CHAPTER - 3

METHODOLOGY

METHODOLOGY

3.1. Blow fly fauna of Central Kerala

Studies were conducted for three years in different sites of Central Kerala to collect the blow flies. The data was generated by attracting blow flies to the decomposing carrion, by using sweep nets and also based on the literature survey. Blow fly species trapped were collected and identified. Blow fly sampling was done during different seasons; summer (April/May), monsoon (July/August) and winter (December/January) during 2019-2021.

The sites chosen were in three different districts: Thrissur. (10° 35′ 34.87″ N, 76° 11′ 22.6″ E), Palakkad (10° 46′ 12″ N, 76° 22′ 48″ E) and Ernakulam (10° 7′ 58.8″ N, 76° 28′ 58.8″ E) of Kerala, India (Fig. 3.1)

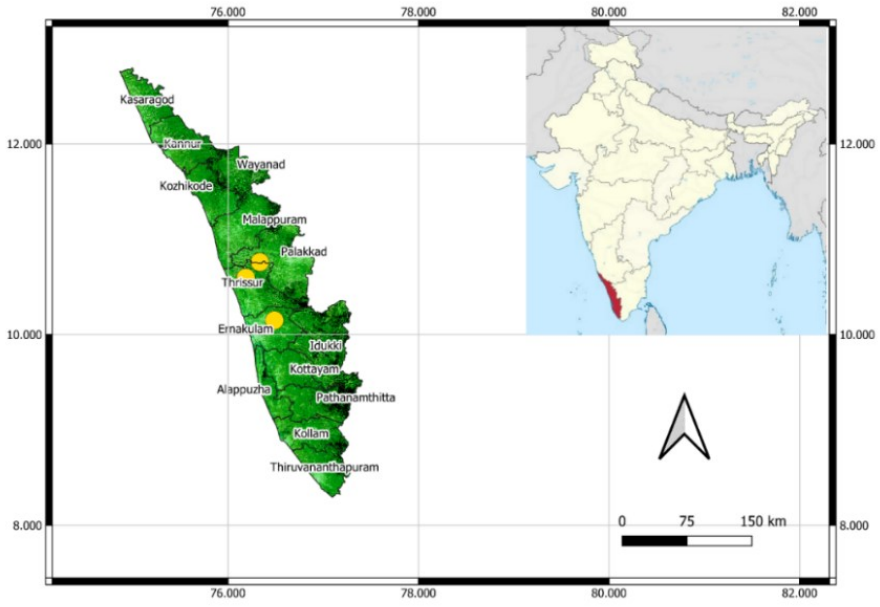

Fig. 3.1. Blow fly collection sites in Thrissur, Palakkad and Ernakulam districts

In addition to the collected and identified species of blow flies of forensic significance, data generation was also done based on published works, case study reports, articles, books and catalogues (Nandi, 2004, Bharti, 2011 and 2017).

3.1.1. Collection and Identification of blow flies

Materials: Aerial/sweep insect nets, collection vials, wide mouth jars with screw caps, eye dropper pippets, pork (collected from meat outlet of Kerala Veterinary and Animal Sciences University), vermiculite/sand, rearing cabinets (size: 2ft ×1ft ×1ft).

The adult blow flies were collected from the sampling sites after trapping with decomposing pork as bait kept in the portable rearing cabinets (size: 2ft ×1ft ×1ft) positioned in the outdoor. Adult flies were collected using fly collecting sweeping net (Fig. 3.2. A-F). Sample specimens were killed by keeping the flies in closed plastic bags having cotton soaked in ethyl acetate and pinned as dry specimens for morphological identification and a few were also preserved in 70% of ethanol for molecular identification.

Preservation

Materials: Insect pins, Insect net, insect boxes, naphthalene balls, needle, pointed watch makers forceps, medium/fine point dissecting curved forceps, paint brush, plastic container, plastic specimen cups, polythene bags, paper labels, marking pencils, paper towels/tissue paper, disposable surgical/polyethylene gloves, funnel, beaker, measuring jar, hand lens, ethyl acetate, ethanol (100%).

For morphological identification, preservation of the adult blow flies were done after killing the collected flies using the liquid killing agent, ethyl acetate. The killed flies

Fig. 3.2. Collection methods of blow flies are shown. A. Bait kept in the portable rearing cabinets. B. Blow flies attracted to the decomposing bait. C. Trapped blow flies in the cabinet. D. Arrangement for collection of trapped blow flies. E. Collection of blow flies in the insect net. F. Segregation and sexing of blow flies

were pinned using stainless steel entomological pin (size no: 2). Pinning was done through thorax between or little behind the base of the forewings and to the right of the midline so that no characters of the body were obscured. Legs were pushed down and away from thorax and wings were turned upwards or sideways from the body. Wings were straightened by using a paint brush dipped in 70% alcohol. The pinned flies were fixed in the standard insect box with Naphthalene balls to prevent the entry of any predatory insects. The killed flies were kept for drying under a table lamp with 60W bulb to remove any moisture. For molecular work, the killed flies were preserved in absolute alcohol (100%).

Morphological identification

Materials: Dissection Microscope, Compound microscope, Camera-Canon and Hand loop (40X). Greenough stereo microscope, Leica S8 APO Stereo Microscope -LEICA-S8APO with digital camera assembly (Leica MC170 HD) and Stereo microscope.

Identification of adult blow flies were done based on keys and characters described in standard literature (Senior-White et al., 1940; Bharti, 2019). Microscopic examinations were done using LEICA-S8APO stereomicroscope with camera attachment.

Molecular characterization

Molecular identification of the species was done using mitochondrial Cytochrome oxidase Subunit I (COI) gene. The DNA sequencing was done at Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, Kerala, India.

Isolation of mitochondrial DNA from the tissues was done using NucleoSpin® Tissue Kit (Macherey-Nagel). Agarose gel electrophoresis was used for checking the quality of the isolated DNA. Gel documentation system (Bio-Rad) was used for capturing the image under UV light after visualizing the gel in a UV transilluminator (Genei).

