CHAPTER II
MATERIAL AND METHODS
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The material for this study was mainly obtained from three brackishwater systems of Kerala, namely, Ashtamudi lake, Cochin backwaters and Korapuzha estuary (Fig.1) between August 1992 and June 1995. In addition to this, some specimens of mud crabs were also collected from the Pulicat lake and estuarine areas of Tharankampady along the Tamil Nadu coast for taxonomic purpose. The mud crabs caught from the inshore waters and landed at the Munambam harbour (Kochi) were also occasionally observed for biological studies. Brief description of Ashtamudi lake, Cochin backwaters and Korapuzha estuary are given below.

Ashtamudi lake: The Ashtamudi lake is a palm-shaped brackishwater system having eight branches as the name implies, situated in Quilon District between Lat. 8°48' N and 9°28'N and Long.76°28'E and 77°17'E. With a total extent of about 32 km² water area, this lake also forms an estuary of river Kallada and remains connected with the sea through out the year. The salinity of the lake ranges between 8 ppt and 33 ppt in most part of the year except during the monsoon period when the water gets considerably diluted due to freshwater inflow from River Kallada (Thresiamma Mathew & Nair, 1980; Suseelan & Kathirvel, 1982).

Cochin backwaters: Cochin backwater (Fig.1) and the connected Vembanad lake extent between Alleppey in the south and Munambam in the north (Lat. 9° 30'-10° 12'N) is the largest estuarine system on the west coast of India. It has a length of about 110 km (Devasia & Balakrishnan, 1985). The width varies between a few hundred meters to about 14.5 km and covers approximately an area of 233 km.
Fig. 1. Map showing study area. A-C, observation Centres:
A - Kavanadu, B - Pallipuram, C - Elathur.
M - Munambam.
Hydrographic parameters of Cochin backwater was investigated in great detail by Qasim and Gopinathan (1969). The Cochin backwater is subjected to strong tidal influence from the sea and mixing of freshwater from river systems in the south and north, thus providing estuarine condition with higher salinity gradient towards the vicinity of barmouth. During the south west monsoon season almost freshwater conditions prevail throughout the backwater at the surface with saline condition at the bottom where the depth is considerable.

**Korapuzha estuary:** The estuary is located at Elathur, Kozhikode (Calicut) between Lat.11°21’ - 11°24’N and Long.75°44’ - 75°46’E. This perennial estuary receives freshwater from River Agatapuzha from the northern side and River Dannur Puzha from the southern side.

After a preliminary survey of the mud crab fishery of these brackishwater systems, 3 important landing centres, one each in Ashtamudi lake (Kavanadu), Cochin backwater (Pallipuram / Palliport) and Korapuzha estuary (Elathur) were fixed for regular sampling and monitoring of the catch. Each observation centre was visited once a month and observations were taken on fishing methods, crab landings, species composition (by commercial grades) and biological aspects. Size measurements were recorded sexwise for a random sample of 40-100 crabs of each farm (species) depending on their availability. The measurements were taken for carapace width (CW) extending between the tip of the ninth anterolateral spines to the nearest millimeter using vernier calipers and weight of the whole body using a monopan balance to represent the size of the crab.

**Taxonomic studies**

Various morphological characters, colour marking and behavioral pattern were observed for taxonomic purpose. For conformation of specific identity electrophoretic studies were also undertaken using material from both the coasts of India. The electrophoretic technique adopted was that of Davis (1964) with slight
modifications as described below.

Electrophoresis was carried out on horizontal PAGE system (Multiphor II) from Pharmacia. During standardisation of methodology, different percentages of acrylamide concentrations which determine the porosity of separating gel were tried. They were 7%, 8%, 8.5% and 10% and out of these, separating gel of 8.5% acrylamide concentration along with 4% stacking gel was found to give better resolution of proteins and this percentage was used in further analysis.

The following buffers and other reagents were used for electrophoresis.

1. Acrylamide N. N'- methylene bis Acrylamide mix (30%) stock.
   a. Acrylamide - 29.1 gm (29.1 %)
   b. Bisacrylamide - 0.9 gm (0.9 %)

   Both the monomers were dissolved in minimum volume of double distilled water made up to 100 ml and filtered through Whatman No.1, filter paper and stored in amber coloured bottles at 4°C.

2. Separating gel buffer:
   1.8 M. Tris HCl (pH:8.9)
   Tris - 21.9 gm
   TEMED - 250 µl

   Dissolved in minimum volume of double distilled water and adjusted the pH with 2 N HCl to 8.9 and then made up to 100 ml and stored at 4°C.

