UNIVERSITI SAINS MALAYSIA

UNIVERSITI SAINS MALAYSIA

Nocturnal Ovipositioning of Flies

Dissertation submitted in partial fulfillment for the
Degree of Bachelor of Science (Health) in Forensics

Helmi Bin Mohd Hadi Pritam

School of Health Sciences
Universiti Sains Malaysia
Health Campus
16150, Kubang Kerian, Kelantan
Malaysia

2006

CERTIFICATE

This is to certify that the dissertation entitled

"Nocturnal Ovipositioning of Flies"

is the bonafide record of research work done by

Mr. Helmi Bin Mohd Hadi Pritam

during the period **December** to **May**
under my supervision.

Signature of Supervisor:

Name and address of Supervisor: **DR. P.T JAYAPRAKASH**
Pensyarah
Pusat Pengajian Sains Kesihatan
Universiti Sains Malaysia
Kampus Kesihatan
16150 Kubang Kerian
Kelantan.

Date: 11. 05. 2006

ACKNOWLEDGEMENT

First of all, I would like to thank God for giving me the opportunity for me to give credit to. Firstly I would like to thank my supervisor Dr. P. T. Jayaprakash for the continuous guidance and support given and to all the knowledge he has bestowed on to me.

Next, I would like to thank postgraduate student, Mr. Naji Manat Arafat who had aided me whenever I am in doubt particularly in giving me initial guidance in order for me to start the research.

I would also like to give my heartiest appreciation to Mr. Muhamad Zakuwa bin Rodzali, Assistant Director, Research, Department of Islamic Development Malaysia for aiding me in scheduling the times for the experiment in my research.

I would also like to thank my family, particularly my mother and brother for the ongoing support and understanding the hardship and time constraints that constantantly interrupted our family time.

All the experience I obtained so far is in part due to the generosity of my teachers, colleagues, and I would also like to express my gratitude to those whom I forgot to specify here.

Helmi Bin Mohd Hadi Pritam

Forensic Year 4, 72600

CERTIFICATE

This is to certify that the dissertation entitled

"Nocturnal Ovipositioning of Flies"

is the bonafide record of research work done by

Mr. Helmi Bin Mohd Hadi Pritam

during the period **December** to **May**
under my supervision.

Signature of Supervisor:

Name and address of Supervisor: DR. P.T JAYAPRAKASH
Pensyarah
Pusat Pengajian Sains Kesihatan
Universiti Sains Malaysia
Kampus Kesihatan
16150 Kubang Kerian
Kelantan.

Date: 11. 05. 2006

TABLE OF CONTENTS

Tables

Charts

Figures

Appendix

ABSTRACT

Post-mortem interval (PMI) is calculated by ascertaining the age of the immature stages of blow flies demonstrating the longest period of association with decomposed human remains. The time at which the flies oviposit is generally assumed to be during the day time in which the flies are known to be active. A few researchers have indicated that limited number of flies do oviposit during night hours and under artificial lighting although studies on twilight ovipositioning have not been carried out so far. Moreover, previous researchers have indicated the use of mutton purchased from the market which does not preclude the possibility of flies depositing the eggs in the market place. This thesis is the compendium of findings of a research that addressed, for the first time, the possibility of ovipositioning during twilight as well as using beef purchased directly from the slaughter house in Kota Bharu, Kelantan. The control and night experiments were carried out inside the campus of Universiti Sains Malaysia, Kelantan. The results indicate the possibility of limited ovipositioning during the twilight period which further declines during the night period although ovipositioning is found significantly delayed by 4 to 5 days after continuous exposure of the animal model during the twilight and night time while diurnal oviposition in the control animal model does not indicate such delay. It is argued here that the delayed appearance of limited maggots attributable to nocturnal ovipositioning need not be construed as a factor attributing to estimating longer PMI since the maggots on account of immediate diurnal ovipositioning are the ones that are longer and considered for estimating PMI. It is suggested that PMI has to be estimated only by considering diurnal ovipositioning and the possibility of disposal during night has to be left for the investigating agency to establish through evidence other than entomological.