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond et al., 2010). Sequence similarity was searched using NCBI BLAST. BIOEDIT Software version 7.2.5 was used to edit and assemble the sequences. After making sure that the sequence has no CDS error or frame shift problems, the sequence was submitted in GenBank by mentioning the coding region or position in the slot provided. After submission of the sequence in GenBank, NCBI, GenBank accession numbers were received.

Isolation of genomic DNA

Materials: NucleoSpin® Tissue Kit (Macherey-Nagel), T1 buffer, proteinase K, water bath, 1.5 ml micro centrifuge tube, RNase A, B3 buffer, 100% ethanol, NucleoSpin® Tissue column, 2 ml collection tube, Ultracentrifuge, BW buffer, B5 buffer and BE buffer.

Isolation of genomic DNA was done using the NucleoSpin® Tissue Kit (Macherey-Nagel).The tissues separated out from the blow fly specimen was introduced into a 1.5 ml micro centrifuge tube. 25 µl of proteinase K and 180 µl of T1 buffer were added to the

same and were incubated at 56ºC in water bath. It was made sure that the tissue had undergone complete lysis. This was followed by the addition of 5 µl of RNase A (100 mg/ml) and the mixture was incubated at the room temperature for 5 minutes. After adding 200 µl of B3 buffer, further incubation was performed at 70ºC for 10 minutes. Addition of 210 µl of 100% ethanol was done and the mixture was vortexed. The solution mixture was pippeted into the NucleoSpin® Tissue column which was kept in 2 ml collection tube. It was centrifuged at 11000 x g for 1 minute. After transferring the NucleoSpin® Tissue column into fresh 2 ml tube, 500 µl of BW buffer was used for washing. 600 µl of B5 buffer was used for repeated washing. NucleoSpin® Tissue column was replaced in a fresh 1.5 ml tube. 50 µl of BE buffer was used for eluting the DNA.

DNA quality check by Agarose Gel Electrophoresis

Materials: Agarose, 0.25% bromophenol blue, 30% sucrose, 6X TE buffer (Tris – EDTA), 0.5X TBE buffer (Tris-Borate-EDTA), Ethidium bromide, bromophenol dye, UV transilluminator (Genei), Gel documentation system (Bio-Rad) and Electrophoretic Unit.

Agarose gel electrophoresis was used for quality check of the isolated DNA. 5µl of DNA was added with 1µl of 6X loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0). 0.8% Agarose gel was made in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. The DNA samples were loaded to the gel. 0.5X TBE as electrophoresis buffer was employed as the tank buffer. Electrophoresis was done at 75 V till the movement of the bromophenol dye to the anodic end of the gel. Visualisation of the DNA bands was done using UV transilluminator (Genei). UV light was used for imaging using Gel documentation system (Bio-Rad).

COI gene PCR amplification using universal primers of CO1

Materials: Primers; LCO (Forward) (GGTCAACAAATCATAAAGATATTGG) & HCO (Reverse) TAAACTTCAGGGTGACCAAAAAATC, PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems), 2X Phire Master Mix, distilled water, isolated DNA.

For the PCR analysis, 5µL of 2X Phire Master Mix, 4µL of distilled water, 0.25µL of forward primer, 0.25µL of reverse primer and 1 µL of isolated DNA were used.

The COI target primers used were;

LCO (Forward) (GGTCAACAAATCATAAAGATATTGG) &

HCO (Reverse) TAAACTTCAGGGTGACCAAAAAATC;

both in 5' → 3' sequence direction. PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) was used for the PCR amplification.

PCR amplification profile is as given below:

COX1

98° C	-	30 sec	
98° C	-	5 sec	}
45° C	-	10 sec	} 10 cycles
72° C	-	15 sec	
98° C	-	5 sec	}
50° C	-	10 sec	} 30 cycles
72° C	-	15 sec	
72° C	-	60 sec	
4° C	-	∞	

Quality check of the PCR products by Agarose Gel Electrophoresis:

Materials: Agarose, 0.25% bromophenol blue, 30% sucrose, 6X TE buffer, 0.5X TBE buffer, ethidium bromide, bromophenol dye, 2-log DNA ladder (NEB) as the molecular standard, UV transilluminator (Genei), Gel documentation system (Bio-Rad) and Electrophoretic Unit.

1.2% agarose gels were prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 4 µl of PCR product was mixed with 1 µl of 6X bromophenol blue and was loaded in the gel. Using 0.5X TBE buffer, electrophoresis was performed for 1-2 hours at 75V till the bromophenol front reached till the end of the gel bottom. 2-log DNA ladder (NEB) was used as the molecular standard. UV transilluminator (Genei) was used for the visualisation. Gel documentation system (Bio-Rad) was used for capturing the image under UV light.

ExoSAP-IT Treatment

Materials: Exonuclease I and Shrimp Alkaline Phosphatase (SAP) (ExoSAP-IT -GE Healthcare)

0.5µl of ExoSAP-IT consisting of the hydrolytic enzymes; exonuclease I and Shrimp Alkaline Phosphatase (SAP) was mixed with 5µl of PCR products and incubated at 37°C for 15 minutes followed by enzyme inactivation at 85ºC for 5 minutes for the removal of unwanted primers and dNTPs from PCR product mixture to avoid interference in downstream applications.

Sequencing

Materials: Sequencing PCR mix: distilled water, 5X sequencing buffer, forward Primer, reverse primer, sequencing mix and Exosap treated PCR product.

PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems)**,** BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA), ABI 3500 DNA Analyzer (Applied Biosystems), Sequence Scanner Software v1 (Applied Biosystems), Geneious Pro v5.1 (Drummond et al., 2010).

PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) was used for the sequencing.

For the PCR analysis, 6.6 µl of distilled water, 1.9µL of 5X sequencing buffer, 0.3µl of forward primer, 0.3µl of reverse primer, 0.2 µl of sequencing mix and 1 µl of Exosap treated PCR product were used.

