Chapter- 2

Materials and Methods
Test organism: *Oziotelphusa senex senex*
The freshwater crab, *Oziotelphusa senex senex* Fabricius was used as the experimental animal in the present investigation.

**CLASSIFICATION**

<table>
<thead>
<tr>
<th>Phylum</th>
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<tr>
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The freshwater crab, *Oziotelphusa senex senex* is an edible decapod crustacean distributed throughout South India. The crab, *Oziotelphusa senex senex* inhabits normally in the rice fields and also in irrigation canals, tanks and ponds and is known as "rice field crab".

It makes burrows in soft mud along the edges of the paddy fields, and canals and lives in them with partially filled water. They do not inhabit in brackish or marine waters, but can survive for longer periods on land and can be considered as semi-terrestrial animal. The crabs can also tolerate a wide range of salinities. It is carnivorous, feeding on worms, insects and so on, and also indulges in cannibalistic feeding on younger crabs.

### 2.1. Procurement of animals and housing

Intact, inter molt (Stage C4) adult freshwater crabs, *Oziotelphusa senex senex* Fabricius with a body weight of $30 \pm 3$ g and body size of $39 \pm 3$ mm were collected from the rice fields and irrigation canals around Tirupati (13° 36' N, 79° 21' E), Andhra Pradesh, India. The animals were housed 6-8 per glass aquaria (length: width:
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height = 60: 30: 30 cm) with sufficient ambient medium (salinity: 0.5 ppt) and transferred to fresh medium everyday. They were acclimatized to the laboratory conditions (temperature 27 ± 1°C; relative humidity 75% and a light period of 12 h) for 7 days. During their sojourn, the crabs were fed with sheep meat *ad libitum*. Feeding was stopped one day before the commencement of the experiment to avoid changes due to prandial activity.

2.2. Test chemicals

The following test chemicals (Fig. 2.1 A, B and C) were used in the present study: 3-hydroxytyramine (3HT), 5-hydroxytryptamine (5HT), N-Acetyl-5-hydroxytryptamine (Melatonin). 3HT and 5HT were purchased from Sigma Chemical Co. (St Louis, MO) and melatonin was purchased from MP Biomedicals Inc., (France).

Figure 2.1. Structures of the test chemicals

Dopamine  
MW: 180.6

Serotonin  
MW: 212.7

Melatonin  
MW: 232.3
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2.3. Preparation of stock solutions

1000 μmolar stock solution was prepared by dissolving 3HT or 5HT in crustacean saline (Van Harreveld, 1936). Melatonin stock solution (1000 μmolar) was prepared by dissolving melatonin in ethanol and diluted with crustacean saline to obtain a final ethanol concentration of 1%.

The required concentrations of 3-HT, 5-HT and melatonin (10^-5 mol/crab to 10^-12 mol/crab) of the chemicals were prepared by diluting the stock solution with crustacean saline. This was done on the day of the experiment, prior to the injection of the chemical to the animal. The dose range varies from 100 μmolar to 100 pmolar (based on 30% of the body weight being equivalent to hemolymph volume (Reddy and Ramamurthi, 1998) in circulation.

2.4. Eyestalk ablation

In order to deprive the animal of the eyestalk hormones, both the eyestalks were removed from the crabs by cutting off the stalks at the base without prior ligation, but with cautery of the wound after operation.

2.5. Preparation of eyestalk extract

Eyestalk extract was prepared according to the method described by Rao (1965). The aqueous extract was prepared by crushing the eyestalks in a pre-cooled mortar with a pestle in a small amount of distilled water. The suspension was centrifuged at 2500 rpm for 20 min at 0°C. The resultant supernatant was diluted with cold distilled water to give a concentration of 2 eyestalk equivalents per 20 μl.

2.6. Hemolymph collection

The hemolymph was collected through the arthrodial membrane of the coxa of the third pair of walking legs of the crab, using a hypodermic syringe. The hemolymph was immediately used for the determination of total carbohydrate levels.

2.7. Isolation of hepatopancreas and muscle

The crabs were dissected and the hepatopancreas was quickly isolated. The muscle tissue was isolated from the chelate legs. The tissues were blotted on a filter paper to remove adhering body fluid and weighed using an electronic balance to the nearest milligram. Homogenates were prepared separately for the use of estimation of
levels of total carbohydrates, glycogen and determination of activity levels of phosphorylase. Estimation of carbohydrates and enzyme assays were carried out between 8 AM and 11 AM to obviate circadian variations.