KEYWORDS: forensic science, forensic entomology, nocturnal ovipositioning, twilight ovipositioning, Calliphoridae, Sarcophagidae, fly-proof cage.

INTRODUCTION

The term 'Entomology' is derived from the Greek word *entomon* (insect) and *logos* (word, reason) meaning the study of insects (Gupta and Setia, 2004) . The first use of forensic entomology has been cited from the Chinese literature "His yuan chi lu" where death investigator Sung Tzu in the 13th century had mentioned probably the first case in which insects led to the identifying the culprit of a murder. The deceased had been slashed probably by a sickle and Sung Tzu told all the villagers to bring their sickles and lay them down. Flies were attracted to a particular sickle leading to apprehending the culprit who confessed to the crime (Benecke, 2001).

Hundreds of arthropod species, mainly flies (Diptera) and beetles (Coleoptera) are attracted to a corpse (Benecke, 2004). Due to the sheer number and size of the variety of species, athropods have proved useful in estimating the time since death. Out of the the two arthropod families, dipterans are known to colonize a corpse first (Leccese, 2004) and provide an estimation of the time since the presence of a cadaver. To determine the PMI, an assessment of physical conditions of the body must be combined with assessment of the fauna associated with it (Marchenko, 2001). Aside from the entomological fauna, the environmental conditions that affect the succession on the cadaver also play an important aspect in determining PMI. The factors influencing oviposition of flies that have been studied so far range from the presence of semiochemicals to pheromones.

The presence of semiochemicals chemicals such as sulphur and ammonia have been shown to attract certain flies (Byrd and Castner, 2001) and high level of moisture in the air has been shown as a factor inhibiting ovipositioning. Other studies have shown the influence of pheromons in determining spatial distribution of egg laying and found that the female blowflies are often attracted to the same sites to lay their eggs indicating that the first eggs laid provide the stimuli for other flies to oviposit (Byrd and Castner, 2001). Role of olfaction has been studied and odours of putrefying tissue have been found highly attractive to gravid females of primary facultative species, but less attractive to gravid females of obligate species (Byrd and Castner, 2001). Studies on nocturnal ovipositioning have been done in the past by a few researchers (Greenberg, 1990; Greenberg, 1991; Singh and Bharti, 2001; Spencer, 2002)

Night-time is defined as the time in every 24 hour period when it is dark (Cambridge Advanced Learner's Dictionary, 2006). Night is the time when a location is facing away from the Sun, and thus dark (Wikipedia, 2006). While 'daytime' has been defined as the period between the time when the sun rises and the time it goes down (Cambridge Advanced Learner's Dictionary, 2006), a 'day' has been defined as a period of 24 hours, especially from 12 o'clock one night to 12 o'clock the next night (Cambridge Advanced Learner's Dictionary, 2006). Dawn itself is of three different types, namely: dawn or civil dawn, nautical dawn, and astronomical dawn (Wikipedia, 2006). Dusk is defined as the darker stage of twilight, especially in the evening (The American Heritage® Dictionary of the English Language, 2006). And like dawn, dusk is also of three types, dusk or civil dusk, nautical dusk, and astronomical dusk. Twilight is the diffused light from the sky during the early evening or early morning when the sun is below the horizon and its light is refracted by the earth's atmosphere (The American Heritage® Dictionary of the English Language, 2006). From this broad meaning, there are also three types of twilight which are civil twilight, nautical twilight and astronomical twilight (Wikipedia, 2006). So dawn and dusk are both actually twilight and the difference relates to the time of observed in a day based on the definition.

This research is the first of its kind addressing (a) dawn, dusk and nocturnal ovipositioning, (b) the use of an animal model that undergoes a continuum of decomposition for as long as a period of 10 days, (c) the use of an improvised fly proof cage that can be kept open at desired time duration and (d) the possibility of nocturnal ovipositioning in Kelantan and Malaysia. The results of this research, for the first time, reveals that although nocturnal ovipositioning occurs, it is limited and is significantly delayed by more than 4 days when the animal model undergoes a continuum of decomposition, and hence cannot interfere in the estimation of PMI as had been cautioned by Greenberg (1990).