PCR amplification profile for the sequencing is as given below:

96° C	-	2min	
96° C	-	30sec	} 30 cycles
50° C	-	40sec	
60° C	-	4min	
4° C	-	∞	

Clean up of post sequencing PCR

Materials: Distilled water, 3M Sodium acetate, EDTA and 100% ethanol, 70% ethanol, Sequencing plate, Vortex (Mixmate) and Ultracentrifuge.

5 µl of distilled water, 1 µl of 3M sodium acetate, 0.1 µl of EDTA and 44 µl of 100% ethanol were properly mixed. Each well of the sequencing plate having the PCR product was added to 50 µl of the prepared mix. The sequencing plate was vortexed by Mixmate vortex for 30 minutes at room temperature. The mixture was centrifuged at 3700 rpm for 30 minutes. The supernatant was decanted. 50 µl of 70% ethanol was added to each well for washing. The mix was again centrifuged at 3700 rpm for 20 minutes and the previous step was repeated with 70% ethanol. The supernatant was decanted and the pellet was air dried.

The air dried cleaned up product was sequenced in ABI 3500 DNA Analyser (Applied Biosystems).

Analysis of the sequence

Sequence Scanner Software v1 (Applied Biosystems) was used for checking the quality of the sequence. Alignment of sequence and basic editing of the obtained sequences were done using Geneious Pro v5.1 (Drummond et al., 2010).

Sequence similarity check using NCBI BLAST and submission of sequence in GenBank, NCBI

Sequence similarity was searched using NCBI BLAST. To begin with, BIOEDIT software was used to open both the forward and reverse fasta file through open sequence set. Nucleotide positions at the beginning and at the end that showed irregular blue bars were noted. AB1 file was used for BIOEDIT for both forward and reverse sequences. The narrow peak formations with clear peaks were checked. Non-performing peaks were noted which were broad. After noting down the nucleotide positions, extract option for trimming was used to give the nucleotide positions in range. This was done for both forward and reverse extracted sequences.

'VecScreen' of NCBI (https://www.ncbi.nlm.nih.gov/tools/vecscreen/) was used for checking the presence of any vector nucleotides used in the PCR amplification of the COI gene. To check for any similarity of sequence found, the sequence was taken to bio-edit again and those portions were trimmed. Reverse compliment was prepared using the software in the bioinformatics site (https://www.bioinformatics.org/sms/rev_comp.html).

Single Contig file sequence (positive strain 5' to 3') was made using CAP3 Sequence Assembly Programme (http://doua.prabi.fr/software/cap3). ORF (Open Reading Frame/coding region) finder of NCBI (https://www.ncbi.nlm.nih.gov/orffinder/) was used to find the largest ORF.

Blast of the sequence with query range from start and end codon was performed using Blastn of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi? PAGE_TYPE= Blast Search). The alignment and frame shift (presence of red letter at the beginning and end of the sequence) was noted. Bioedit was used to remove the nucleotides till the first red letter

and nucleotide sequences from the last red letters. Blast was conducted again on the obtained sequence.

After making sure that the sequence was with no CDS error or frame shift problems, the sequence were submitted in GenBank by mentioning the coding region or position in the slot provided. After submission of the sequence in GenBank, NCBI, GenBank accession numbers were received.

Molecular phylogeny

Evolutionary molecular phylogenetic analysis of sequences by Maximum Likelihood method

The evolutionary history analysis was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree was shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and were in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

3.2. Seasonal abundance in blow fly population in Central Kerala

3.2.1. Monitoring of climatic conditions

Humidity and temperature were monitored in different seasons; summer, monsoon and winter in the months of April-May (2019-2021), June-September (2019-2021), December (2019-2021) and January (2020-2022). Temperature and humidity were monitored using hygrometer (HTC-1, Model No: AP-IS11A056FBA).

3.2.2. Study on seasonal abundance of blow flies

Studies on the influence of seasonal differences on the population abundance were conducted in three seasons of all three years; summer (April-May (2019-2021), monsoon (June-September (2019-2021) and winter in the months of December (2019-2021) and January (2020-2022). As a part of this, preliminary observations on blow flies were conducted from dawn to dusk to find out the peak activity hours. The study revealed two peak periods of activities; one in the morning, starting from 10:30 a. m. to 12:30 p. m. and another in the evening starting from 4:00 p. m. to 5:30 p. m. during the third day of keeping the bait in the cabinet.

3.2.3. Study sites

Blow fly sampling was done from different sites in Central Kerala during different seasons; summer (April/May), monsoon (July/August) and winter (December/January) during the years from 2019-2021. The sites chosen were in three different districts; Thrissur (Kolangattukara; 10°34'29.4"N 76°11'01.8"E, Choolissery; 10°35'45.0"N 76°11'18.3"E, and Thangaloor; 10°37'35.5"N 76°11'15.3"E), Palakkad (Vaniamkulam-II; 10°45'32.1"N 76°20'04.8"E, Palappuram; 10°45'48.3"N 76°24'54.1"E, Varode; 10°48'50.0"N 76°22'47.2"E) and Ernakulam (Aimuri; 10°08'52.8"N 76°29'18.5"E, Kanjirakkad; 10°07'37.8"N 76°28'03.0"E, Kuruppampady; 10°07'01.8"N 76°30'12.6"E) of Kerala, India. (Fig. 3.3, 3.4, 3.5).

