CHAPTER II
MATERIALS AND
TECHNIQUES OF STUDY
MATERIALS AND METHODS

Maintenance of stock culture

A stock culture of *N. rufipes* was maintained in glass troughs measuring 25cm x 25cm x 11.5cm. in the laboratory on a diet of dried sardine. The original sample was taken from the fish drying centers in Puthiyappa, Calicut. The duration of the present study was from 2004-2006.

Biology

Eggs laid by freshly mated females were enclosed in separate specimen tubes (60 in number) measuring 10.5cm X 2.5cm. The tubes were closed with cotton plugs which were soaked with water every 24 hours to provide moisture for the developing larvae. Dried sardine was provided as food and was changed every second day. Observations were made at 24-hour intervals and larval moults were recorded by noting the presence of exuviae.

Fecundity

Freshly emerged males and females were paired in specimen tubes (measuring 10.5cm X 2.5cm). The mated females (20 numbers) were kept individually in the specimen tubes which were closed with cotton plugs. The cotton plugs were soaked with water every day to provide free water for the beetles. Water is provided, as according to Dick (1937) and Taylor (1964) females with access to free water lay more eggs than with those without and oviposition period is also restricted. Dried sardine (approximately 20 g) was provided as food and oviposition medium. It was removed at 24 hr. intervals and the eggs were counted and fresh pieces of dried fish were provided. To study multiple mating, male and female were kept together till death and for double mating the male was removed after a single mating. When the females
stopped laying eggs, they were mated once again after a gap of 15 days with a freshly emerged male.

Preliminary studies indicated that the females that have stopped laying eggs sometimes resumed egg laying after a maximum gap of 12 days after the first mating. Hence, the gap of 15 days between the two mating.

Longevity

Freshly emerged male and female *N. rufipes* (20 numbers each) were kept in individually in specimen tubes (measuring 10.5cm X 2.5cm). Dried sardine provided as food was changed every 2nd day. The cotton plugs were soaked with water after every 24 hours to provide moisture for the insects. Observations were made every 24 hours and mortality was recorded.

Courtship and Mating behaviour

The working area was lined with a clean piece of filter paper. A petri dish (10cm x 2cm) was kept upside down on the filter paper. This was used as the observation arena. A virgin male was introduced under the inverted petridish and was allowed to acclimatize to the surroundings for at least 5 minutes. A virgin female was then introduced. The ensuing sequences of events were carefully observed. If the male and the female did not interact after 2-3 minutes, a different pair was tried. Five such trials were made.

Preparation of plant extracts

Water extracts

Leaves were dried under the shade for 1 week and then powdered in a grinder. 25 gm leaf powder was mixed in 100 ml water. Boiled for 2-3 minutes in a 1000 ml conical flask. Strained it with muslin cloth. Squeezed gently. The residue in the muslin cloth was then mixed in 50ml water and
boiled for 2-3 minutes. It was again strained and made up to 100 ml. Extracted three times with 100, 50, and 50 ml water to make it 150 ml stock solution (Devasahayam and Leela, 1997). Dry fish pieces were immersed in the filtrate of required concentration for 15-30 minutes, dried in the sun and then supplied to various instars and adults of *N. rufipes*. 10 larvae/adults were kept individually in specimen tubes measuring 10.5cm X 2.5cm along with the treated dried fish. Experiments were replicated three times. The control experimental set up contained dried fish soaked in water and dried.

**Alcoholic solution**

100 ml leaf powder was mixed in 300 ml alcohol, stirred well. If it exists as slurry then added more alcohol. Covered and kept it overnight. Filtered it into a conical flask. Extracted thrice with 150 ml. Combined filtrate was kept in a water bath. Evaporated to dryness. It was taken as 100%. Took 0.5%, 1%, and 2% as per the requirement (Devasahayam and Leela, 1997).

Dry fish pieces were immersed in the filtrate of required concentration for 15-30 minutes. Dried in the sun and then supplied to various instars and adults of *N. rufipes*. 10 larvae/adults were kept individually in specimen tubes measuring 10.5cm X 2.5cm along with the treated dried fish. Experiments were replicated three times. The control experimental set up contained dried fish soaked in absolute alcohol and dried.