REVIEW OF LITERATURE

Most of the researches on the assessment of variables influencing succession of dipterans on cadavers that can be useful during forensic investigation have been based on retrospective analysis of maggots found on corpses at the crime scenes (Omar et al., 1994, Benecke, 1998; Introna et al., 1998; Sukontason et al., 2001; Benecke, 2001; Benecke and Lessig, 2001; Amendt et al., 2004; Archer, 2004; Arnaldos et al., 2004; Arnaldos et al., 2004; Benecke et al., 2004; Campobasso et al., 2004; Klotzbach et al., 2004; Klotzbach et al., 2004; Oliva and Ravioli, 2004; Oliveira-Costa and Mello-Patiu, 2004; Turchetto and Vanin, 2004). Though such studies related to real life situations in as much as there were human cadavers, these studies related to grown up maggots and little information had been inferred about the variables affecting oviposition in the initial phase of dipteran infestation. Interferences on the variables relating to ovipositioning including geographical area (Introna et al., 1998' Wolff et al., 2001; Bharti and Singh, 2003; Arnaldos et al., 2004; Carvalho et al., 2004; Tabor, et al., 2004) are limited to the area in which the study was carried out. Some studies relate to the effects of season on a particular succession of dipterans (Arnaldos et al., 2004) and temperature as a variable (Grassberger and Reiter, 2001; Archer, 2004) where the temperature obtained from the weather station has been corrected so that it can be applied to the cadaver. Moisture, semiochemicals, pheromons, and role of olfaction (Byrd and Castner, 2001) are some of the factors that have been suggested to render the carcass attractable. Environment has also been shown to alter the manifestation and growth of entomofauna which are necrophagous (Campobasso et al., 2001; Marchenko, 2001).

Understanding the process of corpse decomposition is basic to establish the PMI (Campobasso et al., 2001). The time the insects start to develop on a cadaver does not necessarily coincide with the moment of death, and the place where the cadaver was found may not be the one of death (Marchenko, 2001).

Insects are poikilothermic and their larvae have faster life cycle completion in an optimum temperature of 31°C (Grassberger and Reiter, 2001). Larval mortality differed from species to species within *Chrysomya spp.* Larval death was reported at 30°C for *Chrysomya spp.* (Lefebvre and Pasquerault, 2004) while for *Lucilia spp.* mortality was observed at 25°C. Kota Bharu, Kelantan has a temperature range of 22°C to 34°C (Malaysian Meteorological Department, 2006). This will ultimately influence the development of necrophagous species. Nevertheless, the climate in India where Singh and Bharti (2001) conducted the study on nocturnal oviposition can be said to be somewhat similar to that in Malaysia. Temperature and humidity can influence the oviposition (Campobasso et al., 2001; Grassberger and Reiter, 2001; Lefebvre and Pasquerault, 2004) and development of larvae (Dadour et al., 2001; Marchenko, 2001).

Collection, Killing, Cleaning and Preservation of Flies

One of the best methods of killing larvae is by immersion of larvae in hot water and transferring it directly to preservation agent such as 10% formalin and 70% ethanol (Adams and Hall, 2003). This method gives the least variation in maggot lengths (Adams and Hall, 2003). Other methods include direct preservation in 70% alcohol (Byrd and Castner, 2001, Leccese, 2004).

For killing the adult flies which are reared from maggots, ethyl acetate has been used (Dahms et al., 1979). This choice of killing agent has been based on its relatively lesser harm to humans (Dahms et al., 1979). Only one or two drops are needed to charge a jar with vapour of sufficient concentration to kill the flies (Dahms et al., 1979). Excess ethyl acetate has been indicated to cause condensation on the inner walls of the jar which wets and discolours the specimens (Dahms et al., 1979). The procedure for storing insects has also been described (Dahms et al., 1979)

Taxonomical basis for identification of insects has also been described earlier (Division of Medical Entomology IMR, 1971; Kurahashi et al., 1997). The key for microscopic identification of larval species has been published by Sukantason (2004) and by The Institute of Medical Research (IMR), Kuala Lumpur (Division of Medical Entomology IMR, 1980).