3. Stacking gel buffer:
   0.5 M Tris-HCl (pH : 6.8)
   Tris - 6.057 gm
Dissolved in minimum volume of doubled distilled H₂O and adjusted the pH with 2N HCl and made up to 100 ml and stored at 4°C.

4. Ammonium per sulphate (APS) 10% stock 0.1 gm of APS was dissolved in 1 ml of double distilled water. This is used as a stock solution for both separating and stacking gel.

5. Electrode buffer:

0.192 M. Tris-glycine (pH: 8.3)

Weighed out 36 gm of Glycine, dissolved in 2 litres of double distilled water and adjusted the pH to 8.3 by using 2M Tris and made up to 2.5 litres.

6. Sample buffer

Stacking gel buffer - 7 ml
Glycerol (20%) - 2 ml
Bromophenol blue (0.5% stock) - 1 ml

7. Staining solution:

Coomassive Brilliant Blue R - 250 - (0.15 %)

Weighed 0.75 gm of CBB-R 250 and dissolved in 230 ml of Methanol and 230 ml of double distilled water, stirred well for half an hour 40 ml of glacial acetic acid was added and filtered through crude filter paper and stored in amber coloured bottles.

8. Destaining solution:

Methanol - 150 ml
Glacial Acetic acid - 70 ml

Mixed and made up to 1 litre with double distilled water.
The following gel mix was standardised for 8.5% of separating gel.

Separating gel mix - 50 ml

- Acrylamide Bisacrylamide mix - 14.166 ml
- 1.8 M Tris-HCl (pH - 8.9) - 6.25 ml
- D.O.H₂₀ - 4.59 ml
- APS - 25 ml

400 μl of APS from the 10% stock solution was made up to 25 ml with double distilled water so that final concentration of APS in gel mix would be 0.08%.

The above solution was cast into the cassette set for slab gels 2 mm thickness. Two or three drops of Butanol was layered over the gel after pouring the gel to avoid meniscus formation.

Stacking gel: After the polymerisation of the separating gel butanol overlay it was removed and washed with distilled water. Water droplets, if any, was removed by using filter paper strips. Then the following composition of the stacking gel mix was poured over the separating gel.

- Acrylamide - 3.33 ml
  + Bisacrylamide
  - 0.5M Tris HCl - 6.25 ml
  (pH - 6.8)
  - D.D.H₂₀ - 15.295 ml
  - TEMED - 25 μl
  - APS (10% stock) - 100 μl
The gel cassette was kept for ten to fifteen minutes for the polymerisation of stacking gel.

The crab samples were collected and transported in live condition to the laboratory and stored at -20°C in deep freezer. At the time of processing the samples were taken out from the freezer and allowed to thaw to room temperature. About 1 g of body muscle was cut out using scissors and after washing with ice cold double distilled water, tissue was thoroughly minced keeping it in a cavity block kept over ice slab. Minced tissue was transferred into an ice cold homogenising glass tube and one 1 ml of ice cold double distilled water was added and contents homogenised using a motor driven tissue homogenizer with rotating teflon pestle. To the homogenate 1 ml of ice cold double distilled water was added and homogenised again. The homogenate was transferred into ice cold centrifuge tubes and the centrifugation was carried out for 20 minutes at 10000 rpm at 4°C in a high speed refrigerated centrifuge (Savant, USA).

The supernatant obtained after the centrifugation was used for electrophoretic analysis. 35 ml of sample supernatant was mixed with equal volume of sample buffer (1:1 ratio) in a microplate. The polymerised gel was set on the multiphorII electrophoretic system and the pre run was carried out at 75 mA for 10 minutes. The sample (70 ml) was loaded into the well and electrophoresis was carried out at a constant current of 65 mA for 3 hours. The temperature of the cooling plate was maintained at 10°C throughout the electrophoretic run.

Immediately after the electrophoresis, the gel was transferred into a plastic tray containing staining solution and kept immersed in it for about 1 1/2 hours. After staining, the staining solution was removed and destaining solution was added. After about 30 minutes destaining solution was replaced with fresh destainer. Photographs of the protein band on the gel was taken after complete removal of the background stain.
Study of reproductive biology

The crabs were sexed based on the shape of abdomen and number of pleopods. In the case of female crabs, the pleopods were examined for the presence of eggs or egg remnants or their absence on pleopods, colour of egg mass was also noted.

