



Faculty of Medicine and Health Sciences

**Implications of Pesticide-Related Deaths on Forensic Entomological
Investigations and the Elucidation of Secondary Pesticide
Bioaccumulation in Blowfly Tissues**

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Implications of Pesticide-Related Deaths on Forensic Entomological
Investigations and the Elucidation of Secondary Pesticide Bioaccumulation in
Blowfly Tissues

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



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سَمِ اللّٰهُ الرَّحْمٰنَ الرَّحِيْمَ

(In the name of Allah, the Most gracious and the Most merciful)

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ABSTRACT

In forensic entomology, insect's development and succession pattern associated with decomposing carrion can become a useful information in the estimation of post-mortem interval. Several external factors such as environment, corpse's condition and the presence of toxins or chemicals can alter the rate of development, insects' arrival, and the duration of decomposition of the carrion. The objectives of this study were to investigate the effects of pesticides on the development rate of blowflies, carrion decomposition and insect succession pattern. Besides, this study also aimed to detect the presence of pesticides in larval tissue of the colonising insects. Three types of pesticides were used in this study; glyphosate (herbicide), chlorpyrifos (insecticide) and coumarin (rodenticide). Several concentrations of these pesticides were spiked into the meat substrate and then the blowfly eggs were placed on the substrate. The development duration and stages of the blowfly from eggs to adulthood were observed and recorded. The finding showed that the larvae did not survive at the higher concentration of glyphosate and chlorpyrifos-treated meat substrate. Meanwhile, the larvae in coumarin survived until puparial stage, but the adult failed to emerge. For subsequent experiment, the pesticides were orally administered to rats (*Rattus norvegicus*), and then euthanised and brought to the study site, at a secondary forest in Kuching, Sarawak. The decomposition stages and duration were observed and compared with control carcasses. The insect succession pattern was also recorded and collected for species identification. The third instar larvae were also collected for toxicological analysis. From the field experiment, a total of 1,579 insects were collected in three trials of experiments. The insects are belonging to nine families from five orders with *Chrysomya rufifacies* (71.5%) was the predominant species infesting on the carcasses, followed by *Chrysomya megacephala* and *Hemipyrellia ligurriens*. The decomposition of the carcasses was consisted of five stages which include;

fresh, bloated, active decay, advanced decay and dry remains stage. The carcasses decomposition duration was delayed for carcasses in chlorpyrifos group (17-21 days), followed by coumarin (~11-12 days) and glyphosate (~8 days) compared to control group (~7 days). During the early stages of decomposition process, the species that were first to arrive was blowflies (Calliphoridae), followed by flesh flies (Sarcophagidae) and muscid flies (Muscidae). The sequence of insect succession in control and pesticide-treated carcasses were similar. During the active decay stage, the larvae were actively infesting the carcasses' tissues except for chlorpyrifos carcasses as the oviposition did not occur, hence the carcasses did not decay by larvae activities. Only few adult flies observed visiting the chlorpyrifos group. Besides adult flies, the wasps were also seen at the site to predate on the flies. During the late stage of decomposition process, few beetles (order: Coleoptera) were seen at the site along with other insects such as Dermaptera. The third instar larvae on the carcasses were collected and brought to the laboratory for toxicological analysis. The specimens were analysed and successfully detected the trace amount both of the analyte, glyphosate and coumarin using Ultraviolet visible spectrophotometer and Gas chromatography mass spectrometry. In conclusion, the findings obtained from this study has indicated that the pesticides altered the fly larvae development and interfered with the decomposition duration of carrion as seen in the field experiment. The changes of larvae development rate were probably caused by the accumulation of the pesticides inside the larvae tissues in which can be detected and quantified. Therefore, the entomological data in this study is important in estimation of post-mortem interval where the presence of pesticides was suspected.

Keywords: Blowflies, decomposition, pesticides, secondary bioaccumulation

Implikasi Kematian Berkaitan Racun Perosak pada Penyiasatan Entomologi Forensik dan Penjelasan Bioakumulasi Sekunder Racun Perosak dalam Tisu Lalat

ABSTRAK

*Dalam entomologi forensik, perkembangan serangga dan corak suksesi yang berkaitan dengan proses penguraian bangkai boleh menjadi maklumat yang berguna dalam anggaran selang masa kematian (PMI). Beberapa faktor luaran seperti persekitaran, keadaan mayat dan kehadiran toksin atau bahan kimia boleh mengubah kadar perkembangan serangga, kedatangan serangga, dan tempoh penguraian bangkai. Objektif kajian ini adalah untuk menyiasat kesan racun perosak terhadap kadar perkembangan langau, penguraian bangkai dan corak suksesi serangga. Selain itu, kajian ini juga bertujuan untuk mengesan kehadiran racun perosak dalam tisu larva serangga pengkoloni. Tiga jenis racun perosak telah digunakan dalam kajian ini; glifosat (racun rumpai), klorpirifos (racun serangga) dan kumarin (racun tikus). Beberapa kepekatan racun perosak ini dicampurkan ke dalam substrat daging dan kemudian telur langau diletakkan pada substrat tersebut. Tempoh dan peringkat tumbesaran langau dari telur hingga dewasa diperhatikan dan direkodkan. Hasil kajian menunjukkan bahawa larva tidak dapat hidup pada kepekatan glifosat dan klorpirifos yang tinggi dalam substrat daging. Sementara itu, larva yang memakan daging yang dicampur dengan kumarin membesar sehingga ke peringkat pupa, namun gagal ke peringkat dewasa. Untuk eksperimen berikutnya, racun perosak secara oral telah diberikan kepada tikus (*Rattus norvegicus*), dan kemudian dimatikan secara eutanasia dan dibawa ke tapak kajian, di hutan sekunder, Kuching, Sarawak. Peringkat dan tempoh penguraian diperhatikan dan dibandingkan dengan bangkai kawalan. Corak suksesi serangga juga direkodkan dan dikumpulkan untuk pengenalpastian spesies. Dari kajian lapangan, sejumlah 1,579 serangga dikumpulkan dalam tiga ulangan kajian eksperimen. Serangga*

tergolong dalam sembilan famili dari lima order, dengan *Chrysomya rufifacies* (71.5%) adalah spesies utama yang mengkoloni bangkai, diikuti oleh *Chrysomya megacephala* dan *Hemipyrellia ligurriens*. Penguraian bangkai terdiri daripada lima peringkat termasuk; peringkat segar, kembung, pereputan aktif, pereputan lanjutan dan peringkat kering/sisa. Tempoh penguraian bangkai adalah lebih lama dalam kumpulan klorpirifos (17-21 hari), diikuti oleh kumarin (11-12 hari) dan glifosat (~8 hari) dibandingkan dengan kumpulan kawalan (~7 hari). Semasa peringkat awal proses penguraian, spesies yang mula-mula tiba adalah langau (*Calliphoridae*), diikuti oleh lalat daging (*Sarcophagidae*) dan lalat muscid (*Muscidae*). Turutan suksesi serangga bagi kumpulan kawalan adalah sejajar dengan kumpulan rawatan. Semasa peringkat pereputan aktif, larva lalat secara aktif memakan tisu bangkai, kecuali bangkai yang dirawat dengan klorpirifos kerana oviposisi lalat dan penguraian bangkai oleh larva tidak berlaku. Didapati, hanya beberapa lalat dewasa yang melawat bangkai kumpulan rawatan dengan klorpirifos. Selain lalat dewasa, tebuhan yang merupakan serangga pemangsa lalat juga dilihat di tapak kajian. Semasa peringkat akhir proses penguraian, beberapa kumbang (order: *Coleoptera*) dan serangga lain (order: *Dermaptera*) dilihat di tapak kajian. Larva instar ketiga pada bangkai telah dikumpulkan dan dibawa ke makmal untuk analisis toksikologi. Spesimen dianalisis dan telah berjaya mengesan jumlah surih kedua-dua analit, menggunakan ultraungu spektrofotometer dan kromatografi gas spektrometer jisim. Sebagai kesimpulan, hasil kajian telah menunjukkan bahawa racun perosak telah mengubah perkembangan larva dan mengganggu tempoh penguraian bangkai seperti yang dilihat dalam eksperimen lapangan. Perubahan kadar perkembangan larva mungkin disebabkan oleh pengumpulan racun perosak di dalam tisu larva yang dapat dikesan dan diukur. Oleh itu, data entomologi dalam kajian ini adalah

penting dalam anggaran selang masa kematian apabila disyaki terdapat kehadiran racun perosak.

Kata kunci: *Langau, racun perosak, penguraian dan bioakumulasi sekunder*

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LIST OF ABBREVIATIONS

%	percentage
°C	celcius
µl/ml	microliter per millilitre
AR	anticoagulant rodenticides
cm	centimetre
CPF	chlorpyrifos
CYP	cytochrome
EC	European Commission
FMOC-Cl	fluorenylmethyloxycarbonyl chloride
g	gram
g/L	gram per litre
GC	gas chromatography
GCMS	gas chromatography mass spectrometry
Gla	glutamic acid
Glu	carboxy-glutamic acid
HPLC	high performance liquid chromatography
IUPAC	International Union of Pure and Applied Chemistry
kg	kilogram
KOH	potassium hydroxide
L1	larva stage 1
L2	larva stage 2
L3	larva stage 3

LD	lethal dose
MET	Malaysia Meteorology Department
mg	milligram
mg/ml	milligram per millilitre
mm	millimetre
nm	nanometre
NPIC	National Pesticide Information Centre
PMI	Post-mortem interval
RIA	radioimmunoassay
RT	retention time
S.D.	standard deviation
SPE	solid phase extraction
TLC	thin layer chromatography
UK	United Kingdom
US EPA	United States Environmental Protection Agency
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Study background

Entomotoxicology is a relatively new area of forensic entomology that deals with the use of toxicological tests on insects for forensic purposes in order to detect the presence of chemicals and toxins in tissues from deceased bodies. This field also studies how toxins affect insect growth, making it crucial for determining the post-mortem interval (PMI) time (Campobasso et al., 2001).

The entomotoxicology application is useful in forensic investigation as it provides clues in intoxication-related death, especially when there are no residual cadaveric tissues. The field focuses on two problems; which are, when and how the victims die. The former problem can be investigated by studying the growth rates of necrophagous arthropods for PMI estimation, while the latter is an alternative, and sometimes the only way to acquire information on chemical related information from insects instead of body fluids or rotten tissues which can interfere with toxicological analysis (Nolte et al., 1992).

The presence of chemical substance on carrion or dead body is not generally well informed and can be difficult to detect. The chemical product may result in delayed colonisation and the absence of the first arrival of necrophagous species on the carcass/cadaver (Campobasso et al., 2001). Besides that, the chemical that is transmitted to carrion-feeding arthropods via food intake or food chain would affect the development of insects. These scenarios will affect on the estimation PMI, which cause the under-estimation of PMI as the estimation is based on the growth rate of insects (Zou et al., 2013).