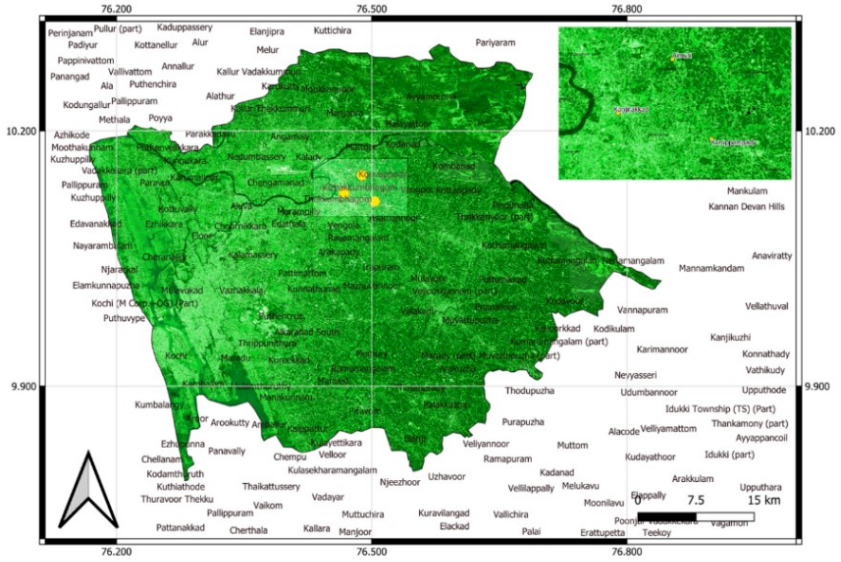

Fig. 3.3. Blow fly collection sites in Ernakulam (Aimuri, Kanjirakkad, and Kuruppampady)

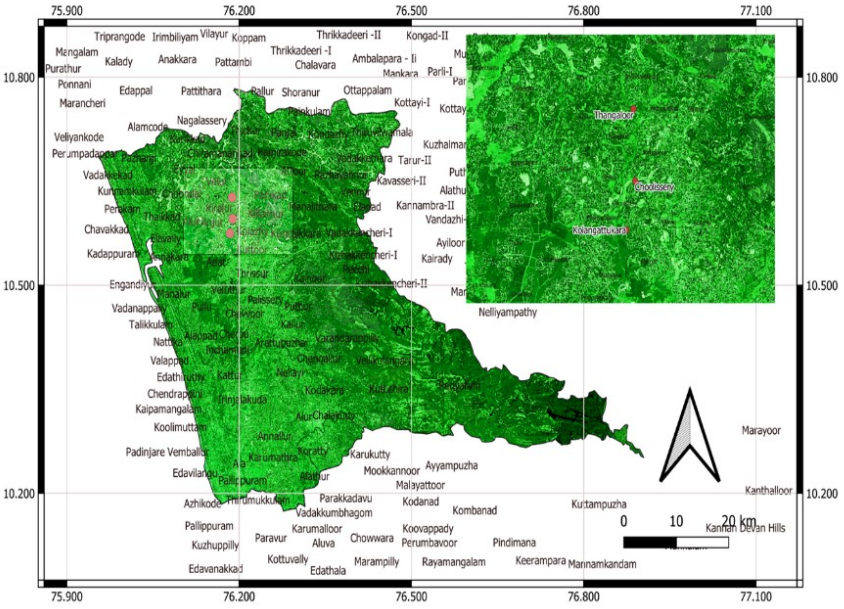

Fig. 3.4. Blow fly collection sites in Thrissur (Kolangattukara, Choolissery, and Thangaloor)

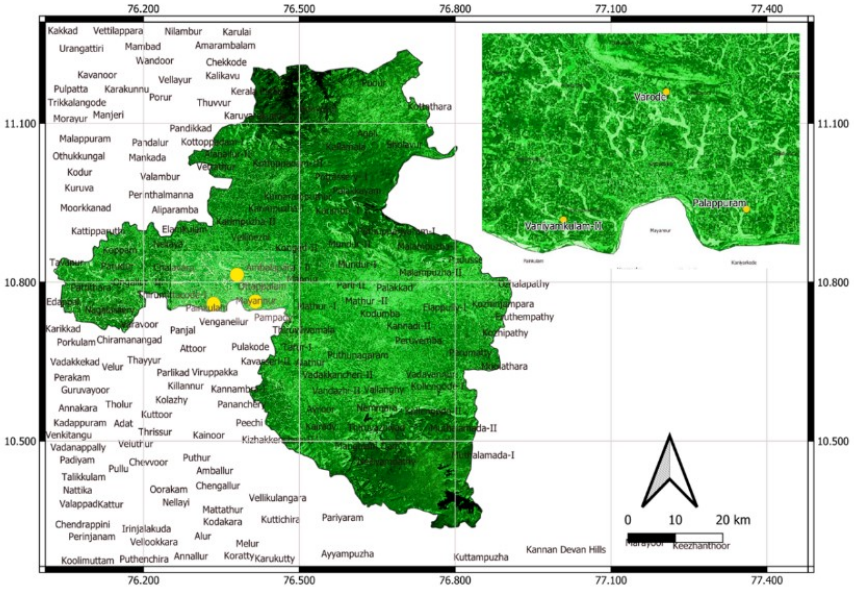

Fig. 3.5. Blow fly collection sites in Palakkad (Vaniamkulam-II, Palappuram and Varode)

Temperature and humidity was monitored using hygrometer (HTC-1, Model No: AP-IS11A056FBA). The insects were trapped in the rearing cabinets (Size: 2ft ×1ft ×1ft) having decomposing pork (*Sus scrofa*) as bait positioned in the outdoor facility (Fig. 3.7. A - B). The collection was done in triplicate in different cabinets. Adult blow fly abundance was monitored by keeping fly net covered over the cabinet. The flies entered into the net during the peak hours as mentioned before were trapped with insect net. Species determination and sexing were done for all the flies trapped in the study. The total number of adult flies were counted and were later segregated into species and then to male and female flies by studying their morphology under Stereo microscope. Studies were done in all seasons to find out the changes in seasonal abundance in the blow fly population.

