CHAPTER II

MATERIALS AND METHODS
# 2 MATERIALS AND METHODS

## 2.1 Sample collection sites

Flies of calliphoridae and sarcophagidae were collected at different seasons from Maharashtra state, located in the West of India along the Arabian Sea. The climate of Maharashtra is typical monsoon climate with tropical conditions prevail all over the state. The winter season is cool dry spell, with clear skies, gentle breeze and pleasant weather prevails from November to February. But the Eastern part of Maharashtra sometimes receives some rainfall. Temperature varies between 12°C-34°C during this season (WRIS Wiki- India online report; Attri and and Tyagi, 2010). The summer season, from March to May, is hot. April and May are the hottest months. Temperature varies between 22°C-45°C during this season. Rainfall starts normally in the first week of July and it is the wettest month in Maharashtra, while August too gets substantial rain (NIDM, Government report, 2012).

The collected samples (Calliphoridae and Sarcophagidae) from different locations has been done by using the standard method and using different forensic entomology tools from different corps or using decaying meat or liver. Adults were collected by using insect net while larvae were collected by forceps. The tools which were used for sample collection as well as other experiment are as follows:

1. Aerial insect collecting net.
2. Forceps.
3. Buffalo’s liver or meat (Fresh/Rotten).
4. Preservative.
5. Insect collecting vials.
7. Small painting brush (no.2).
8. Mask.
10. Troughs.
11. Adult rearing cages.
13. Larvae rearing cages.
15. Electronic balance.
16. Tissue paper.
17. Dissecting microscope.
Rearing of carrion feeding flies at laboratory was problematic, because of the odor of the rotting meat. So that various diets were described for rearing these fly larvae (Singh, 1977). In case of maggot therapy, artificial diets were used to avoid the bad smell (Sherman and Pechter, 1988).

### 2.2 Morphological identifications

Maggots and adult flies were collected from different dead bodies of different animal or by using decaying meat of buffalo purchased from butchery house. Adult flies and maggots were reared in the laboratory in separate culture. Adults were fed daily on fresh liver samples and honey mixed in water. Fresh liver was used as oviposition or larviposition site for females. Maggot culture was provided with fresh liver as a food till the prepupae stage. Prepupae were kept in 500ml beakers containing dry soil which is required for the pupation. Adult flies emerged out from pupae were reared in rearing box size 22× 12×10inches in dimensions length × width × depth respectively and regular culture was maintained. After the collection of the samples, pure cultures for each species was obtained by separating eggs or larvae of one female and cultured them for identification and further experiment.

**Morphological identification of Calliphoridae:** Different stages of life cycle of collected species were dissected to observe the identification marks and followed the published identification keys. Adults and maggots were studied morphologically with the help of stereo-zoom microscope (ERMA Optical works, Tokyo, No. 44883) and light microscope (Magnus Trinocular Microscope MLX-DX, Olympus -India
Morphological characters were photographed by using a 16.1 Megapixel Sony Cyber-Shot digital camera.

Eggs of the flies were studied under the light microscope by using potassium staining technique (Sukontason et al., 2004). Different stages of collected Calliphoridae species were dissected and identified using morphological characters and identification keys (White et al., 1940; Maurice, 1971; Kitchin, 1977; O’flynn and Moorhou, 1980; Queiroz et al., 1997; Spradbery 2002; Sukontason et al., 2003a, 2004a, 2004b, 2005 and 2008; Sukontason et al., 2003b, 2003c, 2006 and 2007; Kamal et al., 2008; Carvalho and Mello-Patiu, 2008; Siriwattanarungsee et al., 2008; Szpila, 2010 and 2012). Also some morphological keys of adult fly were studied under the microscope as below to follow the identification keys to the different species of Chrysomya.