From previous study, there were several chemical substances that can be detected in insect samples such as malathion (Mahat et al., 2009), diazepam (Sadler et al., 1997), morphine (George et al., 2009) and paraquat (Lawai et al., 2015). Besides drugs, other chemicals such as repellent and household products have been tested on the carcasses to study on their effect on forensically important insects. These chemicals, were administered into the carcasses via several mode of administration for example, through spraying or pouring on the surface of the carcass (Charabidze et al., 2009) by oral or intravenous administration (Zou et al., 2013).

The most frequently used insects as a sample in toxicological analysis belong to the family Calliphoridae (blowflies) and Sarcophagidae (flesh flies). These flies feed on corpses and are usually the earliest flies visiting the dead body. These samples were homogenised and can be analysed using various toxicological analyses instruments such as gas chromatography (GC), radio-immune analysis (RIA), thin layer chromatography (TLC), high pressure liquid chromatography (HPLC) and gas chromatography mass spectrometry (GCMS) analysis (Carvalho, 2010).

The transfer of chemical from corpse to insects is the basis of forensic entomotoxicology (Beyer et al., 1980) and it is also the focus of this study. The aim of this study is to investigate the influence of oral-administered pesticides on the insect colonisation and development and also the decomposition stages. The research also aimed to study the effect of pesticide secondary bioaccumulation detection of insect repellent chemical or metabolite in the fly larvae using gas chromatography (GC). Lastly, the fly larvae feeding on pesticide-killed rabbit carcasses were observed on its pathological defects occur within the larval tissue itself.

1.2 Problem statement

Deaths related to drugs have increased in several countries and often the body of the victim remains undiscovered for a period of time (Goff & Lord, 2009). There are many toxicological analyses techniques and procedures available today, but sometimes it is difficult to obtain proper samples because the cadaver may be greatly decomposed or contaminated.

Besides that, there is no blood or urine available for sample and some biological traits may have been altered due to death circumstances (e.g., death by carbon monoxide toxication). Some religious beliefs of the victim's family members forbid the collecting of biological samples from the body. Therefore, under these circumstances, the insects have been used in the absence of body samples for toxicological analysis.

The studies involving forensic entomotoxicology have been conducted worldwide which focuses on determining the effects of xenobiotics (drugs, insecticides, repellent and heavy metals) on various insects' species especially forensically importance fly larvae (Gosselin et al., 2011). Insects are sensitive to certain toxic compounds (Sawaby et al., 2009; Yan-Wei, et al., 2010; de Souza Sandoval and de Ascensão Medeiros, 2013) as the toxins can either speed up or slows down their developmental duration (Bourel et al., 1999; Mahat et al., 2009; Gennard, 2012). Subsequently, the toxic compounds may interfere with the postmortem interval estimation (Mahat et al., 2014).

In Malaysia, the studies on entomotoxicology in forensically important insects in is still underexplored and even more so in Sarawak. These studies are limited to a few drugs and pesticides for example, mitragynin (ketum) (Rashid et al., 2012), malathion (Rashid et al., 2009; Mahat et al., 2012) and paraquat (Lawai et al., 2015; Mahmood et al., 2015).

Therefore, due to variations in pesticide formulation and the reaction of insects towards these chemicals, three pesticides; glyphosate, chlorpyrifos and coumarin were studied to investigate their implications toward insect of forensic importance and its implication on forensic investigation. In general, this study will benefit the forensic entomology field in Malaysia by aiding more information in providing record of insect succession in Sarawak, and its comparison between different pesticide treated in the carcasses. Besides that, this study will elucidate the type of pesticide that will bioaccumulated in the insects and their influence on the insect development upon feeding on the treated carcasses.

1.3 Aim

The aim of this study is to investigate the implication of pesticides on entomological parameters, decomposition of rat carcasses and to elucidate the type of pesticides that bioaccumulate in forensically important flies' tissues.

1.4 Research objectives

- i. To determine the effect of different types of pesticides on developmental rate of forensic importance, blowfly, *Chrysomya megacephala*.
- ii. To determine the effect of glyphosate (herbicide), chlorpyrifos (insecticide) and coumarin (rodenticide) on insect succession patterns and decomposition rate of rat carcasses in secondary forest.
- iii. To study the type of pesticides that will bio-accumulate in blowfly larvae tissues upon feeding on carcass killed by pesticide consumption.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to forensic entomology

Forensic entomology is a study of insects and other arthropods in forensic investigations (Amendt et al., 2007). The insects become part of evidence in legal cases (Hall & Huntington, 1990) in which, the arthropods found on decomposing body are used to determine post-mortem interval (PMI) time (Lee et al., 2004).

According to Lord and Burger (1983), insect specimens must be considered as important as other physical evidence for example, hairs, fibres, fingerprints and blood stains. The knowledge on distribution and biology of the insects are important as it can assist in many types of forensic investigation by providing information on when, where and how the crime was committed or how a person died (Anderson et al., 2002).

Sung Tzu, a Chinese lawyer and death investigator, first described the use of insects in forensic contexts in his work "The Washing Away of Wrongs" in the 13th century. Sung Tzu recalls the case as stabbing near the rice field. One of the farmer's sickles attracts numerous blowflies despite no evident proof located on the sickle. It seems that the flies are drawn to the unseen blood on the sickle. When confronted, the sickle's owner admitted to the crime (Sung-Tz'u et al., 1981).

A case involving skeletonised remains of a child found behind a chimney in France in 1850 was a major breakthrough in forensic entomology field. At that time insect evidence was accepted as a proof that the current residence has nothing to do with the child's death (Bergeret, 1855). This is based on succession analysis of *Sarcophaga canaria*, a flesh fly

and also ticks found on the child. Individual lived in the house seven years prior was caught following the analysis of the remains and insects found (Byrd & Tomberlin, 2009).

The science of forensic entomology was properly established by Yovanovitch, (1888) and Megnin (1894), both were forensic examiners who studied the insect succession on corpses. In the later years, more study on changes in corpses or corpse skeletonisation caused by insects were explored but the data collected were not applied in forensic cases (Hauser, 1926).

Finally, in 1948, the forensic entomology was used to determine the post-mortem interval (PMI) in Europe (Leclercq & Leclercq, 1948). By the early of 1990s, the application of entomology has been used widely as an important tool for forensic investigation (Goff 1991; Greenberg 1991; Catts & Goff 1992; Introna et al., 1998; Campobasso & Introna, 2001).

Studies on insect succession and flies systematic by using animal models have contributed to our knowledge and understanding in this field, forensic entomology. According to Omar (2011), the scientific bases of post-mortem interval (PMI) estimation in forensic investigation has been strengthened by the observation and prediction of insect succession carried out using animal models.

Hence, it can be argued that in determining the PMI time, corpses serve as the biological clock and flies and other insects serve as the clock hands. Arthropods are used in forensic investigations based on the ecological notion of succession (Greenberg, 1991; Omar et al., 1993). The PMI is founded on two main principles: the succession of arthropod species that colonise a corpse follows a reasonably predictable pattern, and the timing of each

species' developmental stages can be predicted. Environmental factors, particularly temperatures, have a significant impact on these principles (Meritt & Wallace, 2009).

Other than to calculate PMI, the entomology data can be used in toxicological examination of necrophagus larvae found on the corpse to identify and estimate drugs or toxicants that may be ingested by the person when alive. Forensic entomology can also be used to investigate people who are alive that needed extra care such as in neglect cases (Amendt et al., 2004).

2.1.1 Areas of forensic entomology

Forensic entomology can be divided into three sub field which are, urban, stored products and medico legal (Lord & Stevenson, 1986). Urban entomology involved insects that are infesting and caused damage to surrounding buildings and infrastructure, which mostly related to litigation such as legal disputes between exterminators and landlord regarding the pests' infestation. The most prominent insect that involved is termites. Besides studying on the insects, urban forensic entomology also involves in toxicology studies focusing on the appropriateness of pesticide application (Tuccia et al., 2018).

Stored products entomology is subsequent invasion of insect pest during harvesting and storage of crops, and insect pests' infestation of food sold by retailers to the public or foodstuffs in domestic kitchen which may cause in prosecution and substantial fines. The time since infestation will be estimated using the developmental stages together with the ambient conditions of the surrounding. The time frames will be used to determine whether the infestation is the fault of the producer, the retailer deliberate contamination of the product (Defilippo et al., 2017).

The last but not least is the medico legal forensic entomology. This subfield of forensic entomology deals with the collection of evidence by studying on insects at the crime scene such as murder or suicide. Due to variations of insect succession in particular places and environmental conditions, the presence of insects on the body can reveal a lot of information regarding the location and time of the crime (Joseph et al., 2011).

The forensic entomology involves the insect's presence and their behavior become the evidence within legal system. This field usually associated with criminal investigation, most frequently homicide (Goff, 2009). The application of insects in medico legal investigation was based on the behavior of the forensically important insects that are attracted to dead body within minutes of death (Greenberg & Kunich, 2002). This behavior of insects enables the forensic entomologist to determine the time since death of the dead body found (Mahat & Jayaprakash, 2013).

In estimating the time since death or also known as post-mortem interval (PMI), the assessment is a combination of physical condition of the body and the assessment of the fauna associated with the body (Smith, 1986). The faunal analysis includes identification of species encountered, the biology of the species and their relation with the surrounding environment (Marchenko, 2001).

Specific studies on the presence and absence of the insects from the body in a particular geographical region and seasonality are important as these variations, not only affecting the type of insects visiting the body, it also affecting the developmental periods of the insects which later can alter the estimation of PMI (Marchenko, 2001).

2.1.2 Post-mortem interval (PMI) estimation

The post-mortem interval (PMI), sometimes known as the "time since death," is a measurement of how long the body has been exposed to the environment. In a legal inquiry, PMI is crucial because it may be used to pinpoint the exact instant when a crime was committed and to ascertain what led to the victim's demise. However, estimation of PMI is challenging due to a few factors that can give a significant impact on decomposition process (Hauther et al., 2015).

The corpse's physical changes, mortis triad (algor, livor, and rigour mortis) and changes in vitreous humour, are typically used by forensic pathologists to estimate the PMI. The estimation of PMI can also be estimated by looking at the soft tissue components (Hauther et al., 2015). The best indicator for PMI estimates initially comes from the predicted changes in the physical and chemical makeup of the body following death (Henssge & Madea, 2004).

However, as the time of death increases, the changes observed on the dead body became less useful information to estimate PMI. Besides, in a situation where the body is greatly decomposed or the body was concealed, dismembered, explode or burned, the body analysis becomes more complicated and PMI estimation will become harder (Vanin et al., 2013).

Usually, more accurate results are obtained using ecological information. Insects are attracted to decomposing body due to the biochemical changes that occur in soil underneath the body as a result of the decay product exposure (Bornesmissza, 1957). Therefore, in the absence of biological samples for analysis or disfigured body to determine the stages of decomposition, entomological approach was carried out by analyzing the development of

blowfly larvae infesting the body and the presence and absence of insects throughout the decomposition process which also known as insect succession pattern (Tuccia et al., 2018).