3.3. Life history of blow flies in carrion

3.3.1. Outdoor rearing of blow flies

The rearing of adult flies were done in the outdoor rearing facility in Thrissur (Kolangattukara; 10°34'29.4"N 76°11'01.8"E and Thangaloor; 10°37'35.5"N 76°11'15.3"E)

A

B

C

D

E

F

G

H

Fig. 3.6. Outdoor rearing facility: A-D. Rearing cabinets with bait and vermiculite E. Hygrometer hung in the facility, F. Round rearing plastic containers with pork and vermiculite, G. 10%Sugar Solution, 1.5%(v/v) multivitamin syrup solution in plastic containers with wicks as diet for blow fly, H. Glass made rearing cabinet with rearing containers, diet and vermiculite

A

B

C

D

E

Fig. 3.7. Rearing methods I: A-B. Blow fly attracted to carrion C-D. Identified female blow fly of F1 generation released into the fresh bait, E.1st instars in the decomposing bait

A

B

C

D

E

Fig. 3.8. Rearing methods II: A. 2nd Instars on the bait, B. 3rd Instars on the bait, C. Migration of the larva for pupation D. Slow moving adult blow flies just emerged from pupae E. Fast moving emerged adult flies

during summer (April-May, 2019-2021), monsoon (June-September, 2019-2021) and winter (December, 2019-2021 and January, 2020-2022) seasons. Temperature and humidity were monitored using hygrometer.

Materials: Milk powder, 10% Sugar Solution, and 1.5 % (v/v) multivitamin syrup solution. Bait: Pork meat (collected from the meat outlet of Kerala Veterinary and Animal Sciences University), Vermiculite/Sand, Rearing Cabinets (Glass) of size: 2ft ×1ft ×1ft with steel mesh framed with Aluminum, Hygrometer (HTC-1, Model No: AP-IS11A056FBA) and Exhaust fan.

Pure culture of the insects was accomplished after rearing for two generations followed by identification and the next generation was considered F1 generation in the study. Rearing was performed in the rearing cabinets in the outdoor facility having decomposing minced pork (*Sus scrofa*) (Byrd and Tomberlin, 2019, Wang et.al, 2017, Bernhardt .et.al 2017, Whitaker, 2014, Catts and Goff, 1992) as bait (Fig.3.6.A-H). To facilitate oviposition, round plastic containers (500 ml) with 10cm diameter opening were used for keeping 100 gm. of pork over a layer of vermiculite kept in polythene pouch. The F1 generation male and female flies were released into the containers (Fig.3.7.C-D). The containers were covered with muslin cloth tightened with rubber bands. The adult insects were provided with milk powder, 10 % (w/v) sugar solution, 1.5 % (v/v) multivitamin syrup solution and water *ad libitum* using cotton wicks kept through the perforated lid of plastic containers. The decomposing pork was served as a reflex stimulus for the adult female fly to lay eggs and also served as a food source for the larvae (Fig. 3.8).

3.3.2. Preservation

Materials: Low tension larval forceps, needle, pointed watch makers forceps, medium/fine point dissecting curved forceps, paint brush, plastic container, plastic vials, plastic specimen cups, paper labels, marking pencils, paper towels/tissue paper, disposable surgical/polyethylene gloves, funnel, beaker, measuring jar, hand lens, 95% ethanol, ethanol (100%), formaldehyde, glacial acetic acid, and Kahle's Solution (30 ml of 95% ethanol: 12 ml of formaldehyde: 4ml of glacial acetic acid: 60 ml of distilled water)

Eggs laid on the bait were collected using a fine brush and transferred to watch glass having 70% ethanol. It was kept for 10 minutes to promote the dissolution of egg mass. Different larval instars were collected and boiled for two to three minutes at 80°C to prevent the shrinkage of larvae and to kill the microbes present in the gut of larvae and thus preventing darkening of the specimens in alcohol (Tantawi and Greenberg 1993, Adams and Hall, 2003). The specimens were preserved in 70% ethanol to facilitate the measurement of length and weight. For long term preservation, the boiled larval specimen were stored in Kahle's Solution.

3.3.3. Morphological identification of eggs and different stages of larval instars

Materials: Microscopic slides, cover slips, sharp blade, test tubes, spirit lamp, DPX mountant, compound microscope, Westwox Optik-Model: JXL No: 77126, with camera attachment (Keowa CE-500X), 10% (w/v) of KOH, alcohol grades: 50%, 70%, 80%, 95% and absolute ethanol (99%), 1% glacial acetic acid, Xylene and distilled water.

Morphological examination of 1st, 2nd and 3rd Instars to study cephalopharyngeal skeleton and posterior spiracle was done using potassium hydroxide enabled clearing. The alcohol preserved larvae were cut at the middle of second thoracic segment and at 11th

segment using a sharp blade under LEICA-S8APO stereomicroscope to study the cephalopharyngeal skeleton and posterior spiracle by digesting the anterior and posterior portions as per the methods stated by Sukontason et al., (2004). The cut anterior and posterior portions were treated with 10% (w/v) of KOH solution in a watch glass for 48 hours and were cleaned 4 times using distilled water. This was followed by treating the specimen in a mixture of 1% glacial acetic acid and 35% ethyl alcohol in a watch glass for 30 minutes. Serial dehydration of the specimens was done using alcohol gradient (50%, 70%, 80% and 95%) and absolute ethanol by placing the specimen in each for 30 minutes. The specimens were treated in xylene for 60 seconds and mounted on a microscopic slide with a coverslip using DPX mountant. The cephalopharyngeal skeleton, anterior and posterior spiracles were observed and photographed using compound microscope and Westwox Optik-Model: JXL No: 77126, with camera attachment (Keowa-CE-500X).

3.3.4. Ultrastructure study of larvae using Scanning Electron Microscopy

Materials: Distilled water, alcohol grades; ethanol-70 & 99.5%, carbon double tapes, aluminium stubs, ultra sonicator, sputter unit (JFC 1600, Japan) and Scanning Electron Microscope (JEOL Model JSM-6390 LV, JEOL Ltd. Japan).