Selection of Animal Model

Many researchers have used animal models to simulate a decompositing cadaver (Omar et al., 1994; Anderson, 1999; VanLaerhoven and Anderson, 1999; Oliva, 2001; Singh and Bharti, 2001; Wolff et al., 2001; Spencer, 2002; Bharti and Singh, 2003; Omar et al., 2003; Archer, 2004; Carvalho et al., 2004; Leccese, 2004; Tabor et al., 2004). The choice of animal differs with the objective of the study. If the objective of the study requires estimation of time taken for a cadaver to decompose and skeletonize, researchers often used white pigs (*Sus scrofa*) adult of suckling cadaver (Archer, 2004; Carvalho et al., 2004; Leccese, 2004; Spencer, 2002; Tabor et al., 2004; VanLaerhoven and Anderson, 1999, Wolff et al., 2001) as the pigs tend to resemble human beings in bulk, in the quantity of body hair and the size of the back. Dipteran infestation has been studied using mutton (Singh and Bharti, 2001) and beef (Oliva, 2001). Rabbit carcass has also been used (Bharti and Singh, 2003). In Malaysia, earlier researchers have utilized cadaver of a Cynomolgus monkey, *Macaca fascicularis* (Omar et al., 1994, Omar et al., 1994). Opportunist study has been conducted using illegally killed bear cubs (Anderson, 1999). Food material used for rearing larval forms included prawns, cow liver and mixed fruits (Omar et al., 2003) as well as fish (Omar et al., 2003). In this research, fresh beef weighing 500 gm obtained from the slaughterhouse was used as the animal model since this research was restricted to aspects relating to oviposition.

Forensic Entomological Research in Malaysia

In Malaysia, very few studies have been done in forensic entomology. Retrospective analysis of forensically important entomological specimens from human cadavers has been done (Lee, 1989; Omar et al., 1994). The studies revealed *Chysomya megacephala* as the dominant species followed by *Chysomya rufifacies* (Lee, 1989). Lee (1989) noted that from all the larvae specimens taken from human cadavers to be processed at the Institute of Medical Research (IMR) *Chrysomya spp.* were found in 76.2% of all the cases studied. These findings are in agreement with the two studies by Omar (Omar et al., 2003, Omar et al., 2003) wherein also *Chysomya megacephala* and *Chysomya rufifacies* were found most prevalent.

Previous Studies on Nocturnal Ovipositining of Flies

It is well known that PMI is estimated taking into consideration the day time lapsed on a restrospective analysis (Benecke and Lessig, 2001; Arnaldos et al., 2004; Arnaldos et al., 2004; Benecke et al., 2004). Blowflies are diurnal species and usually rest during the night (Byrd and Castner, 2001). The eggs are not usually laid at night and therefore are laid the next day (Byrd and Castner, 2001). Some researchers have studied the nocturnal ovipositioning behavior of dipterans (Greenberg, 1991; Greenberg, 1990; Singh and Bharti, 2001; Spencer, 2002; Tessmer et al., 1995). Table 1 and 2 show the summary of the findings made by previous researchers.

Table 1: Summary of the published studies and experiments concerning nocturnal ovipositioning.

Author	Year experiment performed	Location	Trial period	Number of nights in total	Positioning of bait: ground or elevated	Number of sites utilised
Greenberg	1990	Chicago, U.S.A	June, July, August	17	On the ground under a bush & under an alley light	2
Greenberg	1991	Chicago, U.S.A	July, August	6	On the ground under a bush & under an alley light	2
Tessmer *et al.*	1995	Southern Louisiana, U.S.A	July, August	7	30cm elevated above ground	7
Singh & Bharti	2000	Patiala, India	March, September	14	Elevated, 6 feet in the air on a platform	1
Spencer	2002	Bournemouth, Britain	August, September	9	ground and on a 60cm elevated platform	2

Table 2: Abstract of the findings on previous nocturnal oviposition studies.