2.8. Carbohydrate metabolism

2.8.1. Estimation of total carbohydrates

The levels of total carbohydrates were estimated as total anthrone positive substances by the method of Carroll et al. (1956). A 5 % (W/V) homogenate of hepatopancreas and muscle tissues was prepared in trichloroacetic acid (10% W/V). The homogenates were centrifuged at 3000g for 15 min at 4°C. To 0.2 ml of TCA supernatant, 4 ml of anthrone reagent was added in a test tube and kept in a boiling water bath for 15 minutes. After cooling, the developed colour was read at 620 nm using a spectrophotometer (Hitachi U 2000) against a reagent (anthrone) blank. Total carbohydrate content was expressed in mg of glucose/gm wet weight of the tissue. A standard was prepared using the same procedure as described with a known amount of glucose (Analar grade) and its optical density was used for the calculation of total carbohydrates in the samples.

To 0.5 ml of the hemolymph 0.5 ml of 10% TCA (W/V) was added in a test tube. Then the contents were mixed well, centrifuged at 3000 g for 15 minutes and the clear supernatant was used for hemolymph total carbohydrate estimation. The levels of total carbohydrates in the hemolymph was determined essentially similar to the method described above using 0.2 ml of hemolymph (1:1 hemolymph : 10% TCA) instead of tissue homogenate.

2.8.2. Estimation of glycogen

The glycogen content was estimated according to the method described by Carroll et al. (1956) using anthrone reagent. To 1.0 ml of TCA supernatant (prepared for estimation of total carbohydrate), five volumes of 95% ethanol were added with thorough mixing. The contents were kept in a refrigerator (4°C) over-night for precipitation of glycogen and centrifuged for 15 minutes at 3000 rpm. The precipitate was dried by placing the tubes in an inverted position at room temperature for 10 minutes. The precipitate was then dissolved in 0.5 ml of distilled water. To 0.2 ml of this solution, 4.0 ml of anthrone reagent was added in a test tube. After mixing the
contents, the tubes were kept in a boiling water bath for 15 minutes and cooled immediately. The developed colour was read at 620 nm using a spectrophotometer (Hitachi U 2000) against a reagent (anthrone) blank. The amount of the glycogen was read from the standard graph prepared with D-glucose and expressed as mg glucose/gm wet weight of the tissue.

2.8.3. Assay of tissue phosphorylase

The activity of glycogen phosphorylase was assayed in hepatopancreas and muscle tissues by the method of Cori et al. (1955), in the direction of glycogen synthesis, by the determination of the amount of the inorganic phosphate liberated from glucose-1-phosphate.

The hepatopancreas and muscle tissues were homogenized (5% W/V) in aqueous medium containing sodium fluoride (0.1 M) and ethylene diamine tetra acetic acid (EDTA) (0.037 M) (pH 6.5) as recommended by Guillory and Monmaerts (1962). The homogenate was centrifuged at 2500 rpm for 15 min at 4°C and the supernatant was diluted to 4 times (1:3) with cysteine hydrochloride (0.03 M) and β-glycerophosphate (0.015 M) buffer at (pH 6.5). The diluted enzyme (0.4 ml) was added to 0.2 ml of 2% glycogen solution and the reaction was initiated by the addition of 0.2 ml of glucose-1-phosphate (0.016 M) to one tube for estimating the active phosphorylase ('a') activity. To the other tube in addition to 0.2 ml of 2% glycogen, 0.2 ml of 1:1 mixture of 0.016 M glucose-1-phosphate and 0.004 M adenosine-5-monophosphate (5'AMP) was added to estimate the total phosphorylase ('ab') activity. The contents were incubated at 35°C for 15 minutes, for determining total phosphorylase and 30 minutes for active phosphorylase. The reaction was arrested by the addition of 5 ml of sulphuric acid (5N H₂SO₄). The released inorganic phosphate (Pi) was estimated by the method of Fiske and Subbarow (1925). The phosphorylase activity was expressed as μ moles of Pi liberated/mg protein/hr.

2.8.4. Estimation of inorganic phosphate

To 1.0 ml of solution, 1.0 ml of 2.5% ammonium molybdate solution was added. The contents were mixed well and 0.4 ml of 0.2% of 1-2-4-amino naphthosulphonic acid (ANSA) reagent was added. The color developed was immediately diluted to 10 ml with distilled water and allowed to stand for 5 minutes.
The blue colour developed was measured at 720 nm in a spectrophotometer (Hitachi U 2000) against reagent blank.

