1965; Mohamed and Diwan, 1994); *P. merguiensis* (Tuma, 1967); *P. monodon* (Motoh, 1978; Joseph, 1996); *Metapenaeus dobsoni* (Vasudevappa, 1992) and *P. semisulcatus* (Bose, 1995). The present study shows that the general morphology of the male reproductive system of *Metapenaeus monoceros* is similar to that reported in penaeids in the above mentioned works. However, the number of testicular lobes in different species show considerable variation. In the present study the testis of *Metapenaeus monoceros* was found to possess 6 or 7 lateral lobes. Vasudevappa (1992) reported that testis of *M. dobsoni* is composed of an anterior lobe, 6 lateral lobes and one posterior lobe. *Penaeus indicus* has only 4 lobes on either side of the testis (Subrahmanyam, 1965; Mohamed and Diwan, 1993). In *Parapenaeopsis stylifera* there are only 3 lobes on either side (Shaikhmahmud and Tambe, 1958).

Chow *et al.* (1991b) reported that the testis of *Penaeus vannamei* and *P. setiferus* are composed of 8 lobes on either side. Huq (1980) studied the number of testicular lobes in 6 *Penaeus* species and reported that smaller individuals tend to possess fewer lobes than larger ones, and that the numbers of lobes were different between left and right sides and between species. Chow *et al.* (1991b) opined that intraspecific variations in the number of testicular lobes of *P. setiferus* and *P. vannamei* should not be ruled out.

In the present study the vas deferens of *Metapenaeus monoceros* was found to consist of 4 main segements viz. proximal vas deferens, mid vas deferens, distal vas deferens and terminal ampoule. This was in agreement with the general plan of organization of vas deferens in *Penaeus setiferus* (King, 1948; Ro *et al.*, 1990); *P. kerathurus* (Malek and Bawab, 1974a,b); *Metapenaeus dobsoni* (Vasudevappa, 1992); *P. indicus* (Mohamed and Diwan, 1993); *P. semisulcatus* (Bose, 1995) and *P. monodon* (Joseph, 1996). From these studies it was seen that in penaeids, vas deferens had similar morphological regions with characteristic size and shape. Chow *et al.* (1991b) divided the proximal vas deferens in *P. setiferus* and *P. vannamei* into anterior proximal vas deferens and posterior proximal vas deferens.

5.2.2 Maturity stages

Unlike females, the male penaeids give few visible clues regarding the stages of development of the testis. Moreover, the size at first maturity in males is much smaller than that for females and information regarding the maturity stages in male shrimps is scanty. Therefore very few workers have described any well-defined maturity stages in males. However, Subrahmanyam (1965) described five maturity stages in *Penaeus indicus* by relating variations in the opacity and size of the testis to the animal size. Castille and Lawrence (1991) classified males of *P. aztecus* and *P. setiferus* into three maturity stages viz. immature, developing and mature, based on the size and appearance of the

terminal ampoule. Parnes *et al.* (2004) used the appearance of spermatophores through terminal ampoule for classification of the males of *Litopenaeus vannamei*. In the present study, male specimens of *Metapenaeus monoceros* were classified into three stages viz. immature, maturing and mature based on the external morphology of testes, vas deferens, petasma and appendix masculina. Of the different criteria used, the size and colour of the terminal ampoule, appendix masculina and petasma have been found to be more reliable and useful as the same can be observed externally without sacrificing the shrimp. Joseph (1996) also classified *Penaeus monodon* males into three stages.

5.2.3 Anatomy of male reproductive system

In *Metapenaeus monoceros* the sperm duct or vas deferens consists of four distinct regions viz. a narrow proximal portion called the proximal vas deferens (PVD), a thickened middle portion called the medial vas deferens (MVD) which tapers to form a distal, relatively long, narrow portion called the distal vas deferens (DVD). The DVD terminates in a greatly dilated and muscular terminal ampoule. This basic plan of sperm duct has been observed in other penaeids like *Penaeus kerathurus* (Malek and Bawab, 1974a,b); *P. setiferus* (King, 1948; Chow *et al.*, 1991a); *P. vannamei* (Chow *et al.*, 1991a); *Metapenaeus dobsoni* (Vasudevappa, 1992); *P. indicus* (Mohamed and Diwan, 1993); *P. semisulcatus* (Bose, 1995) and *P. monodon* (Joseph, 1996). Malek and Bawab (1974a) further divided the medial vas deferens into a blind pouch, ascending and descending MVD, which has been followed by the subsequent authors. All these regions are clearly discernible in fully mature *M. monoceros*, while not so distinct in immature ones. Detailed anatomical studies on the structural modifications of the vas deferens have been carried out in connection with the spermatophore formation in penaeids, *P. kerathurus* (Malek and Bawab, 1974a,b); *P. setiferus* (Ro *et al.*, 1990); *P. vannamei* (Chow *et al.*, 1991a); *M. dobsoni* (Vasudevappa, 1992); *P. indicus* (Mohamed and Diwan, 1993) and *P. monodon* (Joseph, 1996).

In *M. monoceros* the PVD which was a short and slender tube connecting the testis to the MVD, consisted of an outer connective tissue sheath and an inner muscle layer. The inner lumen of this duct was lined by a layer of basophilic columnar epithelial cells as in other penaeids like *P. setiferus* (King, 1948; Ro *et al.*, 1990; Chow *et al.*, 1991a,b); *P. vannamei* (Chow *et al.*, 1991a); *M. dobsoni* (Vasudevappa, 1992); *P. semisulcatus* (Bose, 1995) and *P. monodon* (Joseph, 1996). However, in *P. indicus* Mohamed and Diwan (1993) reported that no glandular epithelial cells were seen in this part of the sperm duct. In immature *M. monoceros* the innermost lining of epithelial cells was absent. In the penaeoid shrimp *Pleoticus muelleri*, Diaz *et al.* (2002) reported the vas deferens to consist of four parts viz. the collecting tubule, proximal, medial and distal parts. In *P. muelleri* the collecting tubule that is lined by myoepithelial cells is anatomically similar to the PVD in the present study and in most other penaeids.