The invertebrate fauna found on dead body usually consists of mostly Diptera and Coleoptera in which their presence is unique at different stage of decomposition. The presence and development of insects infesting the dead body is influenced by the environmental factors such as temperature and humidity (Turchetto & Vanin, 2004). Therefore, the period of insect activity can reflect the time of the body exposed to the environment since the eggs were deposited less than an hour after the body exposed to the environment (Mohd-Shukri, 2019).

Based on the colonisation interval (CI) time, forensic entomologists determine the minimum PMI time. The time from the moment the eggs are laid until the body is found is known as the colonisation interval (CI), sometimes referred to as the period of insect activity. The true PMI cannot be established experimentally. Physical factors like the body's location (indoors or outdoors) or whether it is dressed can have an impact on CI (Figure 2.1) (Vanin, 2018). In addition, the succession of arthropod species found on and inside the body is another PMI-dependent phenomenon (Schoenly & Reid, 1987). The succession model provides details on the amount of time that passed between a person's passing and the specific arthropod species and stage. Therefore, it can be used to calculate both the lowest and maximum PMI (Vanin, 2018).

The crime scene investigator must choose a model; insect development or insect succession in order to estimate PMI from entomological data. In order to match a case, this entails comparing the results of experimental data to those of previous death investigations (Goff, 1993), however, the experimental data is used most frequently in comparisons (Wells

& Lamotte, 2001). In the laboratory, small containers are typically used to breed flies and measure their larval growth rate. As long as specific variables match those on the scene, larval growth in a laboratory environment effectively simulates growing in the field. During the early 2000s, developmental information has been gathered on many fly species and growth environments (Byrd & Butler, 1996; Highley & Haskell, 2001; Nabity et al., 2006).

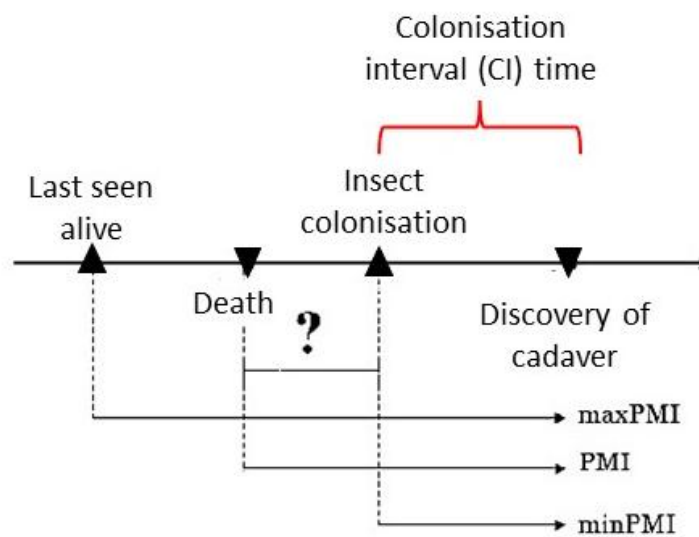


Figure 2.1: Schematic presentation of minimum PMI, PMI and maximum PMI (Tuccia et al., 2018).

The carrion succession insects are part of the ecological study, however recently the studies focus more on forensic investigation field (Wolff et al., 2001; Archer, 2003; Martinez et al., 2007; Cruise et al., 2018). Some experiments involved human corpses (Rodriguez & Bass, 1983; Wang et al., 2017) which are difficult to obtain legally and most published data involving human decay came from case studies (Smith, 1986; Lord, 1990; Introna et al., 1998; Sukontason et al., 2001; Salleh et al., 2007; Syamsa et al., 2015). Therefore, researchers usually employ non-human carcasses (such as pigs, rabbits, rats) in order to include replication and repeated sampling in a succession study (Anderson & VanLaerhoven,

1996; Hewadikaram & Goff, 1991; Schoenly et al., 2006; Chen et al., 2014; Combey et al., 2017).

2.2 Stages of carrion decomposition and associated insects

The body undergoes intense changes after death both in its chemical and physical composition which can be used to calculate the PMI time. The changes, especially chemical changes attract the detritivores, organism that consume decay organic matter. These organisms also can provide information in estimating PMI. The stages of decomposition in terrestrial environment consisted of fresh, bloated, active decay, advanced decay and dry remain stage (Gunn, 2019) as shown in Figure 2.2.



Figure 2.2: Physical appearance of pig carcasses in different stages (Goddard et al., 2012).

Immediately after death, insects are the first organisms arrived on a body. They colonise the body in a predictable sequence as the decomposition process progress from fresh to skeletal (Henssge & Madea, 2004). Each stage in decomposition process attracted different type of sarcosaprophagous arthropods especially insects, some of them attracted to

the dead body itself as a food source and a substrate for oviposition, the other attracted to the large groups of insects that infesting the body tissues that they used as food resource (Anderson, 2004).

Insects continue to colonise the body until it no longer attractive. When these insects migrate from the remains, they perpetually leave evidence of their presence behind, such as cast larval skin and pupal cases. At the same time, the remains now enter the later stage of decomposition which attract later colonisers such as the soldier flies and carrion beetles that attracted to the remains during advanced decay and dry remain stage. Therefore, when investigating the forensic entomologist not only study the insects on the remains but also identify traces left by the earlier colonisers. They will also identify species that are absent but expected to be present based on the succession sequence. Through this information, the estimation of PMI can be made (Anderson, 2004).

2.2.1 Fresh

The skin and mucous appear pale immediately after death due to the ceasing of blood circulation and settling of blood to dependent regions. As the blood circulation ceased, the tissues and cells deprived of oxygen and begin to die. Normal metabolism decreased after death, making the body cool. The cooling is referred to as *algor mortis*, literally means the coldness of death (Gunn, 2019). Excess carbon dioxide causes the cellular process switch from aerobic to anaerobic causes the decrease and increase of certain metabolites. Then, as the membrane in cells ruptures due to the enzymes released by the membranes, the metabolites inside the cell distributed in between tissues.

Hypostasis, also known as livor mortis, is typically seen 20 to 120 minutes after death. The condition known as hypostasis results in a purple or reddish-purple colouring of

the skin and is brought on by the buildup of blood and fluid in the veins and capillaries of the dependant body or organs. Oedema (fluid accumulation) also occur on skin during this time as the blood plasma also settles at the dependent region. For example, if the person is lying on their back, then the hypostasis will develop on their back and body surfaces that close to the ground, whilst a person who died by hanging, will develop hypostasis in their limbs (Figure 2.3) (Gunn, 2019).



Figure 2.3: Hypostasis, also known as *livor mortis* appears under the skin in the most superficial layers of dermis of the dependent parts of the body (Presnell et al., 2020).

Immediately following death, the person's muscles typically start to droop and their joints start to relax, increasing their height by up to three centimetres. Yet after three to four hours, the body stiffens for roughly a day. By firmly tugging the affected limbs, the condition that causes muscles and limbs to stiffen can be broken. After 36 hours, rigour mortis gradually diminishes. (Gunn, 2009).

Blowflies are the early colonisers and can reach the body within minutes (Khalik et al., 2015). They are attracted to odour although it also was thought that vision, colour and presence of other conspecifics on the remains also play a big role (Hall & Haskell, 1995).

The remain will be more attractive once a female adult fly started to lay eggs, only then many other females laying large number of eggs in one area (Barton-Browne et al., 1969).

Blowflies are diurnal species and usually rest at night. Therefore, eggs usually not laid at night and body that exposed to the environment at night may attract flies on the following day (Haskell, et al., 1997). Although fly eggs not laid at night, they usually laid eggs in dark areas during daytime for example, under wrappings, in closet, dark basements and in containers (Erzinclioglu, 1996).

The blowflies continue to infest the corpse or carcass, and lay eggs on it even after the decomposition process change from fresh to bloated stage. Sometimes fire ants also can be seen on the body as they attracted to the eggs' fly as their source of food (Azwandi et al., 2013). The fresh stage ends when the body started to bloat.

2.2.2 Bloated

The corpse or carcass swells after 24 hours or more depends on the environmental factors such as temperature, weather and so on (discussed further in section 2.3.2, pg. 21). The decomposing tissues release substances and gasses which make the skin discoloured and blistered starting on the abdomen in the area above the caecum. The front body bloat and the tongue may protrude and fluid from the lung oozes out from the mouth and nostrils. Along with the physical changes, terrible odour release from the body as a result from bacterial metabolism in the body. The end product of bacterial metabolism such as, hydrogen sulphide and mercaptans are the main source of the odour. Methane (which does not smell) also produced and contributed to the body swelling (Gunn, 2019).

As the odour intensify, more flies especially blowflies and flesh flies attracted to the body (Anderson, 2004). The internal temperatures of carcass or corpse increased at this point of time due to the arthropod activities and the decomposition process. At the beginning of day-2, predators of Diptera can be seen on the carcass, and fly eggs usually hatched at this time. Although it may not be visible due to the size, first instar larvae usually have started infesting the body tissues at this stage (Tullis & Goff, 1987).

Bloated stage ends when the balloon-like abdomen deflates due to the penetration of abdominal wall by larvae. The internal temperatures continue to rise as more larvae concentrated in the body and forming a mass (Tullis & Goff, 1987).

2.2.3 Active decay

According to Gunn (2019), the bloat stage itself is said to be ‘actively decaying’ and during this time the soft body parts rapidly disappears as a result of autolysis, microbial and arthropods activity. The body collapse on itself as the gasses are no longer retain by the skin. The arthropods activity continues on and at this stage, it can be observed that the larvae have moulted into second and third instar. At the end of this stage, the high internal temperatures observed will drop significantly and the larvae can be seen migrate away from the carcass to a drier ground to pupate.

2.2.4 Advanced decay

The carcass is left with bones, cartilage, hair, small portions of tissue and a large amount of wet and vicious material which known as by-products of decay. Obese people tend to decay faster than those of average weight due to the greater amount of liquid in tissues which favours the dissemination of bacteria in the body (Compabasso et al., 2001).

2.2.5 Dry remain

The remain stage characterised by the bones left on carcass with little cartilage remaining and other vicious material has dried up. The transition of remain/dry stage from advanced decay stage is gradual with decreasing number of adult and immatures of Diptera population (Tullis & Goff, 1987). The arthropods visiting and infesting the carcass or corpse are summarised in Table 2.1.

2.3 Factors affecting the insect succession pattern and carrion decomposition rate

As discussed in previous section, the putrefactive process can be separated into several phases which include; fresh, bloated, active decay & advanced decay and dry remains. These phases are a series of events that combine and overlap one another until all organic materials gone. It is important as it acts as a marker in estimating PMI time (Campobasso et al., 2001).