2.8.5 Validity of the Experimental Procedure

2.8.5.a. Aliquots for enzyme assay

Aliquots were selected for the enzyme assay, such that the initial rates were approximated as nearly as possible, yet providing sufficient product to fall within a convenient range for detection by spectrophotometric measurement.

2.8.5.b. Enzyme units

The soluble protein content of tissue homogenate (enzyme source) was estimated using folin phenol reagent (Lowry et al., 1951). This was used for the expression of enzyme activities in standard units that is in \( \mu \) moles of product formed /mg protein/h

2.8.5.c. Substrate requirements

All the enzyme assays were made under the conditions following zero order kinetics. Substrate concentration was maintained at a higher level, such that it was not a limiting factor for the enzyme activity.

2.8.6.d. Lambert-Beer's Law

All the products of the reactions were measured by using the colorimetric procedures in which the optical density (absorbance) of the resulting colored complexes was proportional to the concentration of the reaction products.

2.8.7.e. Enzyme Nomenclature

The nomenclature of the enzymes followed in the present study was according to the Report of the Commission of Enzymes of the International Union of Biochemistry (Pergamon Press, Oxford, 1961).

2.9. Molting studies

2.9.1 Determination of molt stages

The molt stages were determined according to the method described by Reddy (1991) for this crab, *Oziotelphusa senex senex*. Molt stages were determined based on the setal development in the mastigobranch of 3rd maxillepede. The changes in the
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setal development were observed under a phase contrast microscope (Olympus, Model BX41TF, Japan).

2.9.2. Determination of Y-organ and mandibular organ indices

The Y-organs and mandibular organs were isolated and immediately placed in ice-cold crustacean physiological saline (Van Harreveld, 1936) to remove any adhesive tissues. The organs were carefully removed from the saline and blotted on paper towels and weighed wet to the nearest milligram using a Shimadzu electronic balance. The Y-organ, and mandibular organ indices were determined using the formula:

\[
\text{Y-organ index} = \frac{\text{Wet weight of the Y-organ (g)}}{\text{Weight of the crab (g)}} \times 100
\]

\[
\text{Mandibular organ index} = \frac{\text{Wet weight of the Y-organ (g)}}{\text{Weight of the crab (g)}} \times 100
\]

2.10. Reproduction

2.10.1. Determination of ovarian index

The crabs from control and experimental groups were weighed and the ovaries were excised, cleaned in crustacean saline (Van Harreveld, 1936), blotted on filter paper and weighed wet to the nearest milligram. The ovarian index was determined using the following formula:

\[
\text{Ovarian index} = \frac{\text{Wet weight of the ovary (g)}}{\text{Weight of the crab (g)}} \times 100
\]

2.10.2. Histological studies of the ovary

The ovarian sections were prepared according to the method described by Bancraft and Stevens (1982). The ovaries were isolated intact, dried on filter paper.
fixed in Bouin's fluid (picric acid: formaldehyde: acetic acid, 75:25:5). After 24 h, the material was washed thoroughly and dehydrated with ascending alcoholic series. After cleaning in xylene, the tissues were embedded in paraffin wax (m.p. 56-58°C). The sections were cut at 7μm thickness, stained with hematoxylin and counterstained with eosin. Different reproductive stages were observed under a phase contrast microscope (Olympus, Model -BX41TF, Japan) and each stage was photographed.

2.10.3. Measurement of oocyte diameter

The diameter of 20 oocytes from each ovary was measured using an ocular micrometer under a compound microscope (Olympus, Model-BX41TF HB, Japan). The measurements were made on the longest and shortest axes of each oocyte, both dimensions were added and the mean was taken as mean oocyte diameter.