The three regions of the medial vas deferens was discernible only in mature specimens of *M. monoceros* whereas in immature ones this part was a simple straight tube. Joseph (1996) reported similar observations in *P. monodon*. In fully mature shrimps, the blind pouch occurred at the junction of the PVD and MVD, which dilated as the ascending limb of MVD and was continued as the descending limb. A septum divided the ascending limb into a larger spermatophoric duct (SD) and smaller accessory duct (AD). In the descending limb this compartmentalization existed only for a short distance and was found to be disappearing towards the distal portion. Anatomical features of penaeid MVD is basically the same as seen in *P. setiferus* (King, 1948; Ro *et al.*, 1990; Chow *et al.*, 1991a); *P. kerathurus* (Malek and Bawab, 1974a,b); *P. vannamei* (Chow *et al.*, 1991a); *M. dobsoni* (Vasudevappa, 1992); *P. indicus* (Mohamed and Diwan, 1993); *P. semisulcatus* (Bose, 1995) and *P. monodon* (Joseph, 1996). The septum that separated the SD and AD was made of connective tissue lined on either sides by epithelial cells and intermittent blood vessels. Typhlosoles were seen projecting into the lumen of the SD and AD. The SD had two large

typhlosoles whereas the AD had two smaller ones. The number of typhlosoles in the SD and AD of penaeids vary. In *P. setiferus*, Malek and Bawab (1974a,b), *P. semisulcatus*, Bose (1995) and *P. indicus*, Mohamed and Diwan (1993) reported one each in the two ducts. In *M. dobsoni*, Vasudevappa (1992) reported two to three typhlosoles in SD and one in AD. In all these species typhlosoles were richly vascularized indicating intense secretory metabolic rate of glandular epithelial cells.

The Distal vas deferens (DVD) of *M. monoceros* was structurally similar to the MVD except that the lumen was not divided into compartments. This is similar to that in other penaeids (King, 1948; Malek and Bawab, 1974a,b; Chow *et al.*, 1991a; Vasudevappa, 1992; Mohamed and Diwan, 1993). The inner lumen was lined with tall columnar epithelial cells but did not form typhlosoles. Joseph (1996) observed that in the DVD of *P. monodon* also typhlosoles were not fully developed. In *P. indicus* Mohamed and Diwan (1993) reported the presence of fully developed typhlosoles. In *M. monoceros* and in other penaeids studied the DVD opens into the pear shaped muscular terminal ampoule. In immature and maturing animals the muscles and secretory epithelial cells were not well developed. But in fully mature ones the terminal ampoule was divided into a number of chambers, each chamber lined by glandular epithelial cells. Terminal ampoule in *P. setiferus* consisted of five interconnecting chambers and in *P. vannamei* four (Chow *et al.*, 1991a). In penaeoidan shrimp *Pleoticus muelleri* also the terminal ampoule had four interconnecting chambers, lined with infolded glandular epithelium.

5.2.4 Spermatogenesis and spermatophore formation

In *Metapenaeus monoceros* testis was composed of minute convoluted tubules or acini in which sperm cells at various developmental stages were encountered. Non-germinative cells called 'nurse cells' were also seen among the germinal components. There are many reports of crustacean testis having

nurse or nutritive cells among the spermatogonia, which perform important functions like providing nourishment, support and some hormones during spermiogenesis (Hinsch, 1960).

The process of spermatogenesis in *M. monoceros* is similar to that reported in other decapod crustaceans (Pochon – Masson, 1983). Spermatogenesis begins in the peripheral germinative layer of the testicular acini when spermatogonia enter into the prophase of meiosis (King, 1948). Spermatogonial cells, after a period of quick growth, transform into primary spermatocytes and then undergo the first meiotic division to become secondary spermatocytes. These in turn undergo the second meiotic division (mitosis) to form spermatids. These spermatids migrate out of their cell boundaries and transform to spermatozoa without further division. This pattern of spermatogenesis has been seen in *P. setiferus* (King, 1948); *Parapenaeopsis stylifera* (Joshi *et al.*, 1982); *M. dobsoni* (Vasudevappa, 1992) and *P. indicus* (Mohamed and Diwan, 1993). In *M. monoceros* a single testicular acinus was found to contain sperm cells at various stages of development. This synchronous pattern of spermatozoan development has been observed in other penaeids (Pochon – Masson, 1983) and *Macrobrachium rosenbergii* (Dougherty and Sandifer, 1984).

The vas deferens of decapods conveys sperm from testis to the exterior in the form of spermatophores. The structure of decapod spermatophores is highly varied. In *Metapenaeus monoceros* it consists of a sperm mass and milky white crystalline structures, whereas in *M. dobsoni* and *Penaeus indicus* it is made of sperm sac, glutinous substance and wing (Vasudevappa, 1992; Mohamed and Diwan, 1993). With regard to the functions of the three different regions of vas deferens viz. PVD, MVD and DVD in moulding the spermatophore and formation of the accessory structure or wing, the present observation agrees with that of Malek and Bawab (1974b) in *P. kerathurus*, Chow *et al.* (1982) in *Macrobrachium rosenbergii*, Subramoniam (1984) in anomuran crabs *Albunea symnista* and

Emerita asiatica, Radha and Subramoniam (1985) in *Panulirus homarus*, Ro *et al.* (1990) in *P. setiferus*, Chow *et al.* (1991a) in *P. setiferus* and *P. vannamei*, Vasudevappa (1992) in *M. dobsoni*, Mohamed and Diwan (1993) in *P. indicus*, Bose (1995) in *P. semisulcatus* and Joseph (1996) in *P. monodon*.

The process of spermatophore formation in *Metapenaeus monoceros* was found to begin when the sperm cells concentrated at the centre of the seminiferous tubules were drained into the PVD where it became a compact sperm mass. The glandular epithelial cells lining the PVD actively secreted an amorphous matrix. This is in agreement with the reports of Vasudevappa (1992) who observed that in *M. dobsoni* the epithelium of PVD secreted a part of the sperm-supporting matrix. In *M. monoceros,* the epithelial cells lining the lumen of PVD had numerous microvilli and the cytoplasm had free ribosomes and mitochondria. Ro *et al.* (1990) also reported that the epithelial cells were in active secretion with numerous mitochondria and vesicles discharged into the lumen. Contrary to the above observations Mohamed and Diwan (1993) observed no glandular epithelial cells in this part of the sperm duct in *P. indicus* and opined that the function of PVD was to transport the mature sperms from the testis to the MVD through peristaltic movement.