The PMI time can be estimated when the decay process is integrated with the environmental parameters and circumstantial condition of the body. The rate of post-mortem decay can be affected by variables which are the intrinsic and extrinsic factors. Intrinsic factors include age, constitution, cause of death and integrity of the corpse. Among the extrinsic factors, the ambient temperatures followed by ventilation and air humidity, clothing and location of the body are factors that are considered significant in PMI estimation (Campobasso et al., 2001).

Table 2.1: The sequence in which the insects arrive and colonise a corpse during decomposition process (Gunn, 2019).

Stages of decomposition	Insects
Fresh	Blowfly adults, eggs and 1 st instar larvae Flesh fly adults and 1 st instar larvae Burying beetle (adults)
Bloat	Blowfly adults, eggs and 1 st , 2 nd & 3 rd instar larvae Flesh fly adults, 1 st , 2 nd & 3 rd instar larvae Burying beetles (adults) and larvae Hister beetle adults and larvae
Putrefaction (Active & Advanced decay)	Blowfly adults, 2 nd & 3 rd instar larvae Flesh fly adults, 2 nd & 3 rd instar larvae No blowfly eggs in advanced decay stage Blowfly and flesh fly larvae leaving the corpse for pupation Hister beetles (adult and larvae) Eristalid fly larvae (liquefied regions) Phorid fly larvae (Later stages of putrefaction) Piophilid fly larvae (Later stages of putrefaction)
Dry remains	No blowfly larvae Startiomyid fly larvae Dermestid beetles (adults and larvae) Tineid moth larvae Pyralid moth larvae

2.3.1 Intrinsic factor

Intrinsic factors refer to the nature of the corpse itself, which include age, constitution, cause of death and integrity of the body. Foetuses and stillborn infants usually have a slower rate of decay because the absence of intestinal flora. This is different from infants that have lived long enough to be fed and breathed will decompose rapidly due to the abundance of intestinal flora. The other factors are as discussed below.

2.3.1.1 Body size and weight

A study by Anthropology Research Facility in Tennessee has reported that the rate of decay does not influence by the body size and weight. This is due to the rapid loss of body

mass in obese individuals with a liquefaction of body fats (Mann et al., 1990). However, based on the subsequent studies by Hewadikaram and Goff (1991), two pig carcasses weighing 8.4 and 15.1 kg respectively has shown greater temperature rises and faster decomposition in heavier pig carcasses, although there is no difference in pattern of succession of associated fauna.

2.3.1.2 Cause of death

Ante mortem condition of the bodies also plays a role in increasing the rate of decomposition. For example, illness at time of death such as septic infection quickened decomposition as the microbes is already colonising and breaking down the body at the time of death (Gunn, 2019).

Another cause of death is open wounds or trauma. Whether the wounds were either sustained at the time of death or later, the open injury allow the entry of air and invertebrates into the body, thus increase the rate of decomposition. The blowflies are attracted to the smell of blood which then lead to body discovery and exploited more quickly. It was also said that those wounds provide a favourable habitat for oviposition as the flies need moist area for the eggs to hatch (Galloway et al., 1989; Haskell et al., 1997).

However, there is also study conducted to compare the rate of decomposition between traumatized and non-traumatized samples. It was reported that there is no significant difference in terms of period for complete decomposition and the flies preferred to oviposit at natural orifices rather than the wound (Cross & Simons, 2010). However, it is important to note that, the wound provide quicker access for the larvae to reach soft tissues, and thus the tissue loss in injured carcass is more rapid although it eventually plateaus reaching the same level as non-traumatized sample (Cross & Simons, 2010).

2.3.2 Extrinsic factor

2.3.2.1 Biogeoclimatic zone

The geographic region or biogeoclimatic zone in which the remains are found is the most important factor that influence the rate of decomposition. The biogeoclimatic zone defines as the habitat, vegetation, soil type and meteorological conditions of the area. This factors obviously affects the type and species of insect presence on the body as well as their seasonal activity (MacGregor, 1999).

Many insects' carrion are ubiquitous and certain groups will colonise first. For example, blowflies (family: Calliphoridae) will arrive first followed by flesh flies (family: Sarcophagidae) and so on, but the individual species involved in decomposition vary from region to region. For example, in Hawaii, the first colonisers were *Lucilia cuprina* (Widemann), *Chrysomya megacephala* and *Chrysomya rufifacies* (Early & Goff, 1986) whilst in Tennessee, a study shows *Lucilia coeruleiviridis* and *Phormia regina* (Reed, 1958).

The abundance and species composition of insects' community varies between regions and this affect the speed of decay. The rate of decomposition proceeds much faster in the tropics where conditions are both warm and humid and slower in cold or dry environment. In the tropics the corpse or carrion can become a mass of moving maggots in 24 hours, whereas it took several days in UK even in the summer (Gunn, 2019).

Temperature and humidity play a significant role in decomposition process and dipteran development. Summer in the tropics has an average temperature of 34 °C and humidity of 56%. These environment conditions are ideal for both bacterial and insect development. In Townsville Australia, the decomposition of pig carcasses took about a week to complete in average daily temperature of 24 °C to 31 °C (Griffiths et al., 2020). A study

on decomposition period in different climate has shown that complete skeletonization of human cadaver in the summer (14.6 °C) occur in less than six weeks, and winter decomposition on the other hand, took longer time which was about four months with mean temperature -7.1 °C (Komar & Beattie, 1998a).

Times of colonisation of insect species and groups also vary greatly with geographical region. In many areas, carrion beetles (Coleoptera: Dermestidae) are known as the late colonisers. They arrived when all that was left was skin and bone. For instance, dermestid larvae were initially discovered in pig carrion in an open grassland in coastal British Columbia 21 days after the animal had passed away, in the early stages of severe decay (Anderson & VanLaerhoven, 1996). Yet, in the interior of British Columbia, dermestid larvae and adults were initially discovered on pig corpses at the bloat stage and the decay stage, respectively (Dillon & Anderson, 1996; Dillon, 1997).

Skipper flies (Diptera: Piophilidae) is another example of insect that arrived in different time at different post-mortem intervals, depending on the region. These flies known to be associated with corpses during advanced decay stage (McAlpine, 1987). This is not always being the case, as these flies were found during the active decay stage of pig carcasses in Thailand (Vitta et al., 2007). In 2008, *Piophilidae casei*, was recovered for the first time in human cadavers in Malaysia. It was predicted that the infestation occurs during active decay stage (Nazni et al., 2008).

Exposure to sunlight is another factor should be considered when estimating the PMI time. It is however, case dependent. If the body is warm due to sun rays, bacterial decay will be promoted, but if the environment is low in humidity with strong wind, then the body would dessicated and mummify (Gunn, 2019). The body that is exposed to sun will attracts

the invertebrates much faster as the body is visible and smellable. Invertebrates such as flies avoid laying eggs on the surface of the body under the sun. This is due to the combination of dessication and UV light that may kill their delicate eggs and larvae. However, the eggs laid under surfaces or beneath clothes where the environment is protected against the sun and have a more humid condition. This will promote the growth of the microbes and larvae; thus, these covered regions usually decay more rapidly than the surface (Gunn, 2019).

Geographical region or biogeoclimatic zone obviously affects the rate of decomposition and flies' arrival and development on the carrion. This means that, data generated in one region not to be used in to determine the time of death at different region. Databases for every biogeoclimatic zone and the insect associated has been developed in United States (Goff et al., 1986; Payne, 1965), British Columbia (Anderson & VanLaerhoven, 1996) to assist in estimation of PMI time.

2.3.2.2 Condition of the body

The body usually discovered in several condition. Whether they are wrapped, clothed, hanged or bury under water, these conditions definitely have an influence on the rate of decomposition and flies' arrival and development on the body. Remains, whether as a whole or dismembered, are frequently found wrapped in some material. This may be an effort to prevent bleeding onto the floor or vehicle, to disguise the remains or to facilitate handling. The extend of the wrapping may interrupt with the insect colonisation pattern of the remains. Corpses are also found in the boots of vehicles, placed in a suitcase, large containers or confined in a lock room. All of these scenarios have their own effect on decomposition, and the rate of decomposition is slowest when the covering is most effective in excluding oxygen, vertebrates and invertebrates. However, once the body started to decay,

the smell will attract the invertebrates especially flies. The flies usually will find their own way through the wrapping (Anderson, 2001).

Besides wrapping, the human body frequently clothed. The number and variety of successional insects are both increased by clothed pig carcasses, according to research done in the late 1990s. Once the clothing becomes soaked with the breakdown fluid, there are more places for oviposition. This causes greater larval masses, which accelerates the breakdown process (Dillon and Anderson. 1996; Dillon, 1997).

Insects also have been shown to move and tear clothing, in which may mislead the investigators into assuming sexual assault has taken place (Komar & Beattie, 1998b). Maggot masses have been able to move clothing from underneath of the body despite the overlying body weight. Physical changes during decomposition process such as during bloated stage could tear off the clothes as well (Komar & Beattie, 1998b).

Another condition that influences the rate of decomposition and insect succession pattern are hanged bodies. Hanged bodies either as a result of suicide or accident could present a unique environment for insect colonisation. Goff and Lord (1994a) stated that hanged bodies altered the insect composition as the soil-dwelling taxa was excluded and thus changing the drying pattern of the body and limited the fly species activities. This reduced the number of insects infesting the corpse or carcass and delay the decomposition process. It was also reported that they are less site for oviposition as the body is hanged, there is no dark, under surface where flies usually laid eggs. The weather such as rain could wash off the maggots as well (Gunn, 2019).

2.3.2.3 Burial

Decomposition of buried corpses four times slower than those on the surface, and the deeper they are buried, the slower the decay (Dent et al., 2004). The type of soil affects the rate of decay as the microbial density varies depends on the type of soil. According to Coleman et al. (2004), the microbial density on the soil (top soil) is 2×10^9 per gram, while at a depth of 1 to 8 meters is about 10^8 per gram (Gunn, 2019).

Heavy clay soils have low oxygen levels as it poorly aerated and thus limiting the microbial activity, and eventually slows down the rate of decay. Acidic soil also slower the microbial activity, however, the low pH dissolves soft tissues and bones. Even a shallow covering of soil prevents the blowflies from colonising the body, hence reduce the rate of decomposition (Gunn, 2019).

Although blowflies couldn't reach the buried corpses or carcass, there is a type of fly known as coffin fly (*Conicera tibialis*) that can locate bodies a meter or more underground. It was also important to know that shallow buried body will be easily dug by other animals such as dogs or foxes and therefore increase the rate of decomposition (Gunn, 2019).

The bodies disposed in the water are often said to decay twice slower than on the ground. This is probably because the low temperature of water. However, unless the bodies buried underwater were weighed down, the bodies will float up on the surface due to the formation of gas in the abdomen. Bodies floating on lake, river or ponds can be colonised by the blowflies' larvae, along with the temperature on the surface, will increase the decomposition process. The blowflies are not found at sea, although they may colonise body washed up on beaches (Gunn, 2019).