2.10.4. Isolation of ovarian vitellogenin

Ovarian vitellogenin was isolated using the protocol described by Tsukimura et al. (2000). In brief, 10% (W/V) ovarian homogenates were prepared in homogenization buffer (pH 7.8) containing 0.1 M sodium chloride (NaCl), 0.05 M Tris base, 1 mM ethylenetriamine tetra acetic acid (ETTA) and 0.1% tween-20 with 10 mg/ml phenylmethylsulfonyl fluoride (PMSF), using an ice cold glass-glass homogenizer. The homogenate was centrifuged at 4000 X g for 5 min. at 4°C. The resultant supernatant was collected and again centrifuged at 20,000 X g for 20 min. at 4°C. To the supernatant, ammonium sulfate (SAS) was added to produce a 25% SAS solution. After mixing the contents for 1 h at 4°C, the homogenate was centrifuged at 20,000 X g for 10 min. at 4°C. The supernatant was collected and SAS was added to produce 40%, 50% and 60% SAS solution sequentially. The pellets of 60% SAS solution was suspended in homogenization buffer and dialyzed three times against homogenization buffer at 4°C for 12 h each. The purified ovarian vitellogenin was stored at -20°C until further use.

2.10.5. Estimation of vitellogenin content

The ovarian vitellogenin content in each purified sample was estimated by the method of Bradford (1976). To 0.1 ml of isolated vitellogenin sample, 0.9 ml of 0.1M phosphate buffer (pH 7.5) was added followed by 5.0 ml of Bradford reagent. After mixing the contents, the absorbance of the colour was measured at 595 nm against the
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reagent blank in a Hitachi Model U-2001 UV-VIS spectrophotometer. A standard curve was prepared against graded concentrations of bovine serum albumin and the optical density values were used to determine the protein concentration in the purified samples. The protein content was expressed as mg/g tissue.

2.10.6. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The quality of isolated ovarian vitellogenin was determined using discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), which was carried out on 10% polyacrylamide (with 1.5M Tris HCl buffer; pH 8.8) separating gel with 6% polyacrylamide (with 0.5M Tris HCl buffer; pH 6.8) stacking gel with an electrophoresis buffer (0.124M Tris buffer; 1.01M glycine and 0.6% SDS) in a mini slab gel unit. The gels were stained with 0.1% coomassie blue G-250 (in acetic acid, methanol and distilled water in a ratio of 10: 50: 40, respectively) and destained with a solution containing methanol, glacial acetic acid and water (20:7:73). The stained gels were used to determine the molecular masses of purified ovarian vitellogenin. The molecular weight markers (lysozyme, 14.1KDa; carbonic anhydrase, 29.0 KDa; ovalbumin, 43.0 KDa; bovine serum albumin, 66.0 KDa and phosphorylase b, 97.4 KDa) were obtained from Genei, Bangalore (India).

2.10.7. Preparation of antisera

Polyclonal antisera were raised in New Zealand white rabbits by injecting equal volumes of purified vitellogenin (700 µg) and Freund’s complete (in the first week) and incomplete (2nd, 3rd and 4th weeks) adjuvant. After one week of the last injection, blood was collected by ear puncture. The blood was kept at room temperature for 1 hour and then at 4°C overnight. The blood was centrifuged at 1000 X g, 4°C for 20 min. and the titer of anti-sera was determined using double immunodiffusion according to the method described by Ouchterlony (1958). Collected anti-sera were stored at -20°C until further use.

2.10.8. Western blot analysis

Western blotting was performed as per the method described earlier (Burnett, 1981). Proteins were transferred onto a nitrocellulose membrane (0.45 µm) from SDS-PAGE gels using a mini Trans-Blot system (BioRad).
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The transfer was conducted at 100 V for 120 minutes in TGM buffer (0.025 M Tris, 0.192 M glycine and 20% methanol, pH 8.3). The membrane was blocked overnight with 5% nonfat dehydrated milk in 150 mM phosphate buffered saline (PBS), pH 7.2. The membrane was then washed 5 times for 10 minutes in PBS containing 0.1% Tween 20 (PBS-T) and incubated with anti-vitellin polyclonal antisera (1:2000 diluted in PBS-T) for one hour at room temperature. The membrane was washed 5 times with PBS-T and incubated for two hours with the secondary antibody labeled with horse radish peroxidase (Cat: KT-21A, Bangalore Genei Pvt. Ltd., Bangalore). The membrane was washed 5 times for 10 minutes with PBS-T. The antigen-antibody complex was identified by the addition of colour developing solution 3',3',4',4' tetramethyl benzidine (TMB).

2.11. Statistical analysis of data

Statistical analyses were necessary to determine the level of significance of the effect of treatment on the experimental group of animals.

The mean, standard deviation (SD), Coefficient of variations (CV) and probability test (Analysis of variance-ANOVA, Students-Newman Keruls) were carried out using SPSS 16.0 version software package for Windows. A ‘p’ value of more than 0.05 was considered as not significant.