Materials and Methods
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The data on rainfall, temperature and humidity were collected from the Meteorological Centre, Observatory, Thiruvananthapuram.

Studies were carried out both in the field as well as in the laboratory. All the ecological studies were done in the field, but studies pertaining to the biological aspects and susceptibility status of An. stephensi were done in the laboratory.

4.1 Field studies

4.1.1 Adult survey

Knowledge on the resting behaviour of mosquitoes is of paramount importance in the planning of effective mosquito control strategies. Moreover, the success of indoor residual insecticidal spray for the control of vector species depends largely on their indoor resting behaviour. In order to study these aspects and also to determine resting density, species composition, biodiversity, relative abundance of different species, seasonal prevalence and biting rhythm, adult mosquito
collections from indoor and outdoor situations, landing collections on man and animal were done.

Stratified, multi-stage random sampling method was adopted for the survey of adult mosquitoes. Three wards were selected from each strata as described under 3.5. These wards formed the first stage of sampling. From each of the sample wards, collection sites comprising of cattle sheds and human dwellings were identified. These formed the second stage of sampling.

4.1.1.1 Indoor resting collection

Mosquitoes resting in human dwellings and cattle sheds were collected between 0600 hrs and 0900 hrs and also between 1800 hrs and 2100 hrs at fortnightly intervals in all the study sites during the period from September 1999 to August 2001. Collections were made with the help of 3-celled torch light and aspirator (WHO, 1975). The mosquitoes were collected by trained persons. The same persons were engaged for the collection throughout the study period for the purpose of genuine analysis and correlation of the data. The collected mosquitoes were transferred to labelled test tubes. Whenever mosquitoes were caught in large numbers, they were released into a half foot cage to prevent crowding in the test tube. The collected mosquitoes were transported
to the laboratory where they were identified up to species level with the help of identification keys. *Anopheles stephensi* were classified as unfed, fully-fed, semi gravid and gravid according to their abdominal conditions. The gravid females were confined singly in cages for oviposition to determine the fecundity and variants of *An. stephensi*. The fortnightly collections were pooled on monthly basis and various indices of mosquito biodiversity were calculated. From the monthly data, man-hour density, abundance and absolute frequency of *An. stephensi* were also determined.

4.1.1.2 Mosquito biodiversity

The diversity among insects has always been of keen interest to Entomologists. The biodiversity studies have great significance in the conservation of genetic resources as well as control of pests and disease vectors. Documentation of species constitutes an integral component of biodiversity studies. The concept of biodiversity involves species diversity, genetic diversity and ecosystem diversity.

Various indices to estimate the biodiversity of animal and plant communities are available in the literature dealing with ecology (Pielou, 1969; Odum, 1991; Service, 1993a; Purvis and Hector, 2000). Diversity indices provide more information about composition and diversity of
species in a community. They can also be used to compare the numbers of different species and their frequencies in different habitats and also in the same habitat. The following indices that are commonly used and relevant to the present study are used to ascertain the diversity of mosquitoes collected from indoor situations such as human dwellings and cattle sheds in the study area.

**Man-hour total density**

It is an important index which represents the total number of mosquitoes collected over the species for a time interval of one hour. It was calculated using the formula:

\[
\text{Man-hour total density} = \frac{\text{Total number of mosquitoes collected}}{\text{Total time spent for collection in hours}}
\]

Man-hour total density reflects the mosquito load in the community. In this study, as the sampling was done in the indoor environments, man-hour total density gives the measure of mosquito prevalence that poses a threat to human and cattle population in the area.