2.4 Forensically important insects

Arthropods, which include insects, arachnids, centipedes, millipedes, and crustaceans, are invertebrate organisms having jointed legs. The largest group of arthropods, the insects, are distinguished by having six legs and a body separated into the head, thorax, and abdomen (Figure 2.4). Insects and other arthropods are the main decomposers that involved in decomposition process. The population and components of insects are unique to particular habitat where the carrion and corpses locate. The insect's presence will be having some type of relationship towards the decomposing body (Goff, 2009).

There are four basic relationships between decomposing body and insects and other arthropods which are, necrophagous insects which directly feed on the corpse. This group include the true flies (Diptera) particularly blowfly (Calliphoridae) and flesh fly (Sarcophagidae) and beetles (Coleoptera: Silphidae and Dermestidae).

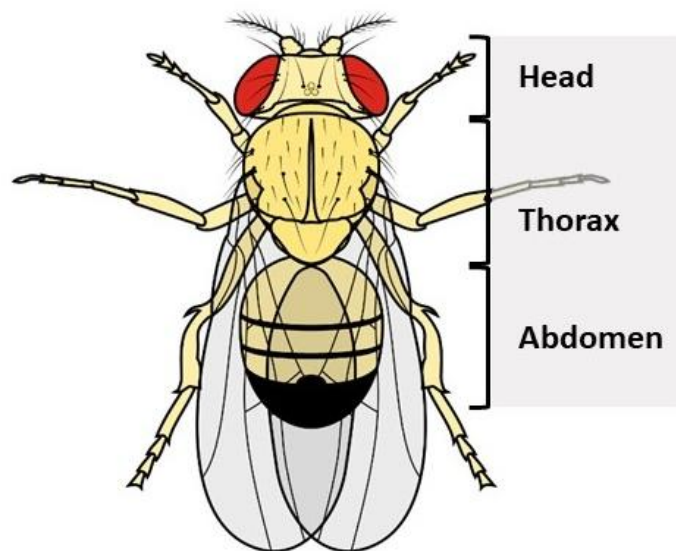


Figure 2.4: The body region of insects (Teleman & Perrimon, 2017).

Secondly, necrophagous animals have predators and parasites, such as beetles (Coleoptera: Silphidae, Staphylinidae, and Histeridae), true flies (Diptera: Calliphoridae, Muscidae, and Stratiomyidae), and wasps (Hymenoptera), which prey on fly larvae and pupa. Thirdly is omnivorous species such as wasps, ants and some beetles which feed on corpse and associated insects. Fourthly is the adventive species where they are using the corpse as their own habitat. For examples, springtails (Collembola), spiders, centipedes and millipedes. The adventive species is the last type of relationship between decomposed body and insects. However, there is one more category that does not have real relationship with the body but still found on the body. They are termed as “accidentals”. This type of insects may have land on the body from surrounding vegetation. They might not attract to the body or associated insects, but they are important as they are able to provide information in the case of post-mortem movement of body (Goff, 2009).

2.4.1 Diptera

The most diversified and numerous organisms on the world is the fly. With the exception of saltwater, they can be found practically everywhere on land and in the majority of aquatic habitats. Wings are an evolutionary adaptation that allows insects to fly when searching for food or trying to find a good place to lay their eggs. This is a crucial consideration for species with forensic value that must swiftly discover and make use of transient resources like carrion (Byrd & Castner, 2001).

Diptera means “two-wings” or also known as true flies can be characterised by having one pair of wings and a pair of balancing organs known as halteres located just back of the base of the wings. Halteres are hind wings that highly modified into a knob-like structures of balancing organ used during flight (Borror et al., 1989).

Flies undergo four-stages of development, which starts from egg, larvae, pupa and adult as shown in Figure 2.5. The process of changing in physical form in each stage known as metamorphosis, in which can be achieved when the insect “shedding its skin” or undergoing moulting at a certain point as it grows. The period of each stage is varying depends on the type of species and external factors such as environment conditions that are present. The time period spends in any particular life stages referred to as a stadium and the insect itself may be called instar especially during larval development (Byrd & Castner, 2001).

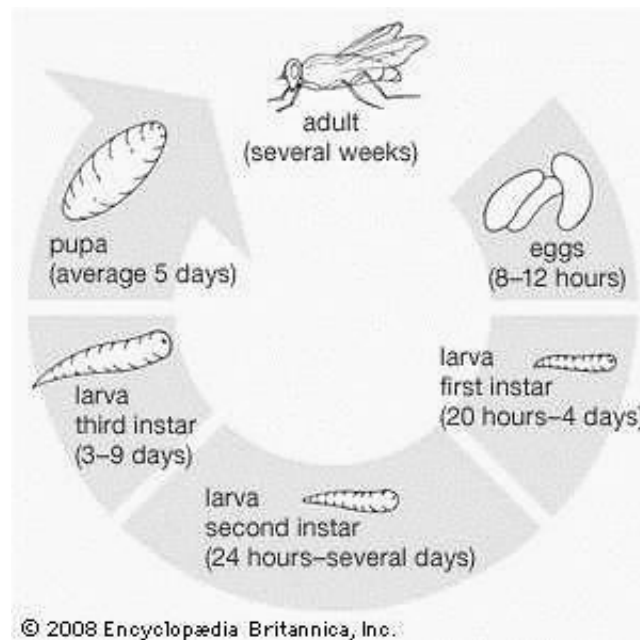


Figure 2.5: The life cycle of flies (Encyclopedia Britannica, 2008).

Fly maggots are soft-bodied, legless and wormlike (Borror et al., 1989). Forensically important flies' third instar larvae, such as blow flies, flesh flies and house flies shed their skin to form into pupa, but they do it inside the old skin of the mature larvae which then shrinks and hardens (Byrd & Castner, 2001). They usually choose a dark place to pupate (Orkin, 2020). The outer skin called the puparium darkens to brown, reddish brown or black

with time depending on species. The morphological characteristics of the larvae are retained on the outer surface of the puparium and are often distinctive enough to allow species identification to be made (Byrd & Castner, 2001). Depending on the species, over the course of three to six days, a full-grown adult fly emerges from the pupae (Orkin, 2020). Adult flies live between 15-30 days (Orkin, 2020). They are important as scavengers, removing decomposing plant and animal materials from the environment. Some are predators and parasites of other insect species and others aid in the pollination of plants (Byrd & Castner, 2001).

Flies have large compound eyes with various types of mouthparts. Mouthparts of flies are adapted for sucking which is in haustellate form (Borror et al., 1989). Haustellate derived from latin verb “haustor” meaning to draw up or suck. They function in several behaviors such as probing/sipping, sponging/lapping, piercing/sucking, etc. In Malaysia, there are several dipteran flies that are of forensically important which are the major decomposers and commonly found around carrion and corpses such as Calliphoridae, Sarcophagidae, Muscidae and etc. (Figure 2.6).

2.4.1.1 Family Calliphoridae (Blowflies)

The flies from family calliphoridae contains more than 1000 species. They can be found around the world. Blowflies along with flesh flies and muscid flies are the most significant species for estimating the post-mortem interval (PMI). Calliphorid flies are attracted to carrion, excrement and some species exploiting open wounds. This family includes the green bottle flies (*Chrysomya sp.*) and blue bottle flies (*Lucilia sp.*) (Anderson, 2001).

**Flies collected in decomposing animals
and human cadavers in Malaysia**

- Calliphoridae (Blow flies)
- Sarcophagidae (Flesh flies)
- Muscidae (Muscid flies)
- Syrphidae (Syrphid flies)
- Piophilidae (Cheese flies)
- Phoridae (Humpbacked/Scuttle flies)
- Stratiomyidae (Soldier flies)
- Milichiidae (Freeloader flies)

Figure 2.6: Forensically important flies collected in Malaysia (Nazni et al., 2012).

Calliphoridae, from the subfamilies Chrysomyinae, Calliphorinae and Lucillinae can be distinguished from other metallic or partly metallic calyptrate Diptera by the presence a row of bristles on the meron and the absence of prominent subscutellum. They also possessed a hairy arista and sharply bent wing vein M (Figure 2.7) though uncommon, non-metallic Calliphoridae subfamily, Melanomyinae have a bare arista (Marshall et al., 2011).

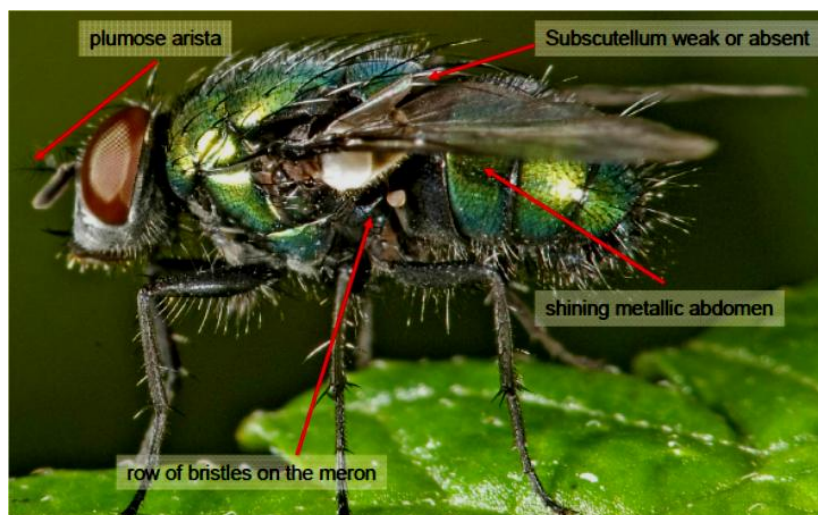


Figure 2.7: Calliphoridae flies characteristics (Marshall et al., 2011).

The blowflies sometimes often confused with metallic Muscidae and Tachinidae. The metallic muscidae normally have a gentle bend in vein M (Figure 2.8a) and never have a row of stout bristles on the meron (Figure 2.8b) while Tachinidae have a prominent subscutellum, they normally have a bare arista (Figure 2.9) (Marshall et al., 2011). There are two ways to estimate the PMI time using the entomological data which are by using the succession data of insects' taxa that changes according to the stage of decomposition process. The other way is by using the development data of the oldest and largest maggots of the first colonising species (Salimi et al., 2018).

Blow flies are among the first to discover and colonise corpses or carrion. In previous study, it has been recorded that calliphorid flies arrived at carcasses within minutes of their exposure (Byrd & Castner, 2001). Adult calliphorids usually range from six to 14 mm in length. The majority of species appeared metallic with several colors ranging from green, blue, bronze or black. On some species, the bright metallic appearance of the fly body sometimes masked by fine powder or dust (Byrd & Castner, 2001). The females laid large number of eggs which are commonly laid at nose, mouth and other natural body opening or open wound that are exposed. The telescoping segment on the tip of female's abdomen extend to form an ovipositor which used to lay eggs (Marshall et al., 2011).

