Materials & Methods

ANIMAL COLLECTION AND MAINTENANCE

The scorpions, *Heterometrus fulvipes* and *Buthus tamulus* used in the present study are abundantly available locally in the nearby (2 to 3 km) fields, forests and hill slopes. *Heterometrus fulvipes* were collected from the field after excavating the burrows without causing any damage to the animal and *Buthus tamulus* were collected from underneath the rocks. Portable UV lights were not used for animal collection because animals are abundantly available and sufficient number of animals can be collected during day time. Animals were brought to the laboratory and maintained in glass containers. The bottom (10 cm) of the containers was filled with soil, collected from the field, for *H.fulvipes* while the containers for *B.tamulus* were provided with the soil and few small rocks. The containers were stored in shaded shelves that were exposed to the natural day (24h light-dark cycles; 32±5°C). A limited number of animals (4) were placed (approximately one animal per one square foot area) in each container to avoid overcrowding and cannibalism. Animals were fed with cockroaches twice a
week. A small petri dish with water was kept in a corner of each container. Gravid scorpions were also collected during April-June and were separately maintained to supply newborn and young scorpions.

**HABITAT**

Burrow construction and morphology were studied both in the laboratory and in the field by carefully excavating the burrows. Frequent trips to different field areas provided the information about the habitats, nature of burrows, texture of soil and the environment around the burrows. To confirm the connections between the burrows of young scorpions with that of the maternal burrow, copper wires connected to a battery and an electric bulb were used. A wire inserted into the burrow of a young scorpion caused a bulb to glow if the wire contacted another wire in the maternal burrow.

**HAIR DISTRIBUTION**

The distribution of different types of hair sensilla on the legs of scorpions was studied in detail under a research trinocular microscope (GTR-II, Japan). Photography and camera-lucida drawings were used for the studies on distribution of hairs in the various segments of the legs. Newborn, second instar and adult animals were used for the different types of studies.

The nomenclature used to identify the various segments of the legs was in accorded with Vachon (1952): coxa, trochanter, prefemur, femur, tibia, basitarsus, and tarsus. The surface of the leg that faces the head when the leg of the scorpion is in its natural walking position is considered as named dorsal (anterior) and the opposite surface is called ventral (posterior).

For studies with the scanning electron microscope (SEM), the legs were separated from newborn, second instar and adult animals and fixed in 10% glutaraldehyde, airdried, coated with gold and then investigated using a Scanning Electron Microscope (ZOEL-35, Japan).

**LEG NERVE PROJECTIONS**

The pattern of leg nerve distribution was studied in adult *H. fulvipes* only. The nerves were exposed through microdissection, stained with 5% methylene blue, washed with water and observed under a research microscope. The distribution pattern was recorded with camera-lucida drawings. The projection pattern of the leg nerves into
the subesophageal ganglion was studied using cobalt chloride back-filling technique (Pitman et al. 1972). The subesophageal ganglion with leg nerves was isolated and placed in scorpion ringer (Padmanabha Naidu 1967). The cut end of the leg nerve was placed in a drop of 4% cobalt chloride while the remaining cephalothoracic nerve mass was allowed to float in a pool of scorpion ringer bordered by vaseline on a perspex plate. The preparations were maintained at 5°C in a refrigerator for 15-20h. The preparations were then separated, washed with scorpion ringer and treated with 1% ammonium sulphide (10 min) to precipitate cobalt sulphide. The preparations were again washed, fixed in Carnoy's fixative (ethanol:chloroform: acetic acid ::6:3:1) for 10 min and finally intensified with silver (Backon and Altman 1977).

The preparations were transferred to distilled water for 5 min and then to developer solution-A (3 g gum acacia, 300 mg citric acid, 10 g sucrose in 100 ml of distilled water; pH 3.0). After 30 min of incubation in developer-A at 50°C in dark, the tissues were transferred to developer solution-C (1% AgNO₃ in soluton-B). The incubation was continued until the preparations became brown due to silver depositing. They were then transferred to warm water (50°C), followed by a cold water rinses for 10 min. The preparations were dehydrated in ethanol series for 1 h each in 30%, 50%, 70%, 90% and absolute, cleared in methylsalicylate for viewing under microscope (GTR-II, Japan). Preparations with nerves well filled with cobalt were selected for camera-lucida drawings and microphotography.