**Identification of Calliphorid flies**

**Identification keys to Chrysomya species**

1) Flies not more than 8mm. in length; eyes widely separated in male; hypopygium inconspicuous .......................................................................................................................... *Chrysomya nigripes*
   - Flies 10mm. in length or longer ........................................................................................................ 2

2) Anterior border of wings deeply infuscated ......................................................... *Chrysomya marginalis*
   - Wings are entirely hyaline .................................................................................................................... 3

3) Prothoracic spiracle white or yellow ......................................................................................... 4
   - Prothoracic spiracle brown .............................................................................................................. 5

4) Prothoracic spiracle yellow ventral side of the body is golden color (golden bluebottle blowfly) ................................................................................................................................. *Chrysomya incisuralis*
   - Prothoracic spiracle white .................................................................................................................. 11

5) Femora swollen in both sexes, but more noticeably so in male; distance between the eyes in both sexes equal to one-fifth total width of head, facets small and uniform, frons parallel-sided, parafrontalia black towards vertex, with silver tomentum anteriorly; ocellar bristles very weak dark reddish-black, fronto-orbital bristles absent in female, Lags: dark brown, femora and tibiai greatly swollen in male, less so in female, the former metallic in certain lights, segments of tarsi in male clearly demarcated from one another........................................
6) Eyes in the male more or less closely approximated; male hypopygium more or less conspicuous .................................................................7

7) Eyes in male separated by four-fifths width of one eye; male hypopygium large...................

.................................................................................................................................................................................................Chrysomya phaonis

8) Parafacialia never brilliant orange .................................................................10
   - Parafacialia and jowls brilliant orange, the latter clothed with golden hairs ...............9

9) Face covered with grey tomentum .................................................................Chrysomya defixa
   - Face dark reddish, jowls grey, black-haired ...................................................... Chrysomya pinguis

10) No black setulae on facial or parafacial around the vibrissa or, rarely, 2 or 3 present; frontal stripe of female not broader at middle of frons, parallel-sided; facets of eye of male larger above than below, but without any distinct line of demarcation; squamae brown. Larvae have complete bands of spines broad, 'on II-VIII,' band' on IX' slightly narrower and sparser; band on X very narrow and sparse, visible only on cleared specimen; most spines single-tipped but a few in the ventral spine bands double-tipped anterior spiracles with 11-14 processes.................................................................Chrysomya saffranea
   - At least several, usually many black setulae around vibrissa, on face and parafacial; facets of eye much enlarged above and sharply demarcated from area of smaller facets below; frontal stripe of female broader at middle of frons, not parallel-sided, Squamae brown.................

.................................................................................................................................................................................................Chrysomya megacephala

11) Prothoracic spiracle white or creamy, Adult small (5-6mm long); face and cheeks wholly yellow; male front femur with prominent, long white hairs ...................... Chrysomya varipes
   - Prothoracic spiracle white, Adult large (7 to 8 mm long); face and cheeks with dense silvery hairs on dark brown to black surface .................................................................12

12a) Anterior thoracic spiracle is open, presence of proepisternal seta (stigmatic bristle). Larvae have complete bands of spines on 11-IV; narrower band on V; many spines with two or three tips; eight fleshy protrusions per segment on IV-XI, those on V-XI having dark spines on their tips and the third to the sixth protrusions on V-XI being covered with dark, round-tipped scales and the space between the fourth and fifth projections being covered with similar scales; anterior spiracle with 9-10 processes........................................Chrysomya rufifacies

12b) Anterior spiracle is closed; absence of proepisternal seta.....................Chrysomya albiceps
12c) Anterior spiracle is closed, presence of proepisternal seta (stigmatic bristle).................
.............. (New Species) ................................................................. Chrysomya indiana

The morphological description of each calliphorid samples using the keys of previous studies mentioned above was done in detailes as below. Identification of Calliphoridae species was done by using the keys of adult and larvae supported with photos for some parts used in the identification. In this study there was a record of a new species of Calliphoridae named as Chrysomya indiana.

Chrysomya megacephala: It is a big-headed fly, shiny, metallic thorax and abdomen. It has yellow gena/ cheeks, anterior thoracic spiracle dark (brown to black). Like the name suggests, head is extremely large and has large conspicuous red eyes. Male eye have much enlarged facets above and smaller facets below with sharply demarcation between these facets. Female head with broader frontal stripe in middle, not parallel sided, ovipositor relatively long, no marked indentation in cheek, several or many, black setulae around vibrissae presented on face and parafacial. Lower squamae are blackish brown to dirty grey.

Larvae of C. megacephala were smooth maggots like most of the calliphorid flies and the anterior spiracles of 3rd instar with about 11-13 papillae.

Chrysomya rufifacies: This calliphorid species is one of hairy maggot blowflies. Adult of C. rufifacies shiny metallic blue-green, face and cheeks with dense silvery hairs on dark brown to black surface, anterior spiracle pale, creamy or white. Lower (posterior) squamae covered with fine hairs above. Anterior spiracle is open and presence of proepisternal seta (stigmatic bristle).