The anatomy of male and female reproductive systems was studied by dissecting mature crabs. After dissection, the gonads and other parts were examined under a dissection microscope for closer study of anatomical features. For studying maturation process, the ovaries were classified into five maturity stages by modifying the methods suggested by Haefner (1977). For histological studies, gonads and other parts of the reproductive systems were cut and fixed in Bouin's fluid from live crabs. The tissues fixed in Bouin's fluid were washed overnight in running tap water to remove the excess picric acid. These tissues were dehydrated using an alcohol series (30-100% alcohol) and cleared in methyl benzoate. The tissues were further cold impregnated with wax shavings in a 1:1 ratio. Subsequently the solvent was evaporated by placing the tissue in an oven at 58°C. The tissues were transferred through two changes of fresh molten wax (Paraffin wax with cersin, BDH, MP 58-60°C). Tissue blocks were prepared by using paper boats or small glass troughs after proper orientation.

Serial sections of block were cut at approximately 6-8 μ thickness using a rotary microtome (Weswox Optik model T-1090A). Sections were affixed on clean glass slides using fresh Mayer's egg albumin-glycerol (1:1 V/V) and flattened by placing on a slide warmer with a drop of distilled water. Subsequently the water was drained off and slides were then used for histological observations. Staining was done by using Harris haematoxylin stain (Preece, 1972) with 1% aqueous eosin as the counter stain. Sections to be stained were first deparaffinized in two changes of xylene and then hydrated through a down series of ethanol grades. They were
then blued using tap water or lithium carbonate. Eosin stained sections were repeatedly washed in 95% alcohol to remove the excess eosin. Slides were further dehydrated in absolute alcohol and cleared in xylene and mounted with DPX or Canada balsm of neutral pH. Mounted slides were examined under a monocular microscope.

Micrometric measurement of oocyte in different stages of maturation were taken using an occular micrometer (ERMA, Japan) calibrated with stage micrometer. As oocyte strongly deviated from a spherical shape, the average of the largest and smallest axes of oocyte found in a maturity stage was taken. Spermatogonial and sperm cell diameter were also recorded in the same manner.

Photomicrographs of histological preparations of ovary and testes were taken using a binocular compound microscope ('Microstar', American Opticals, U.S.A.) and with a camera unit. Appropriate projection eye piece was used and the photographs were taken using 24 x 36 mm ORWO NP 22 (125 ASA, Panchromatic) black and white negative film.

The fecundity was calculated by counting the number of eggs present on the pleopod in ovigerous condition. As the loss of eggs during incubation was not known, only crabs carrying eggs in the early stages of embryonic development were used for this purpose. The egg carrying pleopods were first removed from the crab and immersed them in concentrated solution of sodium hydroxide as suggested by Melville Smith (1987). The eggs became free from the pleopods after 3-6 hours. The eggs were then filtered and weighed to nearest 0.1 mg using an electronic balance (Mettler, PC 440, Switzerland). A sample of the egg mass thus separated was weighed and counted and total number of whole egg mass was determined using the formula.

\[
F = \frac{P}{P1 \times n}
\]

Where \( P \) = the weight of egg mass; \( P1 \) = the weight of the sub sample and \( n \) = the number of sub samples.
n = the total number of eggs in the subsample.

**Growth studies**

Growth in juvenile phase was studied by rearing the baby crab in laboratory. In the case of *S. oceanica*, the study was conducted using animals produced in the laboratory. Fifteen healthy instar-I were used for the study. In the case of *S. serrata* the seed were collected from stake nets operated in the tidal canal of Vypeen Island. The animals thus collected were acclimatized at 15 ppt salinity. From this, 25 animals of about 2 cm carapace width were sorted out and used for the experiment.

All the rearing trials were conducted at 15 ppt salinity. For this filtered sea water diluted with dechlorinated tap water was used. These animals were stocked individually in 40 litre plastic tubs containing 35 litres of diluted sea water (15 + 2 ppt). The tubs were arranged in such a way that all the tubs received uniform light condition. Animals were fed daily with clam meat. Every day before feeding, excess feed and faecal matter were siphoned out and the water was replaced to the original level. The water in the tubs was changed completely once a week. Crabs were observed every day for moulting, and at each moult the carapace width as well as weight measurements were taken as described by Ong (1966). The experiment was conducted during the period May 1994-April 1995.

For analysing growth in natural environment, monthly size frequency distribution was worked out by grouping length measurements in 10 mm size classes. From the size frequency data, modal values were estimated using Bhattacharya (1967) method and using these modes growth parameters K and L were estimated following Gulland and Holt (1959) plot method using FISAT Computer programme (FAO-ICLARM stock assessment tools (ver.1.0)).

Data on growth was also collected from the grow-out culture pond at
monthly intervals. On each observation day 20-25 crabs were collected randomly using the ring nets and carapace width and weight measurements were taken and growth calculated.