When the eggs hatched, the larvae emerged and feed on the decompose tissue surrounding them. The length of larvae may range from eight to 23 mm with white or cream body coloration. The terminal segment typically has six or more cone-shaped tubercles about its perimeter. This segment also comprises of the posterior spiracles, the primary breathing apparatus of the larvae (Figure 2.10).



Figure 2.8: The metallic Muscidae (a) with a gentle vein M on wings and (b) absence of bristles on meron (Marshall et al., 2011).

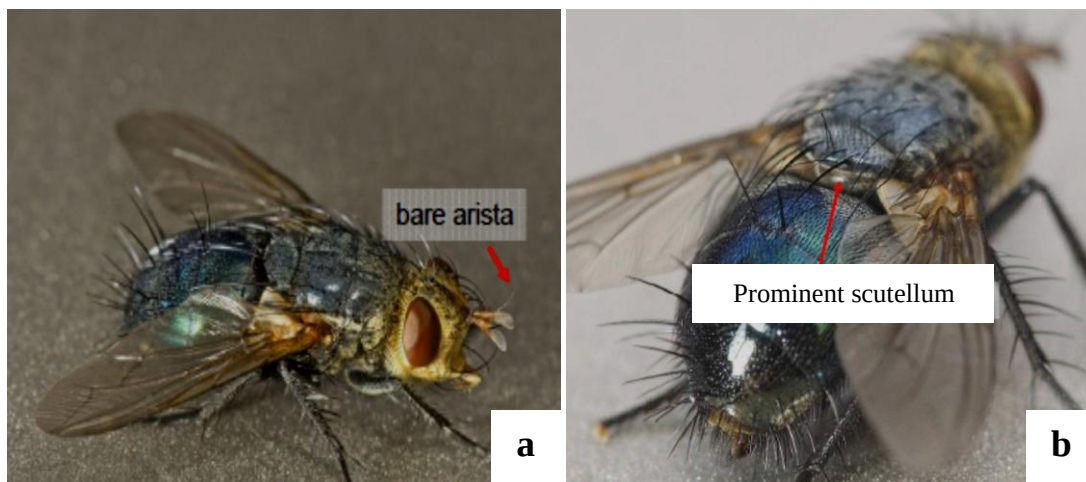


Figure 2.9: Tachinidae flies possessed of (a) bare arista and (b) prominent subscutellum (Marshall et al., 2011).

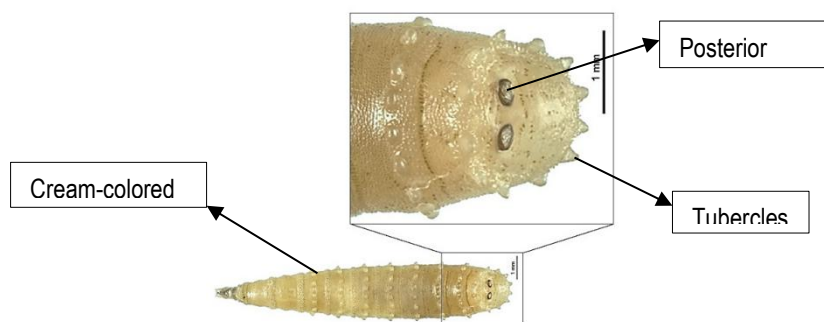


Figure 2.10: The larvae of *Chrysomya chani* showing a cream colored body with tubercles around the parameters. The terminal segment also showing posterior spiracle (Sukontason et al., 2018).

The morphology of the tubercles, the posterior spiracle and the colour of the larvae body can be used to identify the species of blowflies and determination of the larval stage. The larval stage is determined by the number of slits of the posterior spiracle. The posterior spiracle consist of spiracular slits and peritreme which enclosed the spiracles (Figure 2.11). Depends on the species, the peritreme may enclosed the spiracle completely or incompletely (Figure 2.12). The species that are commonly found on the corpses in Malaysia are from subfamilies Chrysomyinae and Calliphorinae as shown in Table 2.2.

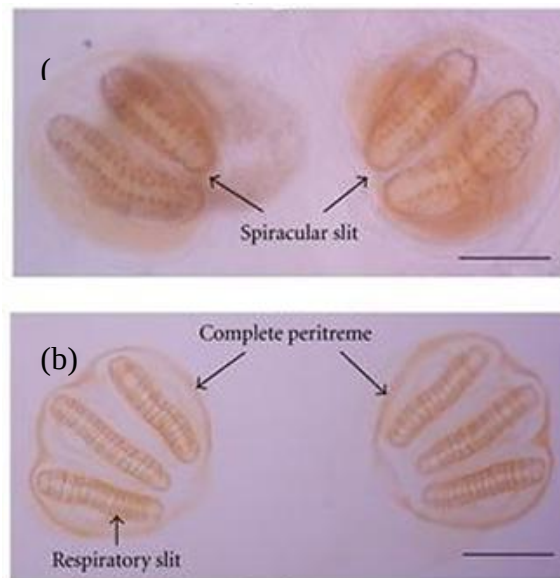


Figure 2.11: The posterior spiracle of *Hemipyrellia ligurriens* showing (a) 2 slits for 2nd instar larvae; and (b) 3 slits for 3rd instar larvae (Bunchu et al., 2012).

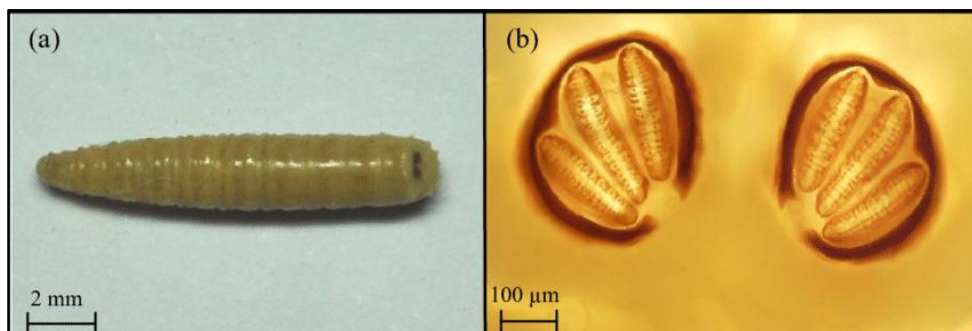


Figure 2.12: The surface body of (a) *Chrysomya megacephala* and (b) its posterior spiracles with incomplete peritreme (Monthatong & Thongchailtriwat, 2015).

Table 2.2: Forensically important blowfly species collected in Malaysia (Nazni et al., 2011).

Family Calliphoridae
Sub-family: Chrysomyinae
<i>Chrysomya megacephala</i> (Fabricus, 1794)
<i>Chrysomya bezziana</i> (Villeneuve, 1914)
<i>Chrysomya rufifacies</i> (Macquart, 1843)
<i>Chrysomya nigripes</i> (Aubertin, 1932)
<i>Chrysomya villeneuvi</i> (Patton, 1922)
<i>Chrysomya chani</i> (Kurahashi, 1979)
<i>Chrysomya pinguis</i> (Walker, 1858)
<i>Chrysomya defixa</i> (Walker, 1857)
<i>Chrysomya thanomthini</i> (Kurahashi et Tumrasvin, 1997)
Sub-family: Calliphorinae
<i>Calliphora fulviceps</i> (van der Wulp, 1881)
<i>Lucilia cuprina</i> (Wiedemann, 1830)
<i>Lucilia porphyрина</i> (Walker, 1838)
<i>Lucilia papuensis</i> (Macquart, 1843)
<i>Lucilia hainanensis</i> (Fan, 1965)
<i>Hemipyrellia ligurriens</i> (Wiedemann, 1830)
<i>Hemipyrellia tagaliana</i> (Bigot, 1877)
<i>Hypopygiopsis violacea</i> (Macquart)
<i>Hypopygiopsis fumipennis</i> (Walker, 1856)

2.4.1.2 Family Sarcophagidae (Flesh flies)

Flesh flies comprise a large family with over 2000 species and approximately 800 species belong to genus *Sarcophaga*. This species can be found throughout the world either in tropical or warm temperate regions. The adults can be characterised by uniform general colour, grey with three longitudinal stripes on mesonotum and checkered abdomen (Figure 2.13) by a row of setae on meron and undeveloped subscutellum. Adults are having a medium to large size (8 – 22 mm) and few species have a smaller size (5 – 8 mm) (Carvalho & Mello-Patiu, 2008). Also, the arista of the antennae is plumose only at the base while in calliphorid flies it is plumose throughout the length (Figure 2.14) (Byrd & Castner, 2001).

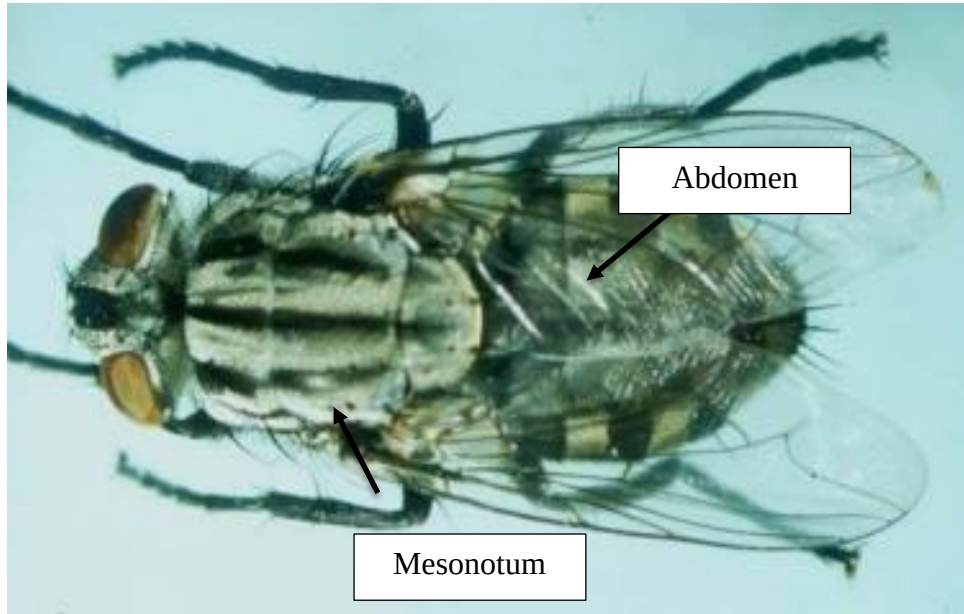


Figure 2.13: Adult *Sarcophaga torvidae* (Family: Sarcophagidae) with stripes on mesonotum and checkered pattern abdomen (Carvalho et al., 2004).