Author	**Bait exposure hours**	**Evidence of nocturnal oviposition**		**Success Rate of evidence for oviposition**
Greenberg 1990	Sunset 20h30	Yes		
	21h00-22h00	No		
	22h00-0h00	No		
	01h00-04h00	Yes		4 in 11 trials= 33%
Greenberg 1991	0h00-03h00	Yes		2 in 6 trials= 33%
Tessmer *et al.* 1995	13h00-20h00	Yes		
	21h00-05h00	No		0 in 14 trials= 0%
	06h00-13h00	Yes		
Singh & Bharti 2001	22h00-03h00	Yes		5 in 14 trials= 35%
		Ground	Elevated	
Spencer, 2002	20h00-22h00	No	No	0 in 9 trials= 0%
	22h00-0h00	No	No	0 in 9 trials= 0%
	0h00-02h00	No	No	0 in 9 trials= 0%
	02h00-04h00	No	No	0 in 9 trials= 0%
	04h00-06h00	No	No	0 in 9 trials= 0%
	10h00-13h00	Yes	Yes	

It is seen that there has been no research addressing the possibility of oviposition during the twilight period. Furthermore, the previous reserarchers (Greenberg, 1990; Greenberg, 1991; Singh and Bharti, 2001; Spencer, 2002) have not included a continuum of decomposition of the animal model in their studies although such inclusion assumes significance in applying the research findings to case situations since the human body undergoes a continuum of decomposition after disposal.

OBJECTIIVE OF THE STUDY

Objective

The objective identified for this research are:

(i) to determine the possibility of nocturnal ovipositioning including the twilight period in Malaysia and

(ii) to asses the influence of nocturnal oviposition in determining PMI by simulating an animal model to undergo continuous decomposition for consecutive days after disposal.

Hypothesis

It is hypothesized that necrophagous dipterans that are known to oviposit during day time may also oviposit during twilight and night period and thus confuse estimation of PMI especially when an animal model undergoes a continuum of decomposition.

METERIALS AND METHODS

I. Materials used for this research

Animal Model

Specific importance was given to the selection of the animal model in order to simulate a fresh cadaver and to follow the changes due to decomposition as closely as possible duly considering the objective of the study which was limited to observing the ovipositioning by flies. Bovine meat weighing 500gm per cage was used. A total of 2 kg of meat was used for each cycle. The meat was purchased at the slaughterhouse at about 0430 hours the animal having been slaughtered one hour earlier (as this was as close to simulating recent death). The meat was bought on the same day the experiment was conducted and was placed in the cage within 30 minutes after purchase.

Improvised Fly-proof Cages

The cage for the animal model used in this research was an indigenous fabrication modified following the basic cage design described by Singh and Bharti (2001). The cage was designed on an elevated platform with a suitably heavy improvised base, a wooden base (30.5 cm X 30.5 cm) that formed the platform with 18 cm high plastic mosquito net on all the four sides and top. The platform was mounted on a wooden pole of about 100 cm height in which the bottom to an extent of 43 cm was embedded into a cylindrical tin container that was filled with cement concrete. This ensured stability preventing toppling of the cage as well as easy movement of the cage. Used wood materials obtained from a previous construction site were used for fabricating the cage. The base (30.5cm by 30.5 cm) served as the platform for placing the animal model and was planked with a 2.5 cm high wall along its periphery in the four sides of the base in order to prevent direct contact of the beef with the net. The mosquito net used to protect the beef from access by the flies was of the commercial type made of plastic and Velcro was used to stick and secure the net with the cage wall. A stapler gun with stapler bullets was used to attach one Velcro strip to the wood while its counterpart Velcro strip was sewn to the mosquito net. The net was made up of two parts. One part, the two side walls was permanently secured while the sewn part, the other two side walls and the top of the cage could be opened and closed using the Velcro. The part of the net that could be opened or closed was sewn at one end with the permanent netting. Figure 5 is the diagrammatic representation of the cage design. On the wooden pole of the cage, an A4 size paper notice was affixed. Figures 1 to 4 demonstrate the cage in situ.