The blind pouch that exists at the junction of the PVD and MVD, helps to convert sperm into a coherent mass with the sticky secretions of epithelial cells lining this part of the duct, and orients the sperm mass (Ro *et al.*, 1990; Joseph, 1996). The spermatophoric duct of MVD of *Metapenaeus monoceros* had two typhlosoles and the secretions of the epithelial cells lining the ascending limb fromed the primary spermatophoric layer (PSL) around the sperm mass. The envelopment of the sperm mass by layers of non-cellular materials appears to be initiated in the MVD (Malek and Bawab, 1974b). The PSL was found to be secreted in the MVD in *Penaeus setiferus* (Ro *et al.*, 1990; Chow *et al.*, 1991a); *P. vannamei* (Chow *et al.*, 1991a) and *P. indicus* (Mohamed and Diwan, 1993) whereas in *P. monodon*, Joseph (1996) observed this layer to be secreted in

PVD. In *M. dobsoni*, Vasudevappa (1992) reported that the acellular secretions of the spermatophoric duct formed the PSL and the secondary spermatophoric layer (SSL) around the sperm mass. In *Pleoticus muelleri* the spermatophoric duct secreted a hyaline layer (Diaz *et al.*, 2002).

The smaller duct in the MVD of *Metapenaeus monoceros*, called accessory duct, contains two typhlosoles whose secretions form the accessory structures of spermatophore. The accessory structures have been referred to as wing by King (1948), Vasudevappa (1992), Mohamed and Diwan (1993) and Joseph (1996). Since a wing proper is absent in the spermatophore of *M. monoceros* (Sasikala and Subramoniam, 1987), the milky white crystalline structures accompanying the sperm mass on extrusion from the terminal ampoule is called accessory substance as is done by Chow *et al.* (1991a) in *P. vannamei* which lacks a wing proper. In *M. dobsoni* a strand like wing is formed in the wing duct (Vasudevappa, 1992)

The ultrastructure of the vas deferens of *M. monoceros* is found to be similar to that of other decapod crustaceans, such as *Libinia* (Hinsch and Walker, 1974); *Homarus* (Kooda – Cisco and Talbot, 1986); *P. setiferus* (Ro *et al.*, 1990; Chow *et al.*, 1991a) and *P. monodon* (Joseph, 1996). The columnar epithelial cells lining the lumen are supported on a basal lamina made of connective tissue supported on muscle layers. These secretory epithelial cells function in the formation of the acellular components of the spermatophore – the different spermatophoric layers and accessory structures. In the present study, the electron micrographs showed that the lumen of the accessory duct of MVD contained a granular secretory product, which formed the accessory structures of the spermatophore, as seen in *P. setiferus* (Chow *et al.*, 1991a).

The columnar epithelial cells lining the DVD of *Metapenaeus monoceros* continued their active secretion although typhlosoles were conspicuously absent. The secondary spermatophoric layer (SSL) was seen covering the PSL in this

duct. In *Penaeus indicus*, Mohamed (1989) observed that the secretions of the highly convoluted typhlosoles of DVD resulted in SSL. Vasudevappa (1992) reported the presence of a compact spermatophore and an elongated wing in DVD of *M. dobsoni*. The DVD dilates to form the highly muscular terminal ampoule which has four inter-connecting chambers lined by highly secretary glandular epithelial cells. The secretions of these epithelial cells form the adhesive globules required to join the two spermatophores extruded from both the terminal ampoules to form a compound spermatophore (Mohamed and Diwan, 1993). Terminal ampoule functions to complete spermatophore maturation (Chow *et al.*, 1991b; Diaz *et al.*, 2002).

5.2.5 Ultrastructure of spermatozoa

The spermatozoal ultrastructure of *Metapenaeus monoceros* conformed to the ground plan of unistellate sperm present in most dendrobranchiates and consisted of a spherical main body and a single spike as seen in *Penaeus setiferus* (Lu *et al.*, 1973), *P. indicus* (Mohamed, 1989), *M. dobsoni* (Vasudevappa, 1992), *Parapenaeus longirostris* (Medina, 1994), *P. monodon* (Joseph, 1996) and *Artimesia longinaris* (Sclezo and Medina, 2003). The main body of the spermatozoan consisted of the central nuclear region, a perinuclear cytoplasmic band and an anterior acrosomal cap. *M. monoceros* share with other panaeoids several presumed plesiomorphic features such as the non–membrane–bound filamentous chromatin, the perinuclear arrangement of the cytoplasmic mass and the absence of centrioles and radial arms (Medina, 1995 a).

According to Medina (1995a) the various decapod sperm morphologies can be grouped into three categories instead of the two classically acknowledged ones. They are: 1. Unistellate sperm of Dendrobranchiates (formerly natantian) in which the chromatin is non–membrane–bound, possessing a membrane–bound acrosomal cap that extends anteriorly to form the spike, 2. Multistellate sperm of

Pleocemata (formerly reptantian), which possesses a complete nuclear envelope and 3. Unistellate caridean (Infra order of Pleocemata) sperm having a non–membrane–bound fibrous spike. *Metapenaeus monoceros* spermatozoan and those of other penaeids fall in the first category.

Like any typical decapod sperm, the sperm of *Metapenaeus monoceros* lacks a nuclear envelope. The nucleus of dendrobranchiate sperm is confined to the main body and is decondensed (Pochon – Masson, 1969; Clark *et al.*, 1973). According to Beach and Talbot (1987) a decondensed nucleus may be necessary in decapods to accommodate the unusual acrosome reaction of this group, where the nucleus is rapidly thrust forward to the egg during the reaction. It is probable that the decondensed state of the decapod nucleus and its relatively fluid state facilitate movement of DNA toward the oocyte during the acrosome reaction. Bulk of the main body of the spermatozoa in *M. monoceros* is composed of loosely packed fibrils (non-membrane-bound nucleus) which is postero–laterally bordered by an amorphous cytoplasmic band as in other penaeids (Clark *et al.*, 1973; Kleve *et al.*, 1980; Medina, 1994; Medina *et al.*, 1994).

Acrosomal complex of *Metapenaeus monoceros* exhibited structures similar to those described in other penaeids like *Penaeus aztecus* (Clark *et al.*, 1973); *P. setiferus* (Lu *et al.*, 1973); *Sicyonia ingentis* (Kleve *et al.*, 1980); *P. indicus* (Mohamed, 1989); *Parapenaeus longirostris* (Medina, 1994); *Penaeus monodon* (Joseph, 1996) and *Artimesia longinaris* (Sclezo and Medina, 2003). Shigekawa and Clark (1986) used the term 'cap region' to denote the combination of acrosomal cap and sub–acrosomal substance. In *M. monoceros* the outermost surface of the acrosomal vesicle is bound by a double membrane consisting of the plasma membrane and the membrane of the acrosomal vesicle proper as seen in all the other penaeids. In *Parapenaeus longirostris* (Medina, 1994), *Penaeopsis serrata* (Medina *et al.*, 1994a) and *Artimesia longinaris* (Sclezo and Medina, 2003) a central protruberance at the inner surface of the

acrosomal cap, located immediately opposite the spike has been reported. This is not seen in the present study.