The first, second and third instar larvae were collected and washed many times in distilled water. To kill the larvae and to prevent deformation changes, the larvae were kept in boiling water (80ºC) for two to three minutes and finally preserved in 70% alcohol (Tantawi and Greenberg 1993, Adams and Hall, 2003). Sample preparation for SEM included dehydration using 99.5% alcohol, followed by ultra sonification and air drying. After being dried at room temperature, larval specimens were gently placed onto stubs fixed with double tape. The specimen was coated with gold using gold sputtering for 10

seconds with 10mA current in the sputter unit (JFC 1600, Japan). Images were taken under JEOL Model JSM-6390 LV, SEM, JEOL Ltd. Japan, in Sophisticated Analytical Instrumentation Facility (SAIF), Cochin University of Science and Technology, Kochi, Kerala. Larval terminology follows Courtney et al. (2000) with a few additional terminologies prepared by Szpila and Villet (2011).

3.3.5. Life cycle

Developmental time of eggs, larvae and pupae

Once the eggs were found, the bait with the eggs was transferred into the new larval rearing round plastic containers (500ml). After eclosion occurred, in total, 1050 larvae were considered per season in which three equal replicates of 350 larvae were transferred separately on 400g of fresh pork kept in the silver foil pack. Each of the pack was kept in transparent round new plastic containers (500 ml) with 10cm diameter opening. The container was kept in the rearing cabinets with steel mesh framed with Aluminum. 200 grams of fresh pork was put into the container during different stages frequently as larval feed. This was continued until the instars reached the non-feeding stage and started pupal migration. Fresh pupae were collected from the vermiculite in the bottom of the rearing cabinet and were transferred to a new rearing round plastic container with moist vermiculite at the bottom and it was kept inside the rearing cabinet for the emergence of the adult fly (Fig. 3.8. D-E). Different larval instars were collected for studying their morphology and length/weight parameters and the developmental time.

Study on preoviposition time, fecundity and incubation time

Fecundity studies were done in all seasons of three consecutive years (2019-2021). In each trial, preoviposition period in days, maximum number of eggs laid by the adult fly

in a day, periodicity for egg laying in days, number of eggs laid by the fly in its life span and incubation period of eggs in hours were observed. Three such trials were conducted for each species for every season of the year.

For obtaining the eggs for the fecundity studies, 50 gm. of fresh pork kept in aluminum foil was placed into round plastic containers (500 ml) with 10cm diameter opening. Female fly of the respective species was released into the container. The flies were provided with milk powder, 10 % (w/v) sugar solution, 1.5 % (v/v) multivitamin syrup solution and water *ad libitum* using cotton wicks kept through the perforated lid of small plastic containers. The containers were covered with muslin cloth tightened with rubber bands. The containers were kept in the rearing cabinet and observed for the next 24 hour period every 30 minutes. The time of laying of eggs were monitored through close observation. Thus, preoviposition period was determined for every replicate per species per season in all three consecutive years.

Egg masses laid by the blow flies on the bait were collected within 24 hours, the meat was removed and examined for eggs laid for fecundity determination. Eggs laid on the bait were collected using a fine brush and transferred to a cavity block having 70% ethanol. It was kept for 10 minutes to promote the dissolution of egg mass. Then ethanol was drained off for egg counting. Dissolution was performed on egg masses by transferring the egg mass to a black fine mesh cloth for a better background contrast. A fine small tissue paper moistened with distilled water was kept over the black cloth having egg mass and was kept for 5 minutes. The eggs were physically separated by gentle manipulation with a 10 mm synthetic paint brush. The eggs were counted using Labomed-CSM2 Stereo zoom microscope.

Study on survival of different stages of blow fly species

Survival studies in triplicate were undertaken in all seasons of three consecutive years. In each trial, survival rate in percentage was calculated for each life stage; egg, 1st Instar, 2nd Instar, 3rd Instar, post feeding stage and pupa till the emergence of adult flies.

For conducting this experiment, three groups of 110-130 eggs were considered in each trial, per season for all three years. The eggs were transferred from the outdoor rearing cabinets and placed in the new larval rearing round plastic containers (500ml). After eclosion occurred, live larvae were counted at each stage. The larvae were then transferred to 200 gm. of fresh pork kept in the silver foil pack. Each of the pack was kept in transparent round new plastic containers (500 ml) with 10cm diameter opening. The container was kept in the glass made rearing cabinets. 200 grams of fresh pork was put into the container as larval feed. This process was continued until the instars reached the non-feeding stage and started pupal migration. Fresh pupae were collected from the vermiculite in the bottom of the rearing cabinet and were transferred to a new rearing round plastic container with moist vermiculite at the bottom and were kept inside the rearing cabinet for the emergence of the adult fly. During rearing, the number of live 1st instar, 2nd Instar, 3rd Instar and pupae which have undergone eclosion were recorded.

3.4. Effect of temperature and humidity on the life cycle of calliphorid flies

3.4.1. Assessment of development rate

Materials: 70% Ethanol, hand loop (40X), dissection microscope, Greenough stereo microscope (Leica S8 APO Stereo Microscope -LEICA-S8APO) with digital camera assembly (Leica MC170 HD), Stereo microscope, Vernier caliper, hot air oven, electronic Balance- Schimadzu - ELB 300.

Seasonal study on the fecundity measures during summer, monsoon and winter seasons during the life cycle of the fly was conducted from the time of mating till ovipositioning. Different parameters like pre-oviposition period, maximum number of eggs laid by adult fly in a day**,** egg laying time interval between successive batches (days) and the total number of eggs laid by the fly in its life span were considered for data analysis. In the rearing cabinet, the observations were made during the period between the mating and the time of oviposition in triplicate trials.

Preoviposition time was noted in each trial**.** Incubation time in all replicates were observed. The phase from the time of oviposition till the emergence of adult fly from pupae was considered for the study of developmental rate. After eclosion occurred, in total 1050 larvae were considered per season in which three equal replicates of 350 larvae were transferred separately on 400g of fresh pork kept in the silver foil pack. Each of the pack was kept in transparent round new plastic containers (500 ml) with 10cm diameter opening. 200 grams of fresh pork were put into the container during different stages frequently as larval feed.