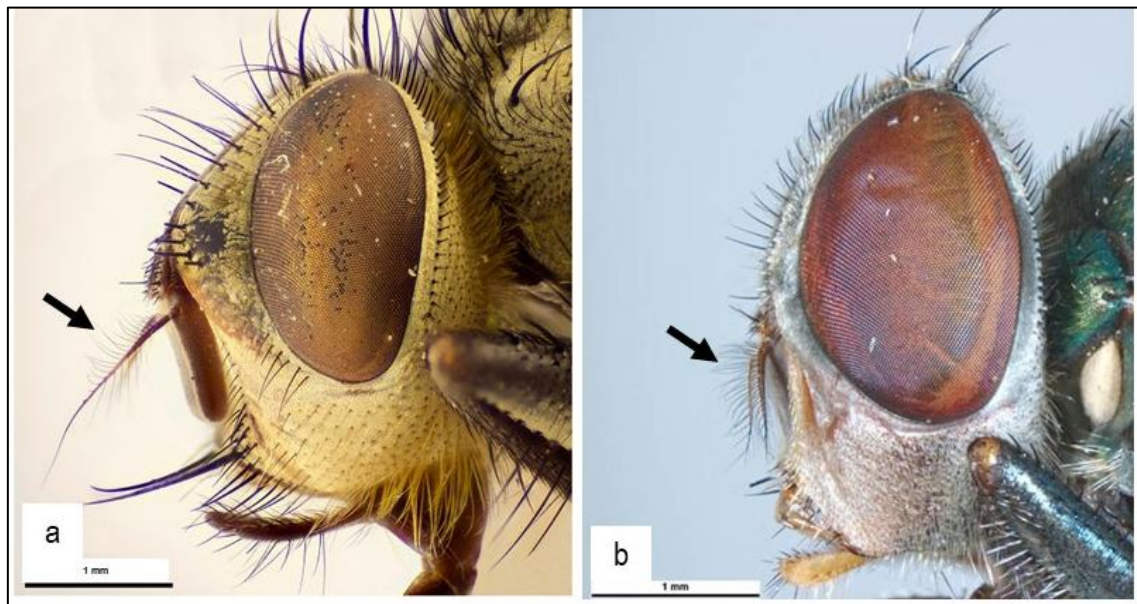


Figure 2.14: The difference of antennae plumose (arrow) between (a) sarcophagid fly (*Sarcophaga maxima* sp.) and (b) calliphorid fly (*Chrysomya rufifacies*) (Meiklejohn et al., 2017; Pest and Diseases Image Library, 2018).

The females are ovoviparous multilarviparous where the females developed large numbers of eggs or in the uterus and deposit the first instar larvae instead of eggs directly on the substrate (Figure 2.15) (Meier et al., 1999) and have a non-teleopic terminalia (Mello-Patiu, 2016). Once the first instar larvae laid on the host, the larvae start feeding immediately. These larvae eat and develop rapidly.

Five days after larviposition, the larvae are in third instar stage and almost ready to pupate. When they are ready to pupate, they leave the carrion and wander to find a suitable location. Pupation starts approximately seven days after larviposition (at 25 °C) and adult flies emerge about ten days after pupation (Figure 2.16) (Diaz & Kaufman, 2011).

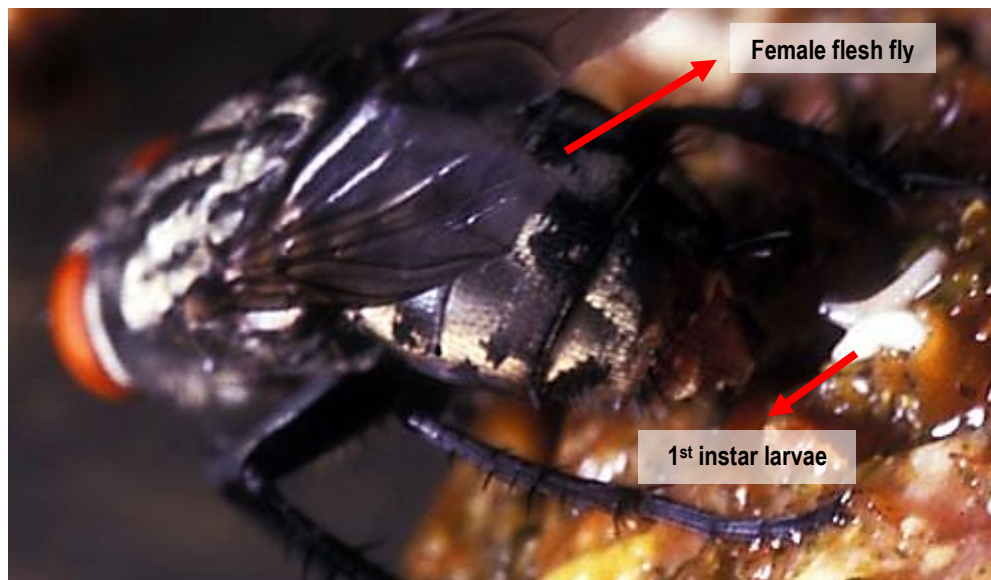


Figure 2.15: Adult *Sarcophaga haemorrhoidalis* (Fallen) (Family: Sarcophagidae) depositing the first instar larvae. Photograph by Jerry Butler, University of Florida (Entomology & Nematology UF|IFAS, 2011).

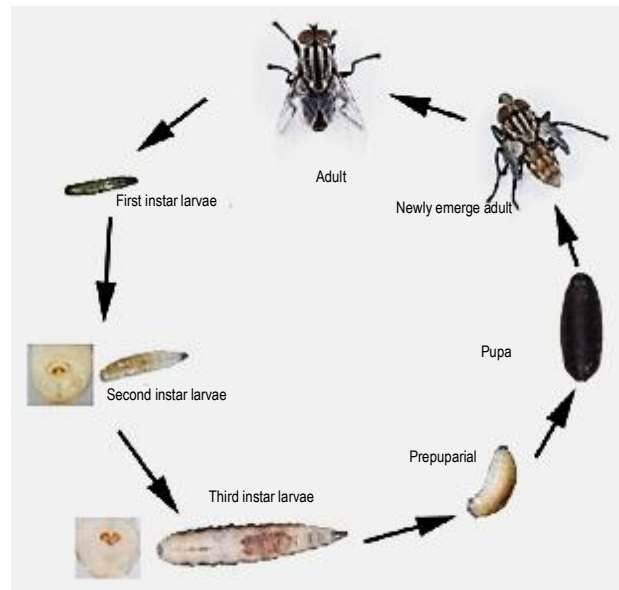


Figure 2.16: The life cycle of *Boettcherisca peregrina* (Family: Sarcophagidae) life cycle (Shang et al., 2019).

Characterisation of genera and species almost fully based on male terminalia and identification of larvae and adult females are notably difficult or still impossible (Mello-Patiu, 2016). It was also said that the similarity of the morphology and lack of keys may contribute to the difficulty in identifying this group of flies (Barros et al., 2008; Mulieri et al., 2010; Vairo et al., 2011).

Sarcophagid adult flies are often found on flowers where they attracted to nectar besides feed on other sweet substance which include sap and honeydew. Sarcophagid in Latin means “flesh-eating” refers to their larvae that feed on animal materials such as carrion or excrement or exposed meats. Due to this behaviour, the larvae have been known to cause myiasis and may involve in transmission of disease. Some other species are parasitic on other

insects (Byrd & Castner, 2001). For example, *Sarcophaga kellyi* which parasitized on grasshopper which was discovered by Kelly (1914).

Sarcophagidae flies are considered forensically important due to its carrion-feeding larvae and scavenging adults (Pape, 1996; Carvalho & Mello-Patiu, 2008; Brown et al., 2009; Mello-Patiu et al., 2009). They became a very useful tool for the forensic entomologist to estimate the minimum post-mortem interval (minPMI) (Oliveira-Costa & Mello-Patiu, 2004).

There are three subfamilies, Miltogramminae, Paramacronychiinae and Sarcophaginae, but Sarcophaginae has species of forensic and medical importance in the Neotropics (Pape, 1996). In Malaysia, the sub-family that are commonly found is from subfamily Sarcophaginae. The list of genus and species identified and found in Malaysia is shown in Table 2.3.

Table 2.3: Species of flesh flies (Family: Sarcophagidae) collected in Malaysia (Nazni et al., 2012).

Family Sarcophagidae
Sub-family: Sarcophaginae
Genus: <i>Boettcherisca</i>
<i>Boettcherisca highlandica</i> (Kurahashi et Tan, 2009)
<i>Boettcherisca karnyi</i> (Hardy, 1927)
<i>Boettcherisca nathani</i> (Lopes, 1961)
<i>Boettcherisca peregrina</i> (Robineau-Desvoidy, 1830)
Genus: <i>Lioproctia</i>
<i>Lioproctia pattoni</i> (Senior-White, 1924)
Genus: <i>Liopygia</i>
<i>Liopygia ruficornis</i> (Fabricus, 1794)
Genus: <i>Parasarcophaga</i>
<i>Parasarcophaga brevicornis</i> (Ho, 1934)
<i>Parasarcophaga dux</i> (Thomson, 1869)
<i>Parasarcophaga taenionota</i> (Wiedemann, 1830)
Genus: <i>Seniorwhitea</i>
<i>Seniorwhitea princeps</i> (Wiedemann, 1830)

2.4.1.3 Family Muscidae (Muscid flies)

Muscid flies includes significant blood-feeding parasites, vectors of disease agent and a pest to humans and domesticated animals. Many species are ubiquitous and synanthropic. They exploit foods and habitats created by agriculture and other human activities (Moon, 2019).

Muscid flies can be divided into several groups based on their habitat and affinities. The first group is the filth flies. Filth flies include house fly whose can be found in variety of filthy organic substrates such as household garbage, manure, manure-soiled and animal bedding. A subset of filth flies are dung flies such as horn fly (*Haematobia irritans irritans*) whose immatures commonly found in cattle droppings. Another group that is belong to the family Muscidae is sweat flies. As the name suggest, these flies are attracted to perspiration (Moon, 2019).

Muscid flies also can be grouped by the nature of their mouthparts which are sponging mouthparts and and piercing-sucking mouthparts. The sponging mouthparts used of non-biting muscid flies to ingest liquids from inanimate substrates and animal tissue. While for biting muscid flies, the piercing-sucking mouthparts can pierce the skin to obtain blood (Figure 2.17) (Moon, 2019). There are 20 genera and about 5000 species in the family Muscidae. Only a few of these genera are significant in medicine and veterinary science. The life stages of dipteran flies are an egg, a larva, a pupa, and an adult (Figure 2.18). The morphology of the eggs, which can appear singly or in groups, is comparable to that of related families. They are elongate-ovate in shape, creamy in colour, and between 0.8 and 2.0 mm length (Moon, 2019).