Estimation of carapace width - weight relationship

Carapace width-weight relationship was estimated using the log form of allometric growth equation \( w = aL^b \), where \( w \) = expected weight, \( L \) = total carapace width and ‘a’ and ‘b’ are constants calculated by the least square method. The difference in CW - weight relationship between sexes was tested by ANOVA using suitable computer programme.

Study of sex ratios

The sex ratios of commercial catches were analysed month-wise and size-wise for both the species and the same were tested using the chi-square analysis as per Panse and Sukhatme (1978).

Assessment of crab landings

On each observation day, the total crab landing at a particular centre was recorded species wise and grade wise. In addition to this, similar information for other fishing days of the month was also recorded from merchants’ diary for as many days as possible. The average daily crab landing was worked out from the data thus obtained and raised to the number of fishing days to assess the monthly total crab landing of that centre.

Larval rearing studies

For studying incubation, larval development and seed production, berried specimens were collected from the wild. Berried specimens of Scylla oceanica were obtained from shrimp trawlers and those of Scylla serrata from Chinese dip nets operating near Cochin bar mouth. Spawners were immediately transferred to water of same salinity in 50 l aerated plastic jars and transported to the field labo-
atory of CMFRI, Narakkal. At Narakkal the spawners were transferred to 2 ton fibreglass tanks filled with clean filtered and aerated sea water. Live clam was opened and given to the animals as feed. Every day half of the water was exchanged with fresh seawater after siphoning out the excess food, excreta and shed out eggs. Continuous aeration was given throughout the incubation period. Development of eggs was monitored by taking small samples from the berry using a small forceps and observing it under microscope. Photomicrographs of developing eggs were also taken.

Only active zoeae were collected from the incubation/spawning tanks for larval rearing studies. In order to facilitate replication of the experiments, zoea were reared in 2200 ml glass troughs of hemispherical shape filled with 2 litres of filtered and aerated sea water. Each trough was stocked with larvae at the rate of 50 numbers/litre. For every larval feed three replicates were tried. Salinity was maintained at 34-35 ppt., pH 7.8-8.2 and temperature 27-30°C. Every day larvae were counted and transferred to fresh sea water using wide bore pipette. Troughs were arranged in such a way to ensure uniform light conditions to all the containers.

During the larval rearing experiments five larval feeds such as *Chlorella marina*, *Brachionus plicatilis*, *Artemia salina* nauplii, egg custard and microencapsulated feed were given individually and in combination. The live feeds were developed in the laboratory. Inoculum for the pure culture of *Chlorella marina* was obtained from CMFRI algology laboratory in sterilized 250 ml conical flask. Filtered sea water of 35 ppt salinity was taken in clean 3 litre Haufkin culture flasks and plugged with cotton. It was sterilized by boiling it on a hot plate for 5 minutes and the water in the flask was allowed to cool over night. To this A, B and C solutions of Conway or Walne's nutrient medium was added using a sterile pipette. Composition of the medium is given below.
Conway or Walne's medium:

Solution A

Sodium nitrate/Potassium nitrate 100 g
Sodium phosphate 20 g
Ferric chloride 1.3 g
Manganese chloride 0.36 g
Boric acid 33.4 g
EDTA 45 g

Dissolved in 1 litre of distilled water.

Solution B (Trace metals)

Zinc chloride 2.1 g
Calcium chloride 2.0 g
Ammonium molibdate 2.0 g
Copper sulphate 2.0 g
Distilled water 100 ml

Dissolved in 1 litre of distilled water.

Solution C (Vitamin stock)

B 12 5 mg
B 1 100 mg

Dissolved in 100 ml of distilled water.

One ml each of solution A and B and 0.1 ml of solution C were added to 1 litre of algal culture medium. To this medium pure culture was inoculated with-
out any contamination and the plugged flasks were kept under direct sun light near the windows. The inoculated *Chlorella* developed into fully grown cultures within 4-6 days and served as stock for mass culture.

Mass culture was done in 50 litre perspex tanks filled with filtered aerated sea water enriched with Conway or Walney’s medium. One litre Inoculum was used for 50 litres of sea water. Culture attained harvestable concentration within 5-7 days. Concentration of the culture was measured using haemocytometer. Every day half of the culture was harvested and replaced the same with fertilized sea water.