**Species richness**

Species richness is the first and oldest concept of species diversity, which is nothing but an indicator of the relative wealth of
species in a community. The number of species per sample is a measure of richness. It does not take into account the proportion and distribution of each species within the community or in an area. The more species present in it, the richer the sample. Species richness as a measure on its own takes no account of the number of individuals of the species present. It gives as much weight to those species which have very few individuals as those which have many individuals. It was calculated using the formula:

$$SR = \frac{s-1}{\log (n)}$$

Where,  
\begin{align*}
SR & = \text{Species richness} \\
s & = \text{Number of species in the sample} \\
n & = \text{Total number of species}
\end{align*}

**Shannon evenness**

Evenness or equitability is a measure of how much even the distribution of different species in a community or area. It considers both the number of species and the distribution of individuals among the species. Evenness ranges from zero to one. When evenness is close to zero, it indicates that most of the individuals belong to one or a few species. When evenness is close to one, it indicates that each species consists of the same number of individuals or none is dominant over the others. Shannon evenness was calculated using the formula:
4.1.1.3 Density, abundance and frequency of *An. stephensi*

Diversity, abundance and frequency of vectors are among the important factors in the epidemiology of malaria. These factors determine the intensity, persistence, etc., of malaria transmission. Therefore, a better understanding of these factors is essential for the formulation of effective strategies for the prevention and control of malaria. There are different indices to determine the density, abundance and frequency of a species in an area or a community (Odum, 1991; Service, 1993a). These indices provide more accurate and adequate information regarding the prevalence, distribution, etc., of a species in a particular area or habitat. In this study, the following indices are used to determine the density, abundance and frequency of *An. stephensi* in the study area.

*Man-hour density (MHD)*

In malaria epidemiology, density of mosquito, particularly vector species is expressed as man-hour density (MHD). It is an important index to measure the relative densities of mosquitoes. It is useful to compare
the prevalence of a vector in the same area between seasons, months or years and to compare it between two different places at the same time. It is also used to assess the impact of vector control measures.

Man-hour density actually expresses the average number of adult mosquitoes collected in human or animal dwelling by hand collection done by standard methods by searches over fixed period of time, per man-hour. Hence, it is otherwise called per man-hour density (PMHD). This is calculated using the formula:

\[
\text{Man-hour density (MHD)} = \frac{\text{Total number of a particular species collected}}{\text{Time spent for collection in hours}}
\]

**Abundance**

Generally, abundance is expressed as the total number of individuals of a particular species out of the total number of quadrats in which the species occur. Therefore, it actually denotes the numerical strength of the species in the productive sites.

In the studies on mosquitoes, quadrat sampling method cannot be employed. Mosquitoes are collected from human and animal dwellings in the selected sites by aspirator method for a fixed period of time, usually 15 minutes during dawn and dusk hours. Hence, in this
study, the number of mosquitoes collected and the positive sites are considered for the calculation of abundance.

Abundance is calculated by using the formula:

\[
\text{Abundance} = \frac{\text{Total number of individuals of the species collected}}{\text{Total number of sites in which the species occurred}}
\]

Absolute Frequency

Absolute frequency (%) may be expressed as the percent of the sample sites in which the species occur. In this case, the number of individuals or the density of the species does not matter. But, the number of sites where the species occur out of the total number of collection sites is taken into account for the purpose of calculation of the index. It shows how much a particular species is scattered or dispersed over an area. This index is calculated by the formula:

\[
\text{Absolute frequency (\%)} = \frac{\text{Total number of sites in which the species occurred}}{\text{Total number of sites of\ collection}} \times 100
\]
4.1.1.4 Outdoor resting collection

Mosquitoes usually take shelter in the bushes, vegetations, tree holes, culverts, crevices, etc. In order to have a better understanding of the outdoor resting behaviour of mosquitoes especially that of *An. stephensi*, all the possible outdoor resting habitats in the study sites were searched on a random basis, covering all seasons during the period from September 1999 to August 2001. Collections were made with the help of flashlight and aspirator. Sweep net collection was also attempted in certain situations.