Figure 1: Full view of cage (closed)

Figure 2: Full view of cage (opened)

Figure 3: Cement concrete filled base

Figure 4: Notice – close-up

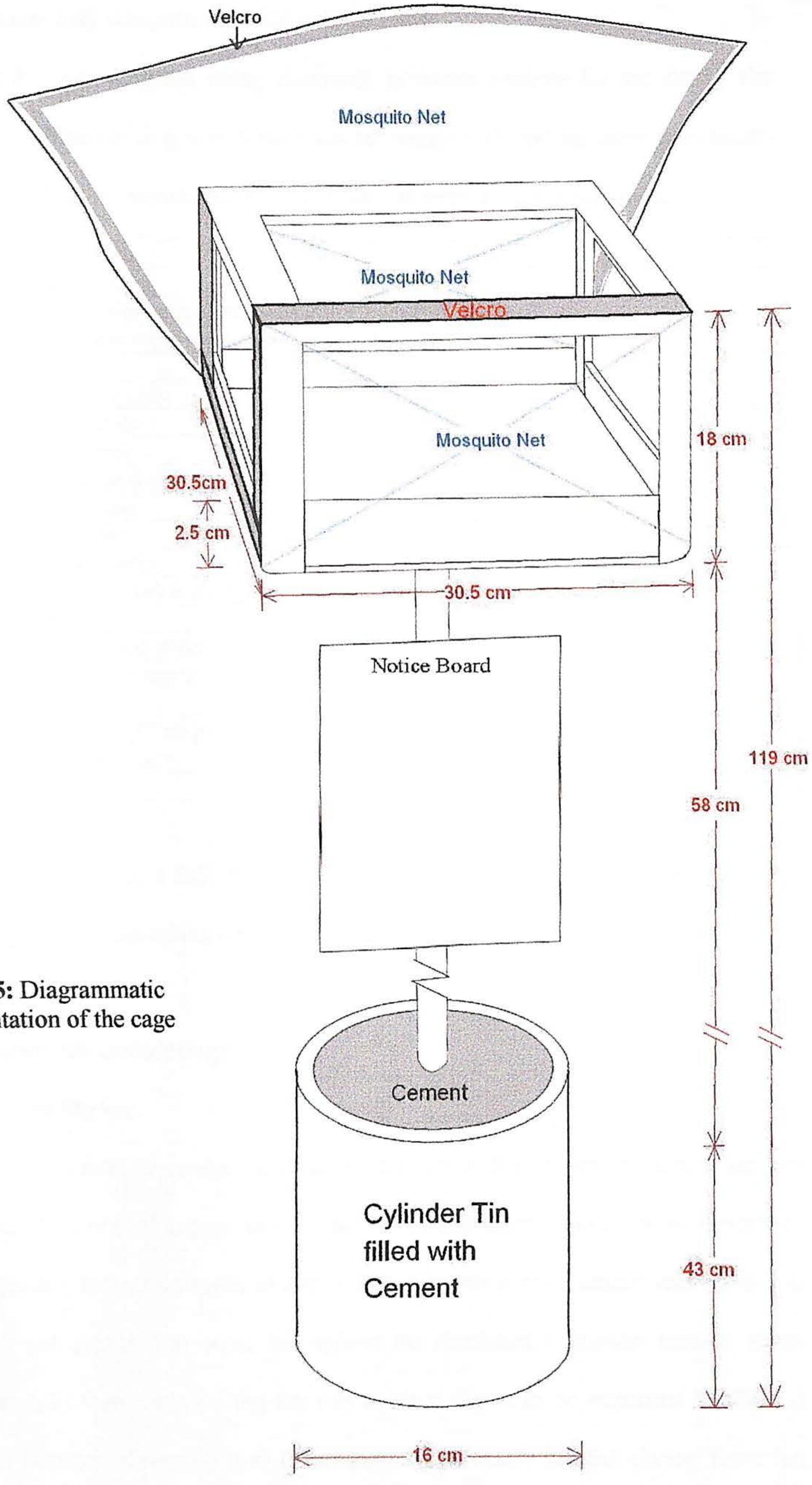

Figure 5: Diagrammatic representation of the cage design.