The larvae of C. rufifacies can be easily identified, the 1st instar larvae smooth without tubercles. The tubercles start developed from 2nd instar larvae, these tubercles are slender in shape have three minute dark spines or crown. Tubercles of 3rd instar larvae well developed and present in both dorsal and ventral surface with three rows of numerous crowns on the tip are present. Ventral papillae/ tubercles indistinct or absent with round scaling at base, smooth distally, absence of hairy like structure at the base of tubercles in the caudal region; Anterior spiracles with 9-12 papillae.

Chrysomya saffranea: Anterior spiracle of adult fly black or blackish, gena orange to reddish yellow, Female head with yellow hairs gena, no black setulae on facial or parafacial around the vibrissae.
Frontal stripes are parallel-sided on female head, not broader at middle of frons, squamae brown and facets of male eyes larger above than below without any distinct line of demarcation.

*Chrysomya indiana* sp. n.

**Type material**

HOLOTYPE (male): India: M.S. / Marathwada region/ Aurangabad, (19.9047N, 75.3102E) 02-VIII-2012, PARATYPES: 1 (male) same locality, same data as Holotype. The holotype deposited to the forensic entomology laboratory, Zoology department, Dr. BAMU, Aurangabad

**Distribution:** Aurangabad city, Maharashtra state- India.

**Etymology:** Named to the respect country of India

**Description**

The egg plastron is narrow but not exactly as elongated as the egg length, micopyle surrounded with very narrow Y shaped plastron, the shell surface shown light colored polygonal in the empty shell.

The 1\textsuperscript{st} instar larvae are smooth, hairy appearance started from the second instar larvae.

At 2\textsuperscript{nd} instar larvae, the maggots develop prominent tubercles encircling the dorsal and lateral side of the body of the maggot, because of these tubercles on maggot stages this species commonly known as hairy maggot species. Anterior spiracles with 10 –11 papillae, dorsal and lateral tubercles of 2\textsuperscript{nd} instar larvae have 8 strong black spines on the apex and have numerous spines surrounding the tubercle from the base to the top; posterior spiracle with light, unclear peritreme and separated two spiracular slit; small hairy like structure appears in the inner side of caudal region and between the bases of the caudal tubercles.

3\textsuperscript{rd} instar was almost similar to the 2\textsuperscript{nd} instar with slight difference as posterior spiracles with distinguishable open peritreme and three spiracular slits; the hairy structure in the caudal region well developed; in the late of 3\textsuperscript{rd} instar the two ends of open peritreme becomes very close to each other.
Adult male has big eyes like other males of hairy maggot flies. Size of the male is smaller than the female, anterior spiracle white colored, closed with the presence of proepisternal seta (stigmatic bristle).

**Morphological identification of Sarcophagidae:** The adult stage was used in the morphological identification of Sarcophagidae. Males were dissected to observe the marks and follow the published identification keys for Sarcophagidae species, which include the external structure, spinulation, characteristic and feature of male terminalia (McAlpine, 1981 and 1987; Smith, 1986; Sugiyama et al., 1990; McAlpine et al., 1993; Povolny and Verves, 1997; Kano and Kurahashi, 2000; Pape and Bänziger, 2000; Nandi, 2002; Spradbery, 2002; Sinha and Nandi, 2002; Sukontason et al., 2003d and 2004c; Dahlem and Naczi, 2006; Carvalho and Mello-Patui, 2008; Hayat et al., 2008; Chaiwong et al., 2009; Sukontason et al., 2003e and 2010; Vairo et al., 2011; Pape et al., 2011; Pekbey et al., 2011; Meiklejohn, 2012). The following steps were followed as identification keys to Sarcophagidae species

**Identification of Sarcophagid flies**

**Identification keys for Sarcophagidae Species**

1) Arista bare or with small fine trichiae; gena narrow, at most 0.2 of eye height; coxopleural streak present; longitudinal vitae on thorax not distinctive; hind coxa bare on posterior surface; male abdominal sternites 2-4 partly concealed by overlapping margins of corresponding tergites; abdomen with transverse bands/median stripe with lateral spots/three distinct spots – no clear checkerboard patterning..........................**Miltogramminae**

- Arista plumose; gena broad, more than 0.2 of eyes height; coxopleural streak absent; thorax generally with three distinct longitudinal vittae; hind coxa setose on posterior surface; male abdominal sternites 2-4 exposed and overlapping lateral margins of corresponding tergites; abdomen with distinct checkerboard patterning (exception of Bleasoxipha with median and lateral stripes)........................................**Sarcophaginae**..............................2

2) Frontal setae in rows parallel or gradually diverging near lunule ........................................3

- Frontal setae in rows strongly diverging near lunule ..........................**Sarcophaga** .........................5

3) Postalar wall bare; males with at least one strong proclinate orbital setae ..........................