4.1.1.5 All night landing collection

Biting behaviour of vector mosquitoes has a significant bearing on the epidemiology of malaria as well as other mosquito-borne diseases. The intensity of transmission in an area depends mainly on the man-vector contact. There are different methods to study the biting habits of vector mosquitoes. But, direct observations are always more reliable and authentic. Therefore, all night landing collections were carried out once in a month during the period from September 2001 to August 2002. The collections were made from 1800 hrs to 0600 hrs with the help of torch light and aspirator.
All night human-landing collections were done in a dwelling house in Fort area. A volunteer was selected for this purpose after proper counselling and he was allowed to sleep in the veranda of the house in the area. Two persons collected all the mosquitoes landing on the man. Similarly, all night animal-landing collections were done in the same area about half km away from the site where human landing collections were conducted. One tamed cow was selected as animal bait and all the mosquitoes landing on or probing to bite the cow were collected. In both the stations, hourly collections were made and the mosquitoes were kept in separate labelled test tubes and they were taken to laboratory. In the laboratory, the mosquitoes were identified and biting rhythm of An. stephensi was calculated.

4.1.2 Larval survey

Larval collections were made once in a month from Fort area for one year from September 1999 to determine the seasonal fluctuations in the breeding of An. stephensi. In order to detect the potential breeding habitats of An. stephensi and also to have a better understanding of the mosquito fauna in the study area, larval surveys were done in different locations on a random basis covering all seasons during the period from September 2001 to August 2003. Different sampling methods were adopted for different habitats (WHO, 1975). For sampling of wells, an
iron bucket of 20 cms top diameter, 14 cms bottom diameter and 20 cms height was used. Five samples were taken from each well, four from the periphery and one from the centre. Successive samples were taken at an interval of 3-5 minutes so as to allow the water to become undisturbed for the larvae and pupae to come to the surface. Sampling in pits, tanks, etc., was done by using a standard dipper of 15 cms diameter and 500 ml capacity. Five to ten samples were taken from each breeding place according to size. For the collection of immature stages from small water collections in leaf axils, tree holes, crab holes, etc., pipetting method was employed. The collected larvae were transferred to a plastic bag filled with water from the same breeding place and transported to the laboratory. The immatures were reared in the laboratory and the emerged adults were identified. The cast off skins of fourth stage larvae were also examined whenever further confirmation was required. Species identification was done with the help of keys of Christophers (1933), Barraud (1934), Puri (1954 & 1960) and Nagpal and Sharma (1995). The mosquitoes of Cx. vishnui subgroup were identified by the key of Reuben et al., (1994). The abbreviations of generic and subgeneric names are from Reinert (2001).
4.1.3 Water analysis

The physico-chemical characteristics of the water in the breeding places have a profound influence on the breeding of mosquitoes. Water samples were collected from the breeding places in Fort area covering all the seasons of the year, i.e., pre-monsoon, monsoon and post-monsoon during 2001-2002. Immatures from the breeding places were also collected concurrently. The water samples were routinely analysed for the physico-chemical characteristics in the Government Analyst's Laboratory, Thiruvananthapuram. The densities of immature stages of An. stephensi were determined for different seasons and correlated with various physical and chemical parameters of the water samples.

4.2 Laboratory studies on An. stephensi

4.2.1 Laboratory colonisation

The establishment of colonies of mosquitoes is an indispensable tool in the study of biology and behaviour of mosquitoes. From such colonies a continuous supply of relatively uniform specimens can be obtained for various experiments in the laboratory. Hence, a cyclic colony of An. stephensi was established and maintained in the laboratory during the study period. Anopheles stephensi collected from the field
were used for raising the colony. Gravid females were confined singly in plastic cups lined with a strip of filter paper and half-filled with water. After oviposition 10-15 eggs from each batch were examined for ridge number. The egg batches having same mode of ridge number were pooled to form the colony. The cyclic colonies were maintained following the procedure of Ansari et al., (1978). The colonies were maintained in 30 cm³ cloth cages in the insectary kept at 28 ± 2° C and 70-80% relative humidity. The adult mosquitoes were provided with water soaked raisins and cotton swabs dipped in 5% glucose solution. The female mosquitoes were fed on restrained rabbit for blood meal.