Like in other penaeids, the spike occurred as an anterior extension of the acrosomal complex as both were encompassed in a single membrane (Clark *et al.*, 1973; Lu *et al.*, 1973; Kleve *et al.*, 1980). In *Metapenaeus monoceros* the spike appeared straight but in *Parapenaeus longirostris*, Medina (1994) reported bent spike which may be because of packing of sperm in ampulla. In the penaeid shrimp *Artimesia longinaris*, Sclezo and Medina (2003) observed that the spike showed a filamentous structure internally due to the organization of the spike materials into a bundle of filaments that are coiled into a loose helix. No organelles were observed in the fully mature spermatozoa of *M. monoceros*. The degeneration of organelles is reported in many decapod crustaceans (Moses, 1961; Langrenth, 1969; Clark *et al.*, 1973). It is not only in decapods, among Malacostraca itself the spermatozoa show considerable modification and loss of organelles (Adiyodi, 1985).

5.3 Biochemical Changes During Ovarian Maturation

The analysis of biochemical constituents during the different maturity stages showed cyclic changes in the accumulation of organic reserves in the hepatopancreas, haemolymph and ovary of *Metapenaeus monoceros*. No definite trend was noticeable in the biochemical constituents of muscle tissue with progression of maturity. The variations in total proteins, lipids, carbohydrates, carotenoids and moisture in tissues like ovary, hepatopancreas and haemolymph could be related to the process of vitellogenesis.

5.3.1 Protein

During the process of vitellogenesis a significant rise in the protein levels in the ovary was seen in *Metapenaeus monoceros*. Protein content in the ovary

showed a gradual increase from stage I to stage IV, when the maximum value was observed. There was a sharp fall in the spent stage.

Main component of crustacean yolk is protein (Adiyodi, 1985). Rise in protein content during the reproductive cycle has been reported in many crustaceans. In penaeid shrimps such an increase in protein concentration of ovary with progression of maturation was reported in *Penaeus indicus* (Mohamed and Diwan, 1992), *Metapenaeus dobsoni* (Vasudevappa, 1992) and *Penaeus semisulcatus* (Bose, 1995). Such observations were also made in *Portunus pelagicus* (Rahaman, 1967; Pillay and Nair, 1973), *Clibanarius clibanarius* (Varadarajan and Subramoniam, 1982), and *Panulirus homarus* (Vijayakumaran and Radhakrishnan, 2002).

In the present study there was no significant difference between the hepatopancreatic protein levels at different maturity stages. The maximum concentration of 54.12 % was seen in the stage III followed by a slight decrease to 53.23 % in stage IV. Hence it could be inferred that the mobilization of proteins from the hepatopancreas during vitellogenesis in *Metapenaeus monoceros* was minimal. This is in agreement with the observation made by Varadarajan and Subramoniam (1982) in *Clibanarius clibanarius*. They observed only a slight fall in the hepatic protein content in the mature stage, which was not significant. Such insignificant change in the hepatopancreatic protein, excludes that organ as a contributor to ovarian proteins. Bose (1995) reported an increase in hepatopancreatic protein during the mature and spent stages of *Penaeus semisulcatus*. However this is in contrast to the trend observed by Dy-Penaflorida and Millamena (1990) in *Penaeus monodon*, Mohamed and Diwan (1992) in *Penaeus indicus* and Vasudevappa (1992) in *Metapenaeus dobsoni* who noticed a reduction in the protein values in mature and spent stages. Castille and Lawrence (1989) reported that the protein contents of hepatopancreas increased during maturation in both *Penaeus aztecus* and *P. setiferus*. According to Spaargaren and Haefner (1994) the hepatopancreatic protein increased only

slightly during early vitellogenesis and decreased during vitellogenesis in the brown shrimp *Crangon crangon*.

Adiyodi (1969) reported that in *Paratelphusa hydrodromous* the hepatopancreas was not the major source of vitellogenin protein but could well be the source of vitellogenic precursor. Rankin *et al.* (1989) also did not detect synthesis of polypeptides in the hepatopancreas of *Litopenaeus vannamei* although the high molecular weight polypeptides of 175-200 KDa were abundant in the vitellogenic ovaries. Shafir *et al.* (1992) through *in vivo* studies demonstrated the intense involvement of hepatopancreas in the vitellogenic process in *Penaeus semisulcatus*. Fainzilber *et al.* (1992), carrying out *in vitro* studies in *Penaeus semiculcatus* also came across vitellin specific *de novo* synthesized protein in hepatopancreas during early vitellogenesis, even though it was only less than 15 % of total protein synthesis. In a study on *Litopenaeus vannamei* Arcos *et al.* (2003) did not observe difference in hepatopancreatic protein contents of immature and mature stages. This observation is in agreement with the present study.

In haemolymph of *Metapenaeus monoceros* maximum protein concentration of 72.8 mg/ml was found in stage II, followed by a gradual decrease in the advanced maturity stages, the difference being significant at $p < 0.01$. This could be due to the transfer of haemolymph proteins to the ovary with the progress in vitellogenesis. In *Metapenaeus dobsoni*, Vasudevappa (1992) noticed that the protein level in haemolymph did not show any change from immature to spent condition. Spaargaren and Haefner (1994) reported the decrease of haemolymph protein content during vitellogenesis in the brown shrimp *Crangon crangon*, as seen in the present study. This contradicts the findings of Mohamed and Diwan (1992) and Quinitio and Millamena (1992) in *Penaeus indicus* in which a steady increase was noticed in the protein levels of haemolymph from the early maturing to the fully mature stage and a decrease in the spent stage. Similar trends were also found in *Penaeus vannamei*

(Quackenbush, 1989) and *Pandalus kessleri* (Quinitio *et al.*, 1989) also. Arcos *et al.* (2003) however, observed a depletion of haemolymph protein with the progress of vitellogenesis in *Litopenaeus vannamei* and suggested that this could be as a consequence of accelerated transfer of protein from the haemolymph to the maturing ovaries.