**Spices oils**

Crude Lemon Grass (LG) oil was collected from Waynad. The crude Lemon Grass oil was fractionated from the Dept. Of Chemistry, University of Calicut in to Lemon Grass A (LG A) oil and Lemon Grass B (LG B) oil.

The other oils such as Cinnamon Leaf oil, Clove bud oil, Black Pepper oil and Turmeric oils were supplied by Synthite Cochin, India.
Spices oils were diluted to 10%, 5%, 2%, 1%, 0.5% and 0.25% concentrations in ethanol and cotton balls approximately 0.5 to 0.75 cm in diameter were wetted with 10 drops each of the diluted solution (each drop is approximately 0.05 ml). The cotton balls were then introduced into the specimen tubes (measuring 10.5 cm X 2.5 cm) containing egg /larvae or adult and dry fishmeal. 10 larvae/adult were housed individually in the specimen tubes. Experiments were replicated three times. The cotton ball was separated from the larvae and food by 1mm wire gauze. Mortality of the respective stages of *N. rufipes* was recorded after 24, 48 and 72 hours after treatment. In the control setup the cotton balls were wetted with absolute alcohol diluted with water as per the concentration.

**Survey**

Population study of the various insect pests were not possible because the pests were located in different area (on bamboo poles, under coir mats, on and inside gunny bags, in the sand, in the waste dumped outside the sheds and also inside the fish that are heaped in the sheds), a uniform method of sampling is not possible and as pooling data from different sampling methods may increase sampling errors it was not attempted. The abundance of each pest species was estimated from an approximate visual count. The survey was conducted in almost all the Coastal districts of Kerala viz., Kasaragode, Kannur, Kozhikode, Malappuram, Trichur, Ernakulam, Alleppey, Kollam, and Thiruvananthapuram. A representative center was selected for each district based on the scale of fish drying being carried out. Monthly sampling and collection were carried out in Thiruvananthapuram, Kollam, Alleppey, Ernakulam and Trichur, Kannur and Kasaragode at 6 month intervals, while it was fortnightly in Malappuram and Kozhikode.
Experiments to determine presence of insecticides in sand

To test the presence of insecticide in sand where fish were dried, sand collected from different drying centres was kept in a glass trough measuring 25cm x 25cm x 11.5cm. *N. rufipes* 2\textsuperscript{nd} instar larvae were introduced into the sand. Dried fish in small pieces were supplied as diet. In a separate control experiment clean sand from the beeches (from location on the beeches were fish are not dried) are also used a separate glass trough. Larval death after 24 hours was noted.

Statistical analysis

**Pearson's chi-square test** ($\chi^2$) was carried out to analyse data on developmental biology. In our case, we need to test whether the environmental factors (temperature & photo period) are influencing on developmental periods of IP and various instars. Temperature, photo period and developmental periods are categorical variables and hence a most appropriate test for independence is $\chi^2$-test of independence. For each category, we formed a two-way table (called contingency table) which gives the observed frequencies of different levels of two factors (eg. For each particular photo period, we formed a two-way table of temperature against developmental periods of incubation period). Using the assumption of independence, the software (Statistica) evaluates the expected frequencies and compute

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\chi^2 = \sum \left( \frac{(O - E)^2}{E} \right)
\]

where $O$ stands for an observed frequency, $E$ stands for the corresponding expected frequency and $\sum$ stands taking the sum total of all computed values in the bracket.
The software also gives p-value of the test and raw percentages. If p-value is less than 0.01, the $\chi^2$-value is highly significant which means that the two factors (raw factor and column) are highly associated. If p-value is greater than 0.01, but less than 0.05, the $\chi^2$-value is significant at 5% level, which means that the two factors (raw factor and column) are associated at 5% level. If p-value is greater than 0.05, the $\chi^2$-value is not significant which means that the two factors (raw factor and column) are independent (no significant association is observed). In significant cases the raw percentages interpret the way the factors are associated.

Toxicity data were analysed by Probit analysis (Finney, 1971). Statistica '99 version was used to carry out all statistical analysis.