The time taken by the eggs for hatching was noted. The freshly hatched larvae were transferred to the new larval rearing chamber and 100 grams of fresh pork was provided as food. Six larvae were collected every six hours and also at different stages of ecdysis for the length meassurements.They were boiled for two to three minutes at 80°C to prevent the shrinkage of larvae, to coagulate the protein and to kill the microbes present in the gut of larvae; and preserved in 70 % alcohol. Larval measurements were made as per the protocol suggested by Adams and Hall (2003) and Donovan et al., (2006).

Seasonal study encompassing the life cycle of the fly with special emphasis on the length and weight of larval instars during summer, monsoon and winter was conducted from the time of egg hatching till the pupation. In the rearing cabinet, the observations were made during the period between the egg hatching and the commencement of pupation on the decomposing meat in triplicate trials.

Time dependent data was recorded in accordance with the length and weight of the instars of blow flies during their developmental stages with special inference on the summer, winter, and monsoon seasons. Length of all instar stages were done using stereomicroscopy with scale *in situ* for the first instar and vernier caliper and dissection microscopy especially for the 2^{nd} and 3^{rd} instars. This was followed by the weight measurement of larvae. The time spent by the species in each life stage was recorded. Larvae of the same batch and phase were boiled for two minutes at 80°C and preserved in 70 % alcohol (Tantawi and Greenberg 1993, Adams and Hall, 2003). The larvae were dried in hot air oven at 50°C for 36 hours. The dried larvae were weighed on an electronic weighing balance. Based on these observations, growth curves were plotted. The development rate assessment was done according to the protocol of Greenberg and Wells (1998). The effect of temperature and relative humidity on preoviposition period, incubation time of eggs and larval period were noted. Larval development especially the length and weight parameters were also studied in detail.

Seasonal study on the survival rate measures during summer, monsoon and winter during the life cycle of the fly was conducted from the time of ovipositioning till the emergence of adult fly. Comparison of survival rate of different stages between seasons and also between years were considered for data analysis.

3.4.2. Laboratory rearing of blow flies under controlled conditions and validation

Materials: 10% sugar solution, 1.5% (v/v) multivitamin syrup solution, pork meat, Vermiculite / Sand, Incubator, 70% ethanol, hand loop (40X), dissection microscope, Greenough stereo microscope (Leica S8 APO Stereo Microscope -LEICA-S8APO) with digital camera assembly (Leica MC170 HD), stereo microscope, Vernier caliper, hot air oven, electronic balance- Schimadzu - ELB 300.

Laboratory rearing was done to validate the outdoor rearing studies conducted on blow flies. The average temperature (T) and relative humidity (RH) recorded in the three seasons were considered for conducting laboratory rearing. The average values of temperature and relative humidity used for laboratory studies were the following. For summer: T-32.28°C, RH-68.23%; for monsoon: T-26.17°C, RH-97.44% and for winter: T-25.19°C, RH-46.32% (Group I -III). A photo period of 12:12 (L: D) h was maintained in the incubator.

Trials were conducted in triplicate per species for a particular constant temperature and relative humidity in incubator (Fig. 3.9. A-D). A total of 375 eggs were considered per species with 125 each in three replicates. 50 gm. of fresh pork kept in aluminum foil were placed into round plastic transparent containers (500 ml) with 10cm diameter opening. Adult male and female fly of the respective species was released into the container. The flies were provided with milk powder, 10 % (w/v) sugar solution, 1.5 % (v/v) multivitamin syrup solution and water *ad libitum* using cotton wicks kept through the perforated lid of plastic containers. The containers were covered with muslin cloth tightened with rubber bands. The decomposing pork was used which served as a reflex stimulus for the adult female fly to lay eggs and also served as a food source for the larvae.

Once the eggs were found, the bait with the eggs was transferred into the larval rearing rectangular plastic containers (Size: 166×124×60mm). The containers were covered with a wet muslin cloth. Wet vermiculite was kept as the bottom layer in the cabinet to assist migration of third instars for pupation and to maintain humidity. 100 grams of fresh pork was put into the container as larval feed. This was continued until the instars reached the non-feeding stage and started pupal migration. Six individuals of different larval instars and pupae were randomly collected every six hours from all the three replicate per species. Fresh pupae were collected and transferred to a new rearing jar with moist vermiculite at the bottom and the jar was kept inside the rearing cabinet for the emergence of the adult fly. Six larvae were collected every six hours and boiled for two minutes at 80°C and preserved in 70 % alcohol for the assessment of length (Tantawi and Greenberg 1993, Adams and Hall, 2003). The time spent by the species in each life stage was recorded. Dry weight was also recorded using precision analytical balance. Based on these observations, growth curves were plotted.

The validation of the outdoor rearing results was done by comparing the data of laboratory rearing under controlled conditions and outdoor rearing data to check the presence of any differences of significance pertaining to length, weight and life cycle duration data for all species.