In the muscle tissue of *Metapenaeus monoceros*, even though a protein level of 75 - 76 % was encountered, it did not show any definite trend in relation to the process of maturation and therefore could not be related to the process of vitellogenesis. This is in accordance with the belief that crustaceans do not generally mobilize energy from the muscles for gonad development (Claybrook, 1983). The irregular pattern observed in the muscle protein content might be due to its involvement in growth and metabolism rather than in reproduction. This observation is in agreement with the results obtained in *Penaeus indicus* (Mohamed and Diwan, 1992), *Metapenaeus dobsoni* (Vasudevappa, 1992) and *Penaeus semisulcatus* (Bose, 1995).

5.3.2 Lipid

Lipid content in the ovary of *Metapaenaeus moncoceros* showed a gradual increase from stage I to stage IV, followed by a sharp decrease in stage V. Gehring (1974) noticed that in *Penaeus duorarum*, total lipids, neutral lipids, phospholipids, triglycerides and sterols increased with gonad maturation with peak levels attained in late maturing stage. Similar variations in the lipid composition during ovarian maturation were also reported in *P.setiferus* (Middleditch *et al.*, 1980), *Penaeus japonicus* (Teshima and Kanazawa, 1983; Teshima *et al.*, 1989), *P.indicus* (Galois, 1984; Mohamed and Diwan, 1992), *P.aztecus* and *P.setiferus* (Castille and Lawrence, 1989), *Pleoticus muelleri* (Jeckel *et al.*, 1989), *P.kerathurus* (Mourente and Rodriguez, 1991), *Metapenaeus dobsoni* (Vasudevappa, 1992) and *Crangon crangon* (Spaargaren and Hafner, 1994). In a study on *Metapenaeus affinis*, Sarojini *et al.* (1986)

reported that the fluctuation of lipid was considerably more than that of protein and carbohydrate. Millamena and Pascual (1990) however, observed a peak ovarian lipid level for stage III in *Penaeus monodon*.

Lipid level in the hepatopancreas of *Metapenaeus monoceros* showed an increasing trend with progressing maturity stages, attaining a peak in stage III, decreasing marginally stage IV and showing a sharp decrease in spent stage. Increase in hepatopancreatic lipid during the entire phase of ovarian development was reported in *Penaeus japonicus* (Teshima and Kanazawa, 1983; Teshima *et al.*, 1989), *P.monodon* (Millamena and Pascual, 1990) and *Metapenaeus dobsoni* (Vasudevappa, 1992). Mohamed and Diwan (1992) observed an inverse relationship between ovarian and hepatopancreatic lipid contents in *P.indicus*, which was not seen in the present study. They suggested that as the ovarian lipid content increased there was a simultaneous decrease in the lipid content of the midgut gland, which was due to the mobilization of stored lipids from the latter. Galois (1984), Teshima *et al.* (1988), Castille and Lawrence (1989) and Palacios *et al.* (2000) also recorded a decrease in total lipids in hepatopancreas during ovarian maturation in penaeids. Hepatopancreas is the principal storage site for lipids in crustaceans (Chang and O' Connor, 1983). Hepatopancreas or midgut gland plays a vital role in the supply of energy reserves, especially lipids for maturation process (Adiyodi, 1985; Teshima *et al.*, 1989). In *Metapenaeus affinis*, Sarojini *et al.* (1986) reported a hepatopancreatic build up of lipid with gonad maturation. She further observed that at the peak of ovarian maturation there was partial mobilization of lipid from the hepatopancreas to the ovary. Castille and Lawrence (1989) reported that the hepatopancreatic lipid content increased with maturation in *Penaeus aztecus*, whereas it decreased in *P.setiferus* and suggested that mobilization of stored lipid from the hepatopancreas to the ovary could be more important in the latter species. In the present study the marginal decrease in hepatopancreatic lipid content from stage III to stage IV could be due to its mobilization to the ovary. This is in agreement with the observation made by Sarojini *et al.* (1986) in

Metapenaeus affinis who reported a partial mobilization of hepatopancreatic lipid to the ovary.

In *Metapeneus monoceros*, the lipid level in the haemolymph also showed increase with progression of maturity, followed by a sharp decrease in the spent shrimps which was due to the transport of this nutrient to the ovary and its utilization in the process of maturation. However, the haemolymph lipid concentration in spent shrimps was marginally higher than that of immature ones. This might be due to the possible re-absorption of lipids from resorbing oocytes and the subsequent transport of the same to storage sites as well as the rapid re-maturation capabilities of *M. monoceros*. A similar trend was reported in *Peneus japonicus* (Teshima and Kanazawa, 1978), *P.indicus* (Adiyodi, 1985; Mohamed and Diwan, 1992) and *M. dobsoni* (Vasudevappa, 1992). Spaargaren and Haefner (1994) reported that the lipid level in haemolymph of *Crangon crangon* increased only slighltly prior to pre-vitellogenesis and decreased during vitellogenesis.

In the present study, the lipid levels of muscle in *Metapenaeus monoceros* showed no relation to ovarian maturation which is in agreement with the observations made by Millamena and Pascual (1990), in *Penaeus monodon*. Mohamed and Diwan (1992) and Vasudevappa (1992) observed in *P.indicus* and *M. dobsoni* respectively, that the lipid levels in muscle increased from immature to mature stages and subsequently decreased in spent stage. Vijayakumaran and Radhakrishnan (2002) noticed significant decrease in the muscle lipid of *Panulirus homarus* both at maturity and after spawning, and discussed its possible role in reproduction.

5.3.3 Carbohydrates

In the present study the total carbohydrates in the ovary of *Metapenaeus monoceros* showed a trend similar to that of proteins and lipids. From the lowest

value in stage I ovary, it almost doubled in stage IV, followed by a sharp reduction in the spent ovary. However, the value in the spent stage was more than that in the immature stage. This trend is identical with the observation made by Sarojini *et al.* (1986) in *M. affinis*, Castille and Lawrence (1989) in *Penaeus aztecus* and *P. setiferus*, Vasudevappa (1992) in *M. dobsoni* and Bose (1995) in *P. semisulcatus*. Mohamed and Diwan (1992) reported that in *P.indicus* the carbohydrate content in the ovary remained more or less static with a small peak in the ripe stage. Adiyodi and Adiyodi (1970b) reported in *Paratelphusa hydrodromous* that the sugars present in the hepatopancreas and haemolymph were also found in some abundance in the ovary during the early stage of vitellogenesis, but disappeared progressively as the proteins in the ovary became conjugated in the course of vitellin formation. Lipovitellin or yolk protein is a lipoprotein conjugated to a sugar moiety (Adiyodi, 1968; Kerr, 1969). In *Clibanarius clibanarius*, Varadarajan and Subramoniam (1982) observed that the carbohydrate levels in the ovary became very low during the fully mature stage. They opined that the carbohydrate levels might be remaining constant but the increase in the quantity of other macromolecules could be decreasing their relative concentration. Spaargaren and Haefner (1994) reported that in the ovary of *Crangon crangon* there was no accumulation of carbohydrates during the process of gonad maturation.