Plastic cups of 6 cm height and 8 cm diameter lined with filter paper and half-filled with water (ovitraps) were introduced into the cages for oviposition. After the eggs were laid, the ovitraps were taken out of the cages and fresh ovitraps were placed in the cages for subsequent ovipositions. The eggs in the ovitraps were retained as such for hatching. After hatching the 1st instar larvae were transferred to an enamel tray of 30 x 25 x 5 cms containing tap water. The larvae were fed on a diet of finely powdered dog biscuit and yeast in the ratio 3:2. The water in the tray was changed everyday and dead larvae were removed. The pupae emerged were collected in separate bowls containing water and introduced into cages for adult emergence.
The egg of mosquito is of considerable biological importance since it is the initial stage in the aquatic life of the mosquito. In order to understand the basic aspects of the biology of eggs, studies were carried out on the morphometry, tanning, incubation period, hatchability and resistance to desiccation of egg in the laboratory.

4.2.2 Morphometry of eggs

Gravid females of An. stephensi collected from different areas were confined in ovitraps for egg laying. After egg laying, 10-15 eggs were carefully taken from the ovitrap with the help of a fine brush and placed on a wet filter paper on a microslide. The eggs were then examined at 100x magnification under bright field illumination of a microscope. The length and breadth of the egg and the length of egg float were measured using ocular micrometer. The ridges on one side of the egg float were also counted. From the above observations, average length and breadth of the egg, length of float, proportion of the egg covered by the float and the mode ridge number were calculated. On the basis of the above results, the variants of An. stephensi in the study area were determined.
4.2.3 Tanning of eggs

When the eggs are laid they will be white. After sometime, the eggs will turn grey and finally black. It is known as ‘tanning’. In order to determine the duration of tanning fully gravid females were confined singly in small plastic cups lined with filter paper and half-filled with tap water. The cups were then covered with nylon net. Immediately after oviposition the female mosquito from the cup was released into the cage and the eggs laid were closely observed with the help of a folding lens for the colour change. Observations were made in 10 batches of eggs. Time of oviposition and time of complete change of colour were noted to find out the duration of tanning.

4.2.4 Incubation period and hatchability of eggs

Eggs laid by females confined singly in ovitraps were floated in water and hourly observations were made till hatching was completed. The number of eggs laid and the number of eggs hatched in each cohort were also observed. From these observations, the incubation period and hatchability percentage were calculated.

4.2.5 Effect of desiccation on egg viability

Although most of the anophelines lay their eggs directly on water, the eggs are likely to be subjected to desiccation due to the
fluctuations of the water level in the breeding habitats. Hence, observations were made to understand the effect of desiccation on the viability of eggs. The eggs laid in the ovitraps were kept as such for 24 hours for conditioning. The eggs were then collected on a wet filter paper. The filter paper along with the eggs was separated into three groups. One group of eggs on the filter paper was kept as such while the other two groups were desiccated. Of these, one group was dried under direct sunlight and the other group was moderately desiccated at room temperature. Known number of eggs from each group were floated in water each day and number of eggs hatched were counted to assess the effect of desiccation on egg viability. Observations continued till there were no more hatch in each batch of eggs.

4.2.6 Morphometry of head capsule of larval stages

The morphometric analyses of head capsule of all the four larval stages were made to study the growth ratio and identify different larval stages. According to Dyar’s law, insects grow in geometric progression by a ratio which is constant in a given species. The length and breadth of the head capsule of all the four larval instars were measured using micrometer. Length and breadth of head capsule were also estimated by using mean geometric progression ratio.
4.2.7 Immature survival

The experiments conducted for the incubation period and hatchability was further continued for this study. The first stage larvae were transferred to separate enamel trays containing tap water. The larvae were fed on standard larval diet as mentioned earlier. Water in the tray was changed daily and larval diet was provided as required. Each day, observations on instarwise larval mortality, pupal mortality, and adult emergence were made. From these observations, instarwise larval survival, pupal survival and sex ratio were worked out.