.................................................................**Tricharaea (Tricharaea) brevicornis**
- Postalar wall setose; males without procline orbital setae ..............................................4

4) Gena and postocular with silver microtrichosity; male mid femur with ctenidium of normal (non-flattened) species .......................................................... **Blaesoxipha**

- Head with bright yellow microtrichosity at gena and silver microtrichosity at postocular; male mid femur with ctenidium of flattened species ....................... **Oxysarcodexia varia**

5) 2nd and 3rd antennomere at least partly yellow ................................................................. 6

- 2nd and 3rd antennomere at most reddish black .................................................................. 9

6) Ground color of terminalia at most reddish black (males: protandrial segment – epandrium- circus; females 6th abdominal tergite) .............................................................. 7

- Ground color of terminalia red or orange ........................................................ **Sarcophaga (Liopygia) ruficornis**

7) Katepisternum and 1st and 2nd abdominal sternites with setulae only black .............. 8

- Katepisternum and 1st and 2nd abdominal sternites with some setulae yellow/white........

.............................................................................. **Sarcophaga (Lioproctia) torvida**

8) Postocular with at least one row of black setulae in addition to black postocular setae, with setulae only yellow / ventrally ....................... **Sarcophaga (Fergusonimyia) bancroftorum**

- Postocular with setulae only yellow/ white.................. **Sarcophaga (Johnstonimyia) lincta**

9) Basal 0.5 of wing vein R1 setulose ..................................................................... 10

- Basal 0.5 of wing vein R1 bare ....................................................................................... 11

10) Propleuron with setulae only black; 1st and 2nd abdominal sternites setulae only black....

........................................................................**** Sarcophaga (Australopierretia) australis**

- Propleuron with setulae mostly yellow/ white; 1st and 2nd abdominal sternites with setulae only yellow/ white .......................... **Sarcophaga (Sarcorohdendorfia) meiofilosia**

11) Ground color of terminalia red or orange (males: protandrial segment – epandrium-circus; females 6th abdominal tergite) .............................................................................. 12

- Ground color of terminalia at most reddish black....................................................... 14
12) Propleuron with only one or at most a few scattered setulae. .........................................................

......................................................................................................................... Sarcophaga (Boettcherisca) peregrina

- Propleuron uniformly bare .................................................................................................................. 13

13) Postocular with setulae only yellow/white; surstylus rounded; cercus in lateral view with posterior projections near apex ........................................... Sarcophaga (Liopygia) crassipalpia

- Postocular with at least one row of black setulae in addition to black postocular setae, with setulae only yellow/white; ventrally surstylus reduced in size and pointed; cercus in lateral view with plateral projections near apex........................................... Sarcophaga (Bercaea) africa

14) Propleuron with only one or at most a few scattered setulae males and females........... 15

- Propleuron uniformly bare (only male) ........................................................................................................ 16

15) Males: vesica membranous with a series of spines; cercus without enlargement at apex; females: 7th abdominal sternite very concave on hind margin ...........................................................

......................................................................................................................... Sarcophaga (Boettcherisca) peregrina

- Males: vesica sclerotised without a series of spines; cercus with enlargement at apex; females: 7th abdominal sternite tear drop shaped...........................................................

......................................................................................................................... Sarcophaga (Fergusonimyia) bancroftorum

16) Katepisternum with setulae mostly black, but at least some yellow/white ......................

......................................................................................................................... Sarcophaga (Johnstonimyia) kappa

- Katepisternum with setulae only black ........................................................................................................ 17

17) At least the ventral surface of the 1st to 4th abdominal tergites with short setulae........

......................................................................................................................... 18

- From 1st to 4th abdominal sternites and ventral surface of tergites with dense very long setulae ................................................................. Sarcophaga (Taylorimyia) aurifrons

18) Juxta aneroventral part with a pair of elongated arms with a bifid tip ......................... 19

- Juxta with long arms bent inwards at 90°; cercus without medial projections near apex.................................................................................................................................... 21
- Juxta with short arms curved inwards; cercus with medial projections at apex...........................