The rotifer *Brachionus plicatilis* used for the study was obtained from local brackishwater ponds. Pond water was filtered through a zooplankton net and the plankton collected was carefully transferred to 1 litre plastic container filled with pond water. Plankton sample so collected was brought to the laboratory and egg bearing parthenogenic females were isolated and slowly acclimatized to *Chlorella* medium taken in a beaker (1 litre) with mild aeration. Within four days the stock was ready. Mass culture of rotifers were done in 10 liter flasks filled with *Chlorella* medium. Rotifer was inoculated at the rate of 10/ml, and within 4-6 days it attained a density of 250 ml. Rotifers were harvested by filtering the culture using a zooplankton net and the same volume was replaced with fresh *Chlorella* medium. Baker’s yeast was also given to the fast multiplying rotifers when *Chlorella* was insufficient to feed them.

*Artemia* cysts were procured from Prime Artemia Incorporated, USA and Ballarpur Industries, Gujarat. Artemia cysts were hatched in 5 litre glass beaker containing sea water of 30 ppt salinity and 8-8.2 pH. Cysts were added at the rate of 2 g/litre and vigorous aeration was provided from the bottom of the container to ensure that the cysts were in suspension. For optimizing hatching continuous illumination (40 watt fluorescent) was provided 20 cm above the water surface.
Hatching was over in 18-24 hours in the case of the US brand and 36-40 hours in the case of the Indian brand. Nauplii were harvested from the bottom of the container after light and air were turned off for 15 minutes and siphoning them to a zooplankton net without disturbing the unhatched eggs settled at the bottom. *Artemia* nauplii were rinsed in fresh seawater before feeding. *Artemia* suspension was made by grinding the nauplii in a mixer for few seconds.

Fresh chicken eggs were used for making egg custard. Egg white and yolk were taken in a bowl and blended with little fresh water. This mixture was then steamed till it hardened well. This steamed egg was passed through 200 micron sieve to produce desired particle size.

Microencapsulated feed used was obtained from SANDERS BRINE SHRIMP COMP. INC., USA, whose composition is given below.

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<tr>
<td>Protein</td>
<td>46%</td>
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<tr>
<td>Lipid</td>
<td>18%</td>
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<tr>
<td>HUFA</td>
<td>2.5%</td>
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<tr>
<td>Ash</td>
<td>0.12%</td>
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<tr>
<td>Moisture</td>
<td>8.5%</td>
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**Crab culture experiments**

Crab fattening experiments were carried out in a brackishwater pond of 0.05 ha (Plate 40a) situated in Vypeen island. The pond was connected to Cochin backwaters by a 5 m wide canal of about 200 m length. The pond had a strong bund of 3 m width at the top. A sluice gate of 1 m width connected the pond to the tidal canal. Average depth of the pond was 1.5 m.

In order to prevent escape of crabs over the bund a perimeter fencing of 1 m height was provided using nylon netting of 20 mm mesh size (Plate 40a).
netting was supported by split bamboo sticks fixed on the dyke at 2 m intervals. The net was fixed in such a way that the lower portion of the same was buried in soil and secured firmly with bamboo pegs. The sticks were planted in slanting manner overhanging the pond to prevent crabs from climbing over the fence. Bund near the sluice gate was reinforced with bamboo matting as the crabs showed a strong tendency to burrow near the sluice gate. A watchman was arranged to keep vigil to prevent poaching. Four fattening trials were conducted between August 1992 and April 1993. The fattening period varied from 45 to 60 days.

During the experiments physico-chemical parameters like temperature, salinity, dissolved oxygen and pH were measured at an interval of 7 days.

Grow-out culture experiment was carried out in a pond of 0.1 ha. area, which was connected to the Cochin backwaters through a 1 m wide sluice gate. A 4 m wide strong bund separated the pond from the backwater on one side and the other 3 sides were surrounded by land mass. The pond had an average water depth of 1.2 m. A perimeter fencing was also given to prevent the escape of crabs as in the case of fattening pond. Since the crabs showed tendency to burrow near the sluice gate, the bund adjoining the sluice gate was reinforced with bamboo mats. Only one culture experiment was carried out in this system, which extended for a period of six months from August 1993 to February 1994. The physico-chemical parameters were also recorded as in the case of fattening experiments.

Analysis of physico-chemical parameters

Temperature: A centigrade thermometer graduated 0-50°C was used to measure water temperature.

pH: pH was determined using a digital pH meter in the laboratory after sampling.

Salinity: Water samples collected from 5 cm below water surface was used for
salinity estimation. The salinity was determined by Mouris titration method (Strickland & Parsons, 1968).

**Dissolved oxygen:** Dissolved oxygen was estimated by “Winkler method” (Strickland & Parsons, 1968).

The proximate composition of crab meat before and after fattening was analysed to find out the possible biochemical change in the crab meat during fattening. Standard methods AOAC (1965) were used for determination of moisture, ash, crude protein, total lipid, crude fibre and nitrogen free extract (NFE).