In the present study the total carbohydrate level in the hepatopancreas did not show any significant ($p<0.01$) change with maturity. However, the maximum concentration was attained in fully mature stage followed by a reduction in the spent stage. Kumar and Agarwal (1983) reported a mobilization of carbohydrate from the hepatopancreas to maturing gonads through the haemolymph in both sexes of *Paratelphusa masoniana*. Adiyodi and Adiyodi (1970b) found that in the hepatopancreas of *Paratelphusa hydrodromous* free sugars like glucose, galactose and sucrose underwent quantitative and qualitative cyclic fluctuations in relation to ovarian cycle. In *Penaeus notialis* there was mobilization of glycogen from hepatopancreas to the gonads (Trujillo and Luna, 1981). Castille

and Lawrence (1991) noticed an increase in carbohydrate concentration in hepatopancreas of *P.aztecus* whereas no such increase was observed in *P.setiferus*.

Haemolymph carbohydrate levels in *Metapenaeus monoceros* showed significant ($p<0.01$) difference with maturity. The value reached its peak in stage IV followed by a decrease in spent shrimps. A similar trend was reported in *Penaeus hardwickii* (Nagabhushanam and Kulkarni, 1980), *P.indicus* (Mohamed and Diwan, 1992), *M.dobsoni* (Vasudevappa, 1992) and *P.semisulcatus* (Bose, 1995). A doubling in haemolymph glucose level was observed by Dean and Vernberg (1965) in *Callinectes sapidus* and by Telford (1968) in *Cancer borealis*, as the crabs became ovigerous.

No definite trend was observed in the variation of total carbohydrate in the muscle tissue in relation to gonad maturation, in the present study. The variation was not significant even at $p < 0.05$.

5.3.4 Carotenoids

In the present study on *Metapenaeus monoceros* the variations in carotenoid levels in the ovary, hepatopancreas and haemolymph were significant ($p<0.01$). Carotenoid concentration in the ovary increased sharply in relation to maturation reaching a maximum value in stage IV and followed by a decrease in the spent stage. However, in hepatopancreas the highest carotenoid concentration was recorded in stage III and thereafter a decrease was noticed in mature and spent ovaries. Peak carotenoid levels in haemolymph were also seen in stage III. Castillo *et al.* (1982) opined that the hepatopancreas plays a major role in the absorption of carotenoids from food and its carotenoid content fluctuates during vitellogenesis. During early maturation, free and esterified carotenoids accumulate in the hepatopancreas and during secondary vitellogenesis they are mobilized from there via the haemolymph to the ovaries

(Vincent *et al.*, 1988; Harrison, 1990). This accumulation of carotenoids in the ovaries during maturation results in the darkening of the colour. In *Carcinus maenas*, Ceccaldi and Martin (1969) observed that carotenoid pigments get mobilized from the hepatopancreas into the haemolymph during vitellogenesis. The ovarian build up of carotenoids with maturation has been reported in *Plesiopenaeus edwardsianus* (Establier, 1966), *Penaeus indicus* (Mohamed and Diwan, 1992), *Metapenaeus dobsoni* (Vasudevappa, 1992), *P.semisulcatus* (Bose, 1995) and *Litopenaeus vannamei* (Cabello *et al.*, 2003). The dark green colouration of the ripe ovary in *Metapeneus monoceros* is due to the high content of astaxanthin pigment, since the green or blue complexes are often found to contain astaxanthin as the prosthetic group (Tanaka *et al.*, 1976; Castille *et al.*, 1982). The increase in carotenoid concentration with ovarian maturation could indicate the ability of carotenoids to bind yolk protein vitellin into a lipo-glyco-caroteno protein complex by which the macromolecules accumulate in the oocyte cytoplasm as a source of food for the embryo (Harrison, 1990). Carotenoids, particularly astaxanthin, are strong scavengers of free radicals and protect the eggs from oxidative deterioration. They also prevent peroxidation of poly-unsaturated fatty acids (PUFA) in the diet. Interestingly, carotenoids also play an important role in providing the necessary reserves in the embryos and pre-feeding larvae for the development of chromatophores and eyes (Dall *et al.*, 1995).

5.3.5 Moisture

Moisture content of the ovary and hepatopancreas showed significant variations and a declining trend during vitellogenesis in *Metapenaeus monoceros*. Both in the hepatopancreas and ovary, an inverse relationship between the moisture content and maturity stages was observed till stage IV, but the water content showed a sharp increase after spawning with the values greater than those of stage I. An inverse relationship between moisture and fat contents of the ovary was reported by Pillay and Nair (1971) and Sarojini *et al.*

(1986) in *Metapenaeus affinis*, Read and Caulton (1980) and Mohamed and Diwan (1992) in *Penaeus indicus*, Vasudevappa (1992) in *M.dobsoni* and Bose (1995) in *P.semisulcatus*. In *M.monoceros*, the continued deposition of organic materials in the ovarian and hepatic tissues during vitellogenesis results in the reduction of water content.

5.3.6 Composition of mature yolk

In crustaceans, yolk is a combination of proteins, lipids, sugars and some steroid hormones (Adiyodi, 1985). The quality and quantity of yolk protein, vitellin has got a direct and measurable effect on larval survival (Quackenbush, 1991). The present study revealed that water formed the largest constituent of the mature ovary of *Metapeneus monoceros* forming over 68 % of the total weight. The major biochemical constituents are presented as a function of dry ovarian weight, which gives a clearer picture regarding changes in the composition without being masked by changes of moisture content (Clarke *et al.*, 1990). Protein with 59.36 % constituted the major organic reserve in the mature ovary of *M.monoceros* followed by lipid, which constituted 31.23 %. Carbohydrate formed only 3 % by dry weight.

In crustaceans, it is generally accepted that the yolk proteins provide the basic structural material needed for tissue build up during embryonic development, while lipids serve as the major fuel component (Adiyodi and Subramoniam, 1983). The low carbohydrate concentration found in eggs of *M.monoceros* excludes this nutrient as a source of energy. Similar observations have been reported for other decapods (Clarke, 1992; Mohamed and Diwan, 1992). Carbohydrates, though in small quantities are essential during embryogenesis for the synthesis of specific compounds such as chitin for exoskeleton (Holland, 1978).