Sarcophaga (Parasarcophaga) misera

19) Postocular with at least one row of black setae in addition to black postocular setae, with setae only yellow / white ventrally ..............................................................20

- Postocular with setae only yellow / white ...................... Sarcophaga (Liosarcophaga) dux

20) Gena with setulae only black ......................................... Sarcophaga (Liosarcophaga) sigma

- Gena with setulae, amix of black and yellow / white ..............................................................

Sarcophaga (Liosarcophaga) kohla

21) Postocular with at least one row of black setae in addition to black postocular setae, with setae only yellow/ white ventrally .................... Sarcophaga (Parasarcophaga) albiceps

- Postocular with setulae only yellow/ white ..............................................................

Sarcophaga (Parasarcophaga) taenionota

Morphological identification of collected Sarcophagid species was done successfully, according to previous identification keys. The terms used for description similar to the terms in different studies mentioned above were used for this identification. More details of description as below.

Sarcophaga ruficornis: Male and female terminalia bright red or orange, with the 2nd and 3rd antennomeres at least partly yellow. Gena, postocular with yellow colour setulae, prescutellar acrostichal setae are absent. In the caudal region of larvae, distinct inner projections between the spiracular slits, lower end of peritreme located at base of middle slit; number of papillae on anterior spiracles 11–15 papillae

Sarcophaga dux: Gena with setulae a mix of black and yellow or white. Postocular with setulae are only yellow or white in color. Parafrontal and parafacial black with silvery to golden pollen, the former with short scattered hairs. On the caudal region of larvae, indistinct inner projections between the spiracular slits, lower end of peritreme located beneath the base of the lower slit number of papillae 14–17.

Sarcophaga peregrina: Body color grey with black markings, parafrontal and parafacial black with silvery to golden pollen, gena black with numerous black hairs,
post gena black with numerous black hairs. Anterior spiracles with number 24–26 papillae in two rows, posterior spiracle widened, distinct inner projections between the spiracular slits tail of the upper end of peritreme was dilated.

2.3 Molecular identification

Molecular identification method was done according to (Wells and Sperling, 2001; Hebert et al. 2003a; Chen et al., 2004; Jefferies et al., 2007 Wells and Williams, 2007; Park et al., 2009; Bajpai, 2010; Meiklejohn et al., 2011, Singh et al., 2014). Samples provided with different sample ID (FZ10= C. megacephala, FZ20= C. rufifacies, FZ30= C. saffranea, FZ40= Chrysomya sp. (new species), FZ1= S. dux, FZ3= S. peregrina and FZ4= S. ruficornis). The flowing materials were used for molecular identification:

1. Adult samples for DNA barcoding, using mtDNA (COI gene)
2. QIAamp® DNA Mini Genomic DNA isolation kit
3. Buffer ATL - QIAGEN proteinase K
4. Universal primers
   - LCO1490, Sequence 5'- GGTCACAAATCATAAAGATATTGG-3' 25 Bases pairs
   - HCO2198, Sequence 5'- TAAACTTCAGGGTGACCAAAAAATCA -3', 26 Bases pairs
5. PCR unit
6. Montage PCR Clean up kit (Millipore).
8. ABI 3730xl sequencer (Applied Biosystems).
9. Sequencing DNA software blast and alignment software (MUSCLE 3.7, MEGA BLAST software, Gblocks 0.91b, PhyML 3.0 aLRT, ClustalX v2.0.12 and Bio Edit Sequence Alignment Editor V7.0.5.3.)
10. Bold system tools online for identification and extracting phylogenetic tree.

Specimens: For barcoding DNA, single specimens of each seven collected species of calliphoridae and sarcophagids were prepared; Chrysomya megacephala, Chrysomya saffranea, Chrysomya indiana (new species), Chrysomya rufifacies, Sarcophaga (Liosarcophaga) dux, Sarcophaga (Liopygia) ruficornis, and Sarcophaga
*Boettcherisca* peregrina. Leg tissues were removed from male adult specimens and kept in Eppendorf tube containing 3 ml of absolute Ethanol. All instruments used to remove legs tissues were cleaned in 70% ethanol, and all steps were done in sterile conditions.