4.2.8 Immature duration

For determining immature duration, eggs were reared individually under ambient conditions and they were observed until the emergence of adults. From the observations, total larval duration, pupal duration and instar duration were determined.

4.2.9 Hypopygial rotation

Immediately after emergence, the males were kept singly in test tubes. The rotation of the hypopygium (genitalia) of each mosquito was observed with the help of a folding magnifier. Observations were made at hourly intervals right from the time of emergence till the hypopygium
was rotated through 180°. The time taken for the completion of 180° rotation was taken as the duration of the hypopygial rotation.

### 4.2.10 Insemination rate

Fifty mosquitoes in the male-female ratio of 1:1 were separated immediately after emergence and released into 30 x 30 x 30 cm cage. These cages were kept in the insectary maintained at 28 ± 2°C and 70-80% relative humidity. Water soaked raisins and cotton pads dipped in 5% glucose solution were provided as food. The experiment was conducted for seven days and was replicated thrice. Spermathecae of all the surviving females in each of the respective day’s replicate cage were dissected in normal saline (0.65%) to examine the presence of sperms. From the observations insemination rate was calculated.

### 4.2.11 Oviposition rhythm

Twenty five gravid females each were separated from cyclic colony and introduced into separate cages. Ovitraps were provided in each cage for oviposition. The ovitraps in the cages were replaced by fresh ones at hourly intervals. The number of eggs laid in the ovitraps at hourly intervals were counted.
4.2.12 Fecundity

Gravid female mosquitoes collected from field as well as from the cyclic colony were confined singly in cages provided with ovitraps. The eggs laid in each ovitrap were counted to determine the fecundity of wild-caught and laboratory-reared mosquitoes.

4.2.13 Gonotrophic cycle

Freshly emerged males and females (25 each) were released into the cage (30 x 30 x 30 cm). The males were offered water soaked raisins and cotton pads soaked in 5% sugar solution. The females were fed on a restrained rabbit for blood meal. From the third day onwards, ovitraps were kept in the cages for oviposition. The number of eggs laid was counted and recorded daily. The duration between the day of emergence and the day of peak oviposition is considered as the time required for the first gonotrophic cycle. The duration between the subsequent peaks is taken as the time required for the successive gonotrophic cycles. The surviving female mosquitoes were offered blood meal to obtain subsequent oviposition.
4.2.14 Susceptibility status to insecticides

4.2.14.1 Adult susceptibility test

Susceptibility status of adults to DDT, malathion and synthetic pyrethroid was determined following the standard WHO procedures (WHO, 1981). Freshly emerged females were collected from cyclic colony and allowed to feed on 5% glucose solution and water soaked raisins. Three day old female adults were exposed to a diagnostic concentration of DDT - 4.0%, malathion 5.0% and deltamethrin 0.025% (synthetic pyrethroid). Three replicates of 20 glucose-fed females were maintained for each insecticide. Control experiment for each insecticide was run parallel. After one hour of exposure to insecticide-impregnated papers, mosquitoes were transferred to recovery chambers provided with cotton pads soaked in 5% glucose solution as food. Mortality counts were made after a 24 hours recovery period. The tests as well as control experiments were conducted at an ambient temperature of 28 ± 2° C and relative humidity of 70-80% under laboratory conditions. If the control mortality is between 5% and 20%, the percentage mortality was corrected by Abbott’s formula.

\[
\frac{\text{% test mortality} - \text{% control mortality}}{100 - \text{% control mortality}} \times 100
\]
4.2.14.2 Larval susceptibility test

Larval susceptibility tests were conducted against temephos as per WHO procedures (WHO, 1981b). Late third stage and early fourth stage larvae collected from the colony were used for the test. Healthy larvae were exposed to the diagnostic dosage of temephos 0.25 mg/litre. Twenty-five larvae were put in 500 ml beaker containing 250 ml of water and the required dose of larvicide. Three replicates and a control experiment were run in parallel. Mortality counts were made after 24 hour exposure period.