5.4 Characterization Of Vitellin

In the present study the yolk protein vitellin of *Metapenaeus monoceros* was identified by monitoring its increasing level of expression on native PAGE with progressing maturity (from stage II to IV) and from their absence in immature (stage I) and spent (stage V) stages as well as in testicular homogenate. The vitellin band so identified was subjected to confirmation by selective staining with Sudan black B, periodic acid schiff and alizarin red. From the present study it was found that vitellin of *Metapenaeus monoceros* was a lipo-glyco-protein which possessed a calcium moiety. It had a molecular weight of 326 kDa. Another protein fraction with a molecular weight of 270 kDa was found to occur in ovaries of all maturity stages and testis samples.

A molecular weight of 326 kDa for *Metapenaeus monoceros* got in the present study is similar to those obtained for other penaeids. Qiu *et al.* (1997) have reported a molecular weight of 350 kDa for native vitellin from *Metapenaeus ensis*, after purifying the protein employing gel filtration and ion exchange chromatography. For *Penaeus monodon* vitellin Quinitio *et al.* (1990) and Chang *et al.* (1993) have reported molecular weights of 540 kDa and 492 kDa respectively. Tom *et al.* (1992) purified and characterized vitellin from *P. vannamei* and *P. semisulcatus* and reported a molecular weight of 289 kDa for the former and 283 kDa for the latter. The molecular weight of the isolated vitellin from six crustacean species ranged from 330 to 370 kDa (Wallace *et al.*, 1967). From the above-mentioned studies it is evident that the molecular weight of native vitellin in most penaeids ranges from 300 kDa to 500 kDa.

Only one form of vitellin was observed in the present study. This was in agreement with the single unit vitellins observed in *P. monodon*, *P. vannamei*, *Parapenaeus longirostris* and *Pandalus kessleri* (Tom *et al.*, 1987; Tom *et al.*, 1992; Quinitio *et al.*, 1989; Quinitio *et al.*, 1990; Chang *et al.*, 1993). Chang *et al.*

(1996) isolated two forms of vitellin of 380 and 500 kDa from the ovary of *P. chinensis*.

Vitellogenesis is the process of hormonally regulated synthesis of yolk proteins namely vitellogenin and vitellin. Vitellogenin that circulates in hemolymph is the precursor of vitellin, the yolk protein proper (Gerber-Huber *et al.*, 1987; Kunkel and Nordin, 1985; Van het Schip *et al.*, 1987; Byrene *et al.*, 1989; Chen *et al.*, 1999; Tseng *et al.*, 2002). In the present study vitellin in *Metapenaeus monoceros* was identified as a lipo-glyco-carotenoprotein with a calcium moiety. Viellogenin and vitellins are of low electrophoretic mobility. Moreover, sudan blck staining, PAS positivity and pigment analysis have identified them as a lipo-glyco-caroteno protein (Adiyodi, 1968; Kerr, 1969). In *Penaeus monodon*, Chang *et al.* (1994) identified vitellogenin as a lipo-glyco-phospho protein.

Vitellin from the mature ovaries of *Metapenaeus monoceros* is dark green, similar to those of other penaeids (Quinitio *et al.*, 1990; Qiu *et al.*, 1997). Hence in native PAGE it was possible to visualize this fraction by its green colour, before staining the electropherograms. As yolk protein vitellin or its precursor vitellogenin in crustaceans contains carotenoids, its presence can easily be identified on the basis of its colour. Association with carotenoid pigments gives a specific colour to vitellogenin and vitellin according to species - violet in *Orchestia gammarella*; red-orange in *Idotea balthica basteri* and *Artemia salina*; orange in several crabs; purple in *Uca pugilator* and green in *Carcinus maenas*, *Ligia oceanica* and several shrimps, thus making it possible to distinguish these female specific fractions by colour, before staining the electropherograms (Souty-Grosset, 1984).

In the present study, a band of high mobility was seen in native PAGE gels with ovarian homogenates of all maturity stages (stages I to V) as well as in testicular homogenate. This band was found to have a molecular weight of 270

kDa and was a lipoprotein with calcium. Qiu *et al.* (1997) also reported a similar faster moving band in the ovarian and testicular homogenates of *Metapenaeus ensis*.

SUMMARY

SUMMARY

Metapenaeus monoceros (Fabricius) is an important component of the crustacean landings of India, forming about 10 % of the shrimp landings. Although presently, aquaculture of this species is confined to the traditional extensive systems like Pokkali fields, it has got potential for culture in improved extensive systems. This is all the more important in the context of the various disease outbreaks in shrimp farms and the tremendous economic losses suffered by the industry. Species diversification, preferably by introducing more indigenous species to shrimp aquaculture has been identified as a major step towards alleviating many a problems plaguing the industry at present. A thorough understanding of the reproductive physiology of the species is imperative for perfecting the hatchery technology of seeds, which is a must before attempting commercial aquaculture. It was in this backdrop that the present study was taken up. The significant findings of the present study are summarized in this section.

- The female reproductive system of *M.monoceros* is structurally analogous to that of the other penaeids with a pair of ovaries, oviducts and a single thelycum located between the fourth and fifth pereopods. Middle lobe of the ovary consists of seven lobules with the oviduct arising from the sixth.

- Based on gross morphology the ovarian development was classified into five stages: Stage I or immature, stage II or early maturing, stage III or late maturing, stage IV or mature and stage V or spent. GSI was found to increase from 0.353 in immature females to a maximum value of 6.98 in mature ones.
- Based on histological studies, the process of oogenesis was classified into five stages: pre-vitellogenic, early vitellogenic, late vitellogenic, vitellogenic and spent, which correspond to the stages I to V based on gross morphology.

- Ovary at pre-vitellogenic stage possessed three types of cells – primary oogonial cells, secondary oogonial cells and pre-vitellogenic oocytes with mean OD of 6.43 µm, 17.64 µm and 21.3 µm respectively. Ovary in the early vitellogenic stage was characterized by perinucleolar oocytes with mean OD of 129.46 µm.

- Late vitellogenic oocytes were characterized by a fully eosinophilic granular cytoplasm and mean OD of 174.38 µm. Vitellogenic ovaries were almost wholly occupied by vitellogenic oocytes with mean OD of 230.56 µm, with patches of oogonial cells in between. Cortical bodies which are characteristic features of fully mature ova of most penaeid species were absent in *M.monoceros*.