3.5. Relation between life cycle and time since death assessment for forensic application

3.5.1. Estimation of Post Mortem Interval (PMI)

Time dependent growth functions / equations are used to represent the biological growth of the larvae and this describes the variations experienced in length in relation to age. The growth in terms of length increases upto the post feeding stage and thereafter, a

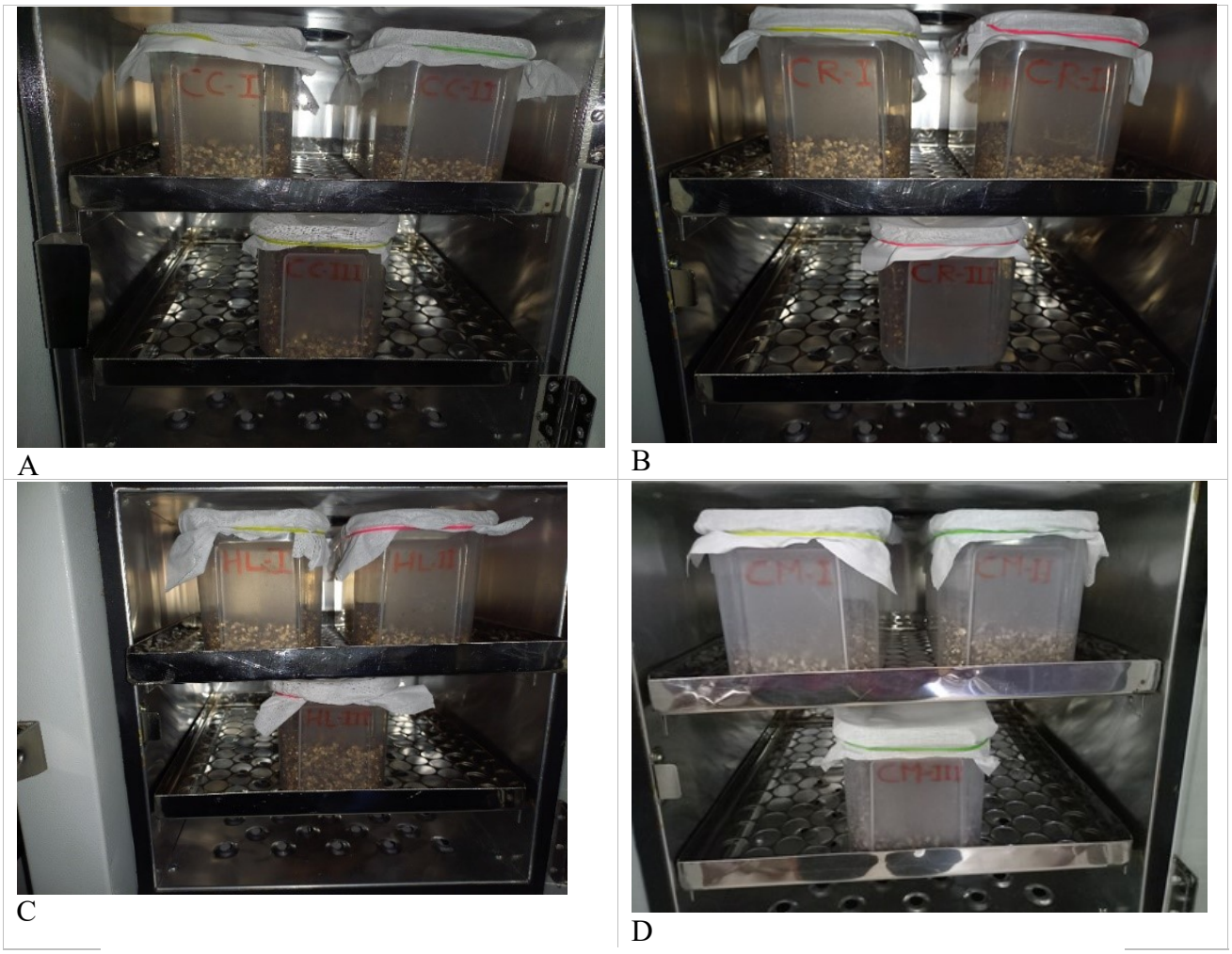

Fig. 3.9. Laboratory rearing showing brood chamber with culture replicates of blow flies

slight decrease was observed. A sigmoid curve was used to represent the evolution of size over time. The pattern of the growth curve (sigmoid) obtained was similar irrespective of the species or seasonal climatic conditions. In this study, the growth models best fitting to the data were used (Kiviste et al., 2002).

For the estimation of PMI, growth biology was represented by a graph plotted between time and length. PMI was estimated in the current study through the development rate with respect to the length of larval instars (Wells and LaMotte, 2017). The construction of growth curves were done based on the age-specific length parameters of larvae versus time taken for the development of each stage of the blow fly species. Regression method was used for constructing a model for the estimation of PMI.

3.6. Statistical analysis of data

Entire statistical analysis was performed using the software SPSS version 21.0. The analysis of variation in length, weight and percentage of survival between years, between seasons, between larval stages and all its interactions were tested by using three-way ANOVA with one factor as year, the second factor as season and the third factor as stage. If the interaction between these factors was found to be significant, pairwise comparison was done between the levels of one factor with different levels of other factor using Least Significant Difference (LSD) test by using least square means.

In the case of fecundity parameters and seasonal abundance, two-way ANVOA with one factor as year and the second factor as season was done to compare between years, between seasons and also to estimate the interaction between years and seasons. Pair wise comparison was done using LSD test by using least square means. The laboratory

rearing data of length and weight of larval instars were subjected to two factor ANOVA to compare between stage and temperature / humidity and also to test their interaction.

The duration of different life cycle stages compared between seasons and years was done using two-way ANOVA. The pairwise comparison between seasons and also between years was done by using LSD test.

For the estimation of PMI, regression equations were constructed as models. (Das and Giri 1979, Draper and Smith, 1988). The regression equations were fitted with length as the dependent variable and duration as the independent variable. Among the different models tested using curve estimation procedure in SPSS, S(sigmoid) function of the form, $E(Yt)=\exp(\beta 0+\beta 1/t)$ was used for predicting length in terms of duration, where E represents expectation, Y_t is the length (mm) at duration 't'(hrs.) and β_0 and β_1 are parameters of the regression model to be estimated. The above model was fitted by logarithmic linearization. Then the model became

$\ln(Y_t) = \beta_0 + \beta_1/t$ where, ln represents natural logarithm.

The best model was selected with the help of coefficient of determination (R^2) and root mean square error. The model having higher R^2 and lesser root mean square error was selected as the best model. To obtain an estimate of development time (Post mortem interval) from these models, reverse prediction procedure was used where the data for the response variable, length, was entered in the equation to obtain duration in hours (t).