**Extraction of DNA:** DNA from each specimen was extracted separately by using QIAamp-DNA Mini kit (QIAGEN-Germany) with the tissue protocol, following the manufacturer’s instruction. Tissue samples were cut into small pieces and placed in a 1.5ml micro centrifuge tube, and 180μl of Buffer ATL. QIAGEN proteinase K was added and at 56°C incubated overnight until the tissue was completely lysed.

**Amplification:** Gene COI fragment was amplified using universal primers LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' 25 base pairs and HCO2198 5'-TAAACTTCAGGCTGACCAAAAAATCA-3' 26 base pair (Folmer et al., 1994). Then 1μL of template DNA was added in 20μL of PCR reaction solution. The LCO1490/HCO2198 primers was used for bacteria, and then PCR reaction performed with initial denaturation at 94°C for 5min and then 35 amplification cycles at 94°C for 45sec, at 55°C for 60sec, and at 72°C for 60sec. Final extension at 72°C for 10min. A positive control (*E. coli* genomic DNA) and a negative control were included in the PCR.

**Purification of PCR products:** Unincorporated PCR primers and dNTPs were removed from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the LCO1490/HCO2198 primers. Sequencing reactions were performed using an ABI PRISM BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase Fluorescence Sequencing enzyme (FS enzyme) from Applied Biosystems.

**Sequencing DNA:** Single-pass sequencing was performed on each template using universal primersLCO14905'-GGTCAACAAATCATAAAGATATTGG-3' 25 base pair and HCO2198 5'-TAAACTTCAGGCTGACCAAAAAATCA-3' 26base pair. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were suspended again in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

The sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The MUSCLE 3.7 software was
used for multiple alignments of sequences (Edgar, 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b. This program eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana, 2007). Finally, the program PhyMLv3.0 aLRT was used for phylogeny analysis and K2P as substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering (Dereeper et al., 2008).

The alignment of Diptera sequences was carried out using the Clustal X v2.0.12 and the alignment file for analysis was prepared with BioEdit Sequence Alignment Editor V7.0.5.3. To avoid interferences in the analyses due to presence of stop codon, the sequences ends were cut. Analysis was, therefore, made with 658bp from COI barcode fragment. Each sequence was submitted to Barcoding of Life Database (BOLD; available online at www.boldsystems.org), record list of submitted project (DBCS) (Appendix: I). All sequences were entered into BOLD separately for each sample (Appendix: II-VIII), where stored and preliminary barcoding analyses were done. Sequences were submitted to Gene Bank through bold system. The BOLD Index Number (BIN) and GeneBank Accession Numbers were provided in (Appendix: IX).

2.4 Assessment of the seasonal effect on life cycle of flies

2.4.1 Life cycle of Calliphorid flies in different seasons

Adult flies were reared in cages size 22 ×12×10 inches covering with muslin cloth. Thermocol covered with white filter paper was used as a base in every cage. Fresh liver was provided daily in one Petri dish and water sweetened with honey in another dish, tissue paper was kept as base in the dish. While larvae rearing cabin was designed to minimize the odor in the laboratory. Chamber was designed to be air tight but had an exhaust and inlets. One end of each pipe was connected to the exhaust and the inlet while the other was outside the laboratory so that the odor will be released outside the laboratory. The open ends of the pipes were closed by fine mesh to avoid the entry of other flies. The exhaust fan used for the CPU box of computer was used. The exhaust fan was switched on so that the odorous air was released outside and the fresh air rushed in the chamber (Mali, 2011). At the upper surface of the chamber one
big aperture was made and tightly closed with transparent glass sealed with adhesive tape which made the observations easy.

After the maintaining of the pure culture of collected species of calliphoridae, as a stock culture, this stock culture was used for collecting the eggs for further experiments. About 100 eggs were collected with the help of fine brush and reared at the laboratory condition in different seasons (summer, rainy and winter season) to study the duration of life cycle and the morphological parameter of each of these species. Larvae were provided with fresh liver daily till the prepupae stage. Prepupae were kept in 500ml beakers containing dry soil which is required for the pupation. Temperature and related humidity were recorded every day at least 2-3 times a day. For measuring the morphological parameter (length, width and weight) killing of larvae was done by using method that results in the lowest change in length of maggots, larvae were immersed in hot water (above 80°C) before the measurement (Adams and Hall, 2003; Amendt et al., 2007; Tantawi and Greenberg, 1993). Prepupae were provided with dried soil in beakers and small containers to insure the dry condition for the pupation. The experiment with same method was repeated 5 times in every season for each species.