- Folliculogenesis which started during early vitellogenic stage was completed in late vitellogenic stage. The basophilic follicle cells which were cuboidal in early vitellogenic stage became flattened, thin and ribbon-like in late vitellogenic stage due to the increasing diameter of oocytes.

- Spent ovary was similar to that in pre-vitellogenic stage except for the larger number of resorbing / atretic oocytes present in the former. Vacuolated follicle cells encircled resorbing oocytes.

- Ultrastructural observations of ovaries at various maturity stages revealed that in early vitellogenic oocytes the nucleoli appeared as electron-dense bodies along the inner periphery of the nuclear wall and the ooplasm was rich in RER, free ribosomes and mitochondria indicative of autosynthesis of yolk. Ultrastructure of late vitellogenic ovaries showed arrangement of nucleoli along peripheral nucleoplasm

of the oocytes. Cytoplasm was abundant in mitochondria, free ribosomes, RER, golgi bodies, yolk spheres and yolk platelets.

- Ultrastructural investigations of ovaries at various maturity stages pointed towards the mode of synthesis of yolk, which was mostly autosynthetic. Oolemma did not develop micovilli or micopinocytotic vesicles.

- The male reproductive system of *M.monoceros* is structurally analogous to that of other penaeids with paired testes, vas deferens, terminal ampoules, petasma and appendix masculina. Based on gross morphology, the male specimens were classified into three stages: immature, maturing and mature.

- In immature animals, vas deferens was a simple tube throughout its entire length but in mature ones it consisted of a proximal vas deferens (PVD), medial vas deferens (MVD) and a distal vas deferens (DVD), which terminated in terminal ampoule. The MVD was further divided into a blind pouch, ascending limb and descending limb.

- Histological sections of vas deferens revealed that it was made up of an outer connective tissue sheath, inner layer of muscle fibres and innermost layer of epithelial cells showing structural modifications to aid in the secretory activity of different parts like PVD, MVD and DVD. In the PVD, epithelial cells secreted an amorphous matrix which packed the sperm mass.

- The ascending limb of MVD was divided into two unequal compartments – the larger spermatophoric duct (SD), which secreted the primary spermatophoric layer, and the smaller accessory duct (AD), which secreted the accessory substances. SD had two large

typhlosoles, whereas AD had two smaller ones. In the descending limb of the MVD the septum that divided the SD and AD, after remaining for some distance disappeared, thus uniting the sperm mass and the accessory layer.

- DVD had tall columnar epithelial cells, which secreted the secondary spematophoric layer around the primary spermatophoric layer. Terminal ampoule of mature males was a highly muscular organ with a layer of circular and longitudinal muscle fibres inside the outer connective tissue layer. Lumen of terminal ampoule was divided into chambers lined with basophilic glandular epithelial cells.

- The secretions of the glandular epithelial cells of the terminal ampoule led to the formation of adhesive globules. Ultrastructural investigations showed that the epithelial cells of the terminal ampoule were highly secretory in nature with cytoplasm having numerous free ribosomes and RER and with vesicles discharged into lumen.

- Ultrastructure of MVD showed that the columnar epithelial cells were supported on a basal lamina made of connective tissue supported on muscle layers. Plasma membranes of adjacent cells showed extensive interdigitations.

- Fully mature spermatozoan of *M.monoceros* consisted of a spherical main body and a spike (unistellate spermatozoan). The main body was made up of a central nuclear region, a peripheral cytoplasmic band and an acrosomal cap, which overlaid the nuclear region anteriorly. From the acrosomal cap projected the extended spike towards the anterior of the spermatozoan. The average length of the spermatozoa was 6 µm.

- Estimation of biochemical parameters like proteins, lipids, carbohydrates, carotenoids and moisture in tissues like ovary, hepatopancreas and haemolymph showed cyclic changes in these metabolites with ovarian maturation. In muscle tissue no definite trend was visible in any of the metabolite with ovarian maturation.

- Moisture levels in ovary and hepatopancreas decreased with progressing maturity stages reaching the lowest value in stage IV. The moisture level in spent ovary was more than that of stage I ovary.

- Total protein in the ovarian tissue showed a gradually increasing trend from stages I to IV followed by a decrease after spawning. Hepatopancreatic protein level variations between maturity stages were not significant showing that mobilization of proteins from this organ during vitellogenesis was minimal. However, haemolymph proteins showed a declining trend with ovarian maturation, which could be due to transfer of proteins to ovary.

- Lipid levels in ovary, hepatopancreas and haemolymph showed an increasing trend till attainment of maturity and a sudden decrease after spawning. In the present study it was observed that the hepatopancreatic lipid mobilization to the ovary was only partial.

- Total carbohydrates in ovary also reached the maximum value in stage IV followed by a decrease. However, the change in hepatopancreatic sugars was not significant.

- Carotenoid level in ovary reached its maximum value in stage IV as seen from the dark green colour. However, the maximum carotenoid

concentrations in hepatopancreas and haemolymph were attained in stage III.

- Native PAGE of ovarian tissue revealed that the yolk protein vitellin showed a steady increase in the level of expression from stage I to stage IV ovaries. Yolk protein vitellin of *M.monoceros* had a molecular weight of 326 kDa. Selective staining of the vitellin band in native PAGE showed that it is a lipo-glycoprotein bound with calcium.

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Appendix : Synonyms of the penaeid shrimps mentioned in text

Name commonly used	Synonym
Penaeus aztecus	*Farfantepenaeus aztecus*
Penaeus brasiliensis	*Farfantepenaeus brasiliensis*
Penaeus duorarum	*Farfantepenaeus duorarum*
Penaeus notialis	*Farfantepenaeus notialis*
Penaeus paulensis	*Farfantepenaeus paulensis*
Penaeus subtilis	*Farfantepenaeus subtilis*
Penaeus chinensis	*Fenneropenaeus chinensis*
Penaeus indicus	*Fenneropenaeus indicus*
Penaeus merguiensis	*Fenneropenaeus merguiensis*
Penaeus penicillatus	*Fenneropenaeus penicillatus*
Penaeus occidentalis	*Litopenaeus occidentalis*
Penaeus schmitti	*Litopenaeus schmitti*
Penaeus setiferus	*Litopenaeus setiferus*
Penaeus stylirostris	*Litopenaeus stylirostris*
Penaeus vannamei	*Litopenaeus vannamei*
Penaeus japonicus	*Marsupenaeus japonicus*
Penaeus canaliculatus	*Melicertus canaliculatus*
Penaeus kerathurus	*Melicertus kerathurus*