List of Equipment and Reagents and Chemicals Used

A toolbox kit was designed using a normal hardware toolbox for use during the observation and collection as it was convenient to transport all the necessary equipments to each study site. List of instruments and reagents that were used are as follows:

1. Latex gloves
2. Pointed forceps
3. Capped plastic vials (3cm diameter x 5 cm height)
4. Clear plastic cups with sieved cup covers
5. Spatula
6. Magnifying glass
7. Ethyl Acetate
8. 80% ethanol
9. Microscope slides
10. Thermometer
11. Petri Dishes
12. Surgical blades
13. Permanent marker pens
14. Pocket knife
15. Notebook and stationery
16. Plastic centrifuge tubes (orange cap – 50 ml)
17. Cotton balls
18. Small pins of 3cm length
19. Styrofoam sheets
20. Fly paper

During daily observations, a digital camera (Canon A 70, 3.2 mega pixels) was used for photographing and videographing the specimens.

II. Research methodology

Selection of Test Period

The period from December to March was chosen for the experiment since this period covers the monsoon season as well as the normal tropical weather in Kelantan . During this period, heavy rain spells of 2 to 3 days duration is experienced and about 3 or 4 such spells are expected to occur throughout the Northeast Monsoon season. Rainy spells, occasionally heavy and lasting for two to three days can be expected for three to four episodes between November and December. The monthly rainfall during these two months range from 600 to 800 mm. In the following three months (January till March), the weather is relatively drier with monthly rainfall of 100 to 180 mm. Between these heavy rain spells, the weather is relatively fair (Rainfall, 2006). The study was conducted in an open field to ensure that trees, bushes or shrubs and buildings do not interrupt the exposure to the elemental forces.

Climate that Prevailed During the Study Period

The study sites were in the open fields of the Universiti Sains Malaysia Health campus, Kelantan, Malaysia. This tropical region lies in latitude 06-10N, longitude b102-17E located about 5 m above sea level with an average temperature of 29 ± 3°C, the relative humidity being about 75 ± 10% and with an annual rainfall of 1.3 to 2.0 m (Malaysian Meteorological Department Ministry of Science, 2006).

All the beef samples were placed in the middle of the platform inside the cage ensuring uniformity of environments for all samples. The control for this experiment was the 'day cage' as it is well known that flies oviposit during the day time. The daily rainfall data during the study period was obtained from the Malaysian Meteorological Department's website (Rainfall, 2006).

Duration of Experiment

Two main cycles extending to a maximum period of observation for 10 days were designed during this research. A period of 10 days was considered sufficient to observe fly oviposition. Since, in the control experiment, oviposition and consecutive larval growth reaches almost completion within this ten days period that nocturnal oviposition beyond such period would not be relevant for interpreting PMI. Periods beyond 10 days will attract other insects to the meat which are not of study interest (Omar et al., 1994;

VanLaerhoven and Anderson, 1999; Byrd and Castner, 2001; Schroeder et al., 2002; Bharti and Singh, 2003; Carvalho et al., 2004). A pilot study was conducted to test the cage for its durability to harsh environmental conditions and the ability of the net to deter flies from ovipositioning on the meat when the case was closed. The test study was also done in order to familiarize with the working conditions during the actual experiment.

Aside from the experiments required for ascertaining nocturnal oviposition, a separate experiment was done during the trial run to find normal fly infestation when the animal model was kept on the ground. This test was done within the second cycle in order to find the maggot species from normal day time ovipositioning.

Table 4: Duration of Experiments

Experiment	Starting Date	Ending Date	Duration	Animal Model
Pilot Study	17th December 2005	24th December 2005	8 Days	700gm Processed beef liver
Cycle 1	9th January 2006	18th January 2006	10 Days	4 x 500gm bovine meat
Cycle 2	31st January 2006	9th February 2006	10 Days	4 x 500gm bovine meat
Open Trial	3rd February 2006	9th February 2006	7 Days	2.0kg bovine meat

Cage Placement

The cages were placed inside the premises of Universiti Sains Malaysia choosing stations so as to ensure total darkness and an inter-distance of at least 50 m in between two cages (Schoenly et al., 1991). Enabled preventing of the fly population from being attracted from one station to another. Furthermore, the cages were placed in a field at least 20 m away from any kind of tree or bushes and also at least 50 m away from the nearest building under human inhabitation. The cages were located at least 10 m away from the nearest road to avoid the odour of the decaying meat from disturbing any passer-by. Each of the stations chosen was completely devoid of any kind of artificial lighting (mainly