**OVERT RHYTHMICITY**

**Burrow Emergence Rhythm**

Several burrows inhabited by *Heterometrus fulvipes* were chosen for the studies on the rhythm of burrow emergence. The burrow emergence and doorkeeping behaviour of the animals were observed from 1700 to 1900h on several days, preferably the days on which the experiments on physiological rhythmicity were also performed in the laboratory so as to obtain the relationship between burrow emergence and physiological rhythmicity.

**Locomotor Activity**

Locomotor activity of the 2nd instar and adult *Heterometrus fulvipes* was measured using an optoelectronic device with replaceable animal chambers to accommodate animals of different size. Movement of the animal caused the chamber to move and
this was recorded with electronic digital counter over the 24h day under natural light-dark conditions and the activity was finally presented graphically.

**PHYSIOLOGICAL RHYTHMS**

Animals were acclimatized to laboratory conditions for several days prior to experimentation. The animals were dissected, and the subesophageal ganglion and ventral nerve cord were carefully removed and placed in scorpion ringer (Padmanabha Naidu 1967). The tissues were blotted and weighed in an electrical balance to the nearest milligram (mg). After weighing, separate homogenates of the required percentages were prepared. The entire process of preparation of tissue samples was carried out in a short time. Twelve different time periods of 24h solar day (i.e. 0600, 0800, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 0000, 0200 and 0400h) were chosen to study the physiological rhythmicity (AChE and ATP-ases activities).

**ESTIMATION OF PROTEIN CONTENT**

Protein content of tissues was estimated by the method of Lowry et al (1951).

**Total Proteins**

In order to estimate the total protein content, 2% (w/v) homogenates of nervous tissue were prepared in 0.25M sucrose solution. To 0.5ml of homogenate, 1ml of 5% TCA was added, and the samples were centrifuged at 1000g for 15 min. The supernatant was discarded and the residue was dissolved in 1ml of 1N sodium hydroxide. To this solution, 4ml of copper reagent was added followed by 0.4ml of Folin-phenol (1:1) reagent. The colour was measured at 600nm in a spectrophotometer against a blank.

The amount of proteins in the sample was calculated after comparison with standard samples prepared from bovine albumin.

**Acetylcholinesterase (AChE) Activity**

(Acetylcholine hydrolase : EC 3.1.1.7).

The AChE activity of SOG and nerve cord was estimated by the calorimetric method of Ellman et al (1961).

One percent (w/v) tissue homogenates were prepared in 0.25M sucrose solution. The reaction mixture contained 3.0ml of 0.1M phosphate buffer (pH : 8.0), 20 µl of 0.075M acetylthiocholine iodide (substrate) and 100 µl of 0.01M DTNB (5,5-
dithiobis-2-nitrobenzoic acid). The reaction was initiated with the addition of 20μl of homogenate. The contents were incubated for 15 min at room temperature, and the colour absorbance was measured at 412nm in a spectrophotometer. The enzyme activity was expressed as μmoles of ACh hydrolyzed/mg protein/h.

**Adenosine Triphosphatase Activity (ATPase)**

(ATP Phosphohydrolase: EC - 3.6.1.3)

Total, Na⁺,K⁺ and Mg²⁺-ATPase activities in the tissues were estimated following the method of Tirri et al (1973).

**Mg²⁺-ATPase**

One percent (w/v) homogenates of tissues were prepared in 0.25M ice-cold sucrose solution. The reaction mixture in a final volume of 1.6ml contained 0.5ml of tris buffer (0.13M; pH 7.4), 0.4ml of substrate-ATP (4 μmoles), 0.5ml of MgCl₂ (0.05M) and 0.2ml of homogenate (enzyme source). The contents were incubated at 37°C for 15 min, and the reaction was arrested by the addition of 1.5ml of 10% TCA. Zero time controls were maintained by adding TCA prior to the addition of homogenate. The contents were centrifuged at 1000g for 15 min, and the inorganic phosphate was estimated in the supernatant fraction following the method of Fiske and Subba Row (1925).