2.4.2 Life cycle of Sarcophagid flies in different seasons

Similar methods which have been done for rearing of calliphorids species were repeated for sarcophagids, except here in sarcophagidae species about 100 larvae were collected and reared at the laboratory condition in different seasons (summer, rainy and winter season). The experiment with same method was repeated 5 times in every season for each species.

2.5 Assessment of the effect of chemicals on life cycle of flies

Two chemicals (sedative drug and insecticide), zolpidem tartarte and dimethoate, were used for their impact on life cycle of calliphorid and sarcophagid flies. Chemical structures of these two compounds are depicted below (Figure: 4).
Zolpidem Tartrate is a sedative-hypnotics drug which is commonly used in treatment of sleeping disorders and anxiety. It is illegally used by people for suicidal purposes (Gock et al., 1999; Ben-Hamou et al., 2011; Darke et al., 2012). Zolpidem is available in 5 mg and 10 mg strength tablets for oral administration. On the other hand, dimethoate is a widely used organophosphate insecticide used to kill insects on contact (Stephenson et al., 2006). In many parts of the world typically in the developing countries, the availability of this insecticide for agricultural purposes makes them one of the most important causes of poisoning by accidental exposure, suicide and sometimes homicide (Sunger and Güven, 2001).

These substances have been selected because of their availability, their common use in suicidal cases. Also evidence from prior investigations showed that the larval growth rate can be altered when the feeding materials are contaminated with chemicals such morphine (Bourel et al., 1996, 1999), diazepam (Carvalho et al., 2001), paracetamol (O’Brien and Turner, 2004), triazolam (Kintz et al., 1990), phenobarbital (Kintz et al., 1990), codeine (Kharbouche et al., 2008), malathion (Mahat et al., 2009) and organophosphate insecticide (Abd El-bar and Sawaby, 2011).

2.5.1 Treatment with zolpidem tartrate

Zolpidem Tartrate Tablets I.P. 5mg, a trade name known as (Zolfresh*5), tablet, Manufactured by (Acme Formulation Pvt. Ltd. India), tablet was first dissolved in 5 ml distilled water until it completely dissolved to make 1mg/ml solution. The liver was chopped in the mixer and 50gm of chopped liver was mixed with the zolpidem (0.05ml, 0.1ml, 0.15ml and 0.2ml) to prepare different concentrations (1ppm, 2ppm, 3ppm and 4ppm) respectively. One sample with only chopped liver was maintained as a control. These fresh samples of fresh liver contaminated with freshly prepared zolpidem solution were prepared on daily basis. For each
concentration 60 1st instar maggots were released on the 50 grams of prepared liver sample to avoid the overcrowding in the containers. Durations required for the development of each stage in different concentration were recorded. Morphological parameters (length, width and weight), temperature and humidity were also recorded. Killing of the larvae was done by using boiling water method to measure the length of maggots in relaxed condition (Tantawi and Greenberg, 1993; Adams and Hall, 2003 Richards et al., 2013; Rosilawati et al., 2014).

2.5.2 Treatment with Dimethoate

Dimethoate used in this study known as (TAFGOR Dimethoate 30% EC) as trade name, manufactured by Rallis India Limited- India. Dimethoate was diluted in distilled water to prepare 1mg concentration by mixing 3.33ml of dimethoate 30% in 1000ml of distilled water. Different concentrations of dimethoate were prepared (0.05mg, 0.1mg, 0.15mg and 0.2mg) and mixed with 50gm of chopped liver to get 1ppm, 2ppm, 3ppm and 4ppm and one set was kept as a control. The experiment was set exactly as for the zolpidem experiment.

2.6 Estimation of minimum PMI

There are many methods used for PMI estimation, most of them with limitations and complicated process. In this study minimum PMI was estimated by using the degree of development of larvae, length and width (Wells and LaMotte, 2001; Sharma et al., 2015; Mali, 2011) Post Mortem Interval directly correlated with the development stage and length, width and weight of the larva, PMI can be determined from the larvae of blow flies and flesh flies using the growth parameter and larval, length and width and weight as a biological clock. The growth parameter of the egg, first, second and third larval instars, total larval period, pupal period and egg-to-adult period was studied in the laboratory. Hence the age of the oldest (longest) developmental stage provides the minimum post mortem interval. For more accuracy of the measurement of length, width and weight previous method of killing larvae was used.