Ouabain is an inhibitor of Na⁺, K⁺-ATPase. In the present investigation, experiments were performed with and without addition of ouabain (10 μmoles in 0.1ml). No significant difference was found in the enzyme activity with addition of ouabain. Similar observations were reported earlier for insect preparations and the investigators choose to assay the Mg²⁺-ATPase activity in the presence of Mg²⁺ and ATP only, and subtract that from the total activity to obtain at the Na⁺,K⁺-ATPase (Tolman and Steele 1976; Sachin and Kroenger 1980).

**Total ATPases**

One percent (w/v) homogenates of tissues were prepared in 0.25M ice-cold sucrose solution. The reaction mixture in a final volume of 2.6ml contained, 0.5ml of tris buffer (0.13M; pH 7.4), 0.4ml of substrate-ATP (4μmoles), 0.5ml of MgCl₂(0.05M), 0.5ml of NaCl (0.05M), 0.5ml of KCl (0.05M) and 0.2ml of homogenate (enzyme source). The contents were incubated at 37°C for 15 min, and the reaction was arrested by the addition of 1.5ml of 10% TCA. Zero time controls were maintained by adding TCA, prior to the addition of homogenate. The contents were centrifuged, and the inorganic
phosphate was estimated in the supernatant fraction following the method of Fiske and Subba Row (1925).

$$\text{Na}^{+}, \text{K}^{+}-\text{ATPase} = \text{Total ATPase} - \text{Mg}^{2+}-\text{ATPase}.$$ 

**Estimation Of Inorganic Phosphate**

To 1.0ml of supernatant, 1.0ml of ammonium molybdate solution (2.5gm in 100ml of 10NH₂SO₄) was added, followed by 0.4 ml of ANSA (1-amino-2 naphtho-4 sulphonic acid) (2.5mg of ANSA, 97.5ml of 15% sodium bisulphite and 2.5ml of 20% sodium sulphate). The mixture allowed to react for 5 min. The blue colour formed was measured at 660nm in a spectrophotometer against a reagent blank. The blank contained 2.0ml of trichloroacetic acid (TCA), 1.0ml of ammonium molybdate and 0.4 ml of ANSA. The enzyme activity was expressed as μmoles of inorganic phosphate formed/mg protein/hr.

**SPONTANEOUS ELECTRICAL ACTIVITY OF LEG NERVES**

The experiments were conducted at 20-22°C in an air-conditioned room. Animals were dissected to expose the leg nerves. The scorpion ringer (Padmanabha Naidu 1987) was used to moisten the nerves. Spontaneous electrical activity from the leg nerves was recorded for 3 min each hour in the period from 1500h to 2200h. Recordings were made with finely tapered silver electrodes. The potentials were fed through AVB-21 preamplifier and displayed on a Nihon Kohden (Japan) VC-11 memory oscilloscope. Photographic recordings were made using Nihon Kohden RLG-6201 camera.

**Validity Of Experimental Procedures**

Cell free extracts of freshly prepared homogenates in cold 0.25M sucrose solution were used for all biochemical assays.

**Enzyme Units**

Enzyme activities were expressed in standard units i.e, μmoles of product formed or substrate cleared per mg protein per hour. For enzyme unit expression, protein estimation was made by the Folin phenol method (Lowry et al 1951). All enzyme assays were conducted after standardization with known quantities of enzyme and substrate.
Lambert-Beer Law

All the products of the reaction were measured by spectrophotometric procedures in which the optical density (absorbance) of the resulting coloured complexes are proportional to the concentration of the reaction product.

Statistical Analysis Of Data

The data analysis was done using one way ANOVA (Analysis of variance) and SNK (Student-Newman-Keul) test for statistical significance. The test procedures were followed as described by Steel and Torrie (1960).