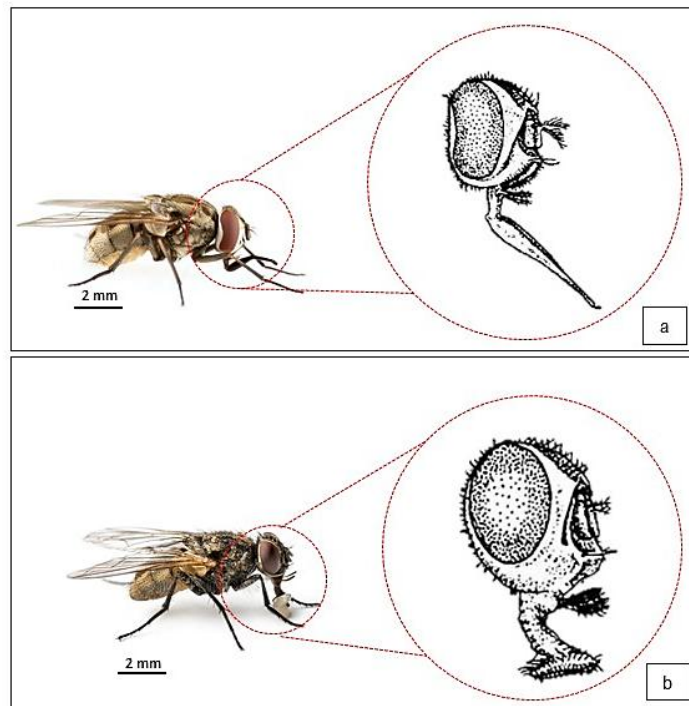


Figure 2.17: The (a) piercing-sucking mouthpart of biting muscid fly, stable fly (*Stomoxys calcitrans*) and (b) sponging mouthpart of non-biting muscid fly, house fly (*Musca domestica*) (Dessi, 2019).

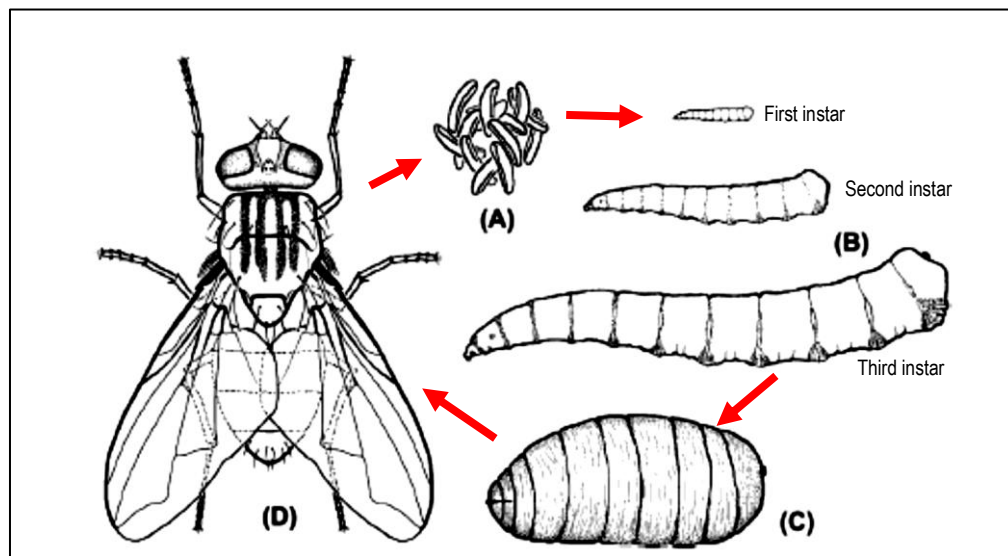


Figure 2.18: The life cycle of housefly (*Musca domestica*) (A) Eggs, (B) Larvae (three instars), (C) Pupa and (D) adult. Traced from drawing by Fritz Gregor in Greenberg, 1971 (Moon, 2019).

Muscid larvae has a typical cylindrical shape of a maggot, tapering from the tail end towards the head and mouth (Byrd & Castner, 2001). It lacks eyes and has minute antennae that resemble papillae. The thorax is legless and consist of eight segments. The muscid pupa consist of a case known as puparium which forms during a process called pupariation. It involves contraction and hardening of the instar integument within (Moon, 2019).

Adult muscid flies are small to medium sized, ranging from 3 – 12 mm long, with wings longer than the abdomen. They tend to be dull gray to dark in colour, although few species have a metallic sheen (Byrd & Castner, 2001). Some species often have dark longitudinal stripes on the thorax, called vitae and dark spots or blotches on abdomen (Moon, 2019). These species can be differentiated from blowflies by taxonomic characteristics of the wings and head (Byrd & Castner, 2001).

The adult non-biting muscid flies obtain sugars from off-host sources such as plant nectaries and honeydew from sap-sucking aphids and scale insects to survive and reproduce. The biting species obtain their proteins through blood, serum, saliva, mucus and lachrymal secretions. For example, horn fly and stable fly obtain nearly all their nutrients from blood (Moon, 2019).

Muscid flies are of forensically importance due to their wide distribution, ubiquitous in nature, and close association to man. They tend to arrive at bodies after flesh flies and blowflies and often lay their eggs in natural body opening and open wounds or in fluid-soaked clothing. The larvae feed directly on carrion, but as their mature they tend to show predacious behaviour where they preying on other species' larvae and eggs. This could affect the fauna composition on the remains (Byrd & Castner, 2001).

In previous study in Malaysia, the muscid flies, adult *Musca domestica* was discovered on pig carcass (Chin et al., 2008) but no maggots were found from their study. However, study conducted by Chen et al. (2010) has discovered maggots of *M. domestica* on day-33 (remain stage) of a decomposed monkey carcass, together with another muscid fly maggots, *Hydrotea* (= *Ophyra*) *spinigera* Stein. It was reported that *H. spinigera* was the dominant coloniser in dry stage. Other muscid flies. were also reported in other part of the world, for example; *Ophyra spp* in Argentina (Patitucci et al., 2010), *Synthesiomyia nudiseta* in Europe (Velásquez et al., 2013).

Other forensically important muscid flies such as; Lesser housefly (*Fannia canicularis*), Latrine fly (*Fannia scalaris*), Bronze dump fly (*Hydrotea aenescens*), Black dump fly (*Hydrotea leucostoma*), House fly (*Musca domestica*) and more are listed in Table 2.4.

Table 2.4: Forensically important muscid flies (Family: Muscidae).

Family Muscidae	Author
<i>Hydrotea</i> (= <i>Ophyra</i>) <i>spinigera</i> (Stein, 1910)	Omar et al. (1994); Chen et al., (2010) & Nazni et al. (2011)
<i>Ophyra chalcogaster</i> (Wiedemann, 1824)	Nazni et al. (2011)
<i>Synthesiomyia nudiseta</i> (Van der Wulp, 1883)	Von Emden (1965); Tantawi et al. (1996); Calderon-Arguedas et al. (2005); Nazni et al. (2008); De Souza et al. (2008); Nazni et al. (2011); Syamsa et al. (2012) & Velásquez et al. (2012)
<i>Musca domestica</i> (Linnaeus, 1758)	Byrd & Castner, (2001); Heo et al. (2008) & Nazni et al. (2011)
<i>Fannia canicularis</i>	Byrd & Castner, (2001)
<i>Fannia scalaris</i>	Byrd & Castner, (2001)
<i>Hydrotea spinigera</i>	Sukontason et al. (2001)

2.4.1.4 Other forensically important flies

Besides blowflies (Family Calliphoridae), flesh flies (Family Sarcophagidae) and muscid flies (Family Muscidae), there are other flies that are also attracted to carrion, excrement and other decaying matters such as Cheese skipper flies (*Piophilidae casei*) which belongs to family Piophilidae which often recovered on human bodies after active decay stage (Chin et al., 2008) and from bodies recovered in aquatic situations (Byrd & Castner, 2001).

Black scavenger flies (*Sepsis* sp.) belong to family Sepsidae occur in large number and often found on dung. Also known as sepsid fly, the larvae develop in variety of decomposing organic matter including carrion and excrement especially during advanced decay stage. Another forensically important flies are, Black soldier flies (*Hermetia illucens*) (Family Stratiomyidae) which also referred to as “wasp fly” due to the wasp-like appearance of the adult. The larvae commonly found in rotting fruits and vegetables, excrement and carrion (Byrd & Castner, 2001). When associated with human remains, they usually found in decay to advanced stage (Kavitha et al., 2013). There are other flies such as scuttle flies and humpbacked fly (Family Phoridae) that also can be found on carrion especially in later stage of decomposition (Byrd & Castner, 2001).

All these flies play their role as scavengers and predators of other species on carrion. They visit the carrion at different stages of decomposition making them a significant tool in the estimation of PMI time. Besides, the unique habitat of certain fly species can be an important indication in forensic investigation.

2.4.2 Coleoptera

The Order Coleoptera is the largest order of insects and contain about 40% of known species in subphylum Hexapoda (Borror et al., 1992). Some of the adult and larvae species secrete chemicals that can irritate the skin and eyes of humans and other animals. The beetles also can induce inhalation allergies when found in stored products and disease to domestic and wild animals when they act as intermediate host for helminth (Krinsky, 2019). More than 380 thousand species of beetles have been described, and currently they are belonging to 165 families (Crawson, 2013).

The beetles in suborder Polyphaga are known for their importance public health and veterinary importance. They are consisting of less than 100 species worldwide and belongs to families: Meloidae (blister beetles), Oedemeridae (false blister beetles), Staphylinidae (rove beetles), Tenebrionidae (darkling beetles), Dermestidae (larder beetles) and Scarabaeidae (scarab or dung beetles). Diseases caused by beetles include eye, ear, skin and nose irritation, respiratory allergie and minor gastrointestinal discomfort (Krinsky, 2019).

Alongside public health and veterinary importance, beetles also play an important role in forensic entomology. Certain literature has described the research on beetles associated with carrion and corpse are under-emphasized due to the assumption that the dipteran flies locate the corpses faster, and thus the estimation of post-mortem interval (minPMI) time are more accurate (Midgley et al., 2010). This is not exactly true as in one observation by Midgley & Villet (2009) have shown that *Thanatophilus micans* (family: Silphidae) was able to locate corpses and breeds within 24 hours of death. In addition, the immature stages of Coleoptera is longer than Diptera, not just during early decomposition but also in later stages of decomposition. During the late decomposition period, most of fly

larvae already left the corpse, leaving mostly beetles. Thus, the potential of beetles to be utilised in PMI estimation is equal to those of flies (Midgley et al., 2010).

Coleoptera derived from Greek words “koleos” which defined as “sheath” and ptera meaning “wings”. It refers to the front wings (elytra) that function as protective covers for the membranous hind wings (Figure 2.19). The hind wings usually longer than the thickened, hard and brittle front wings. The hind wings usually folded under the front wings at rest and are the only ones used for flight. The beetles body structure consists of three regions which are head, thorax and abdomen (Borror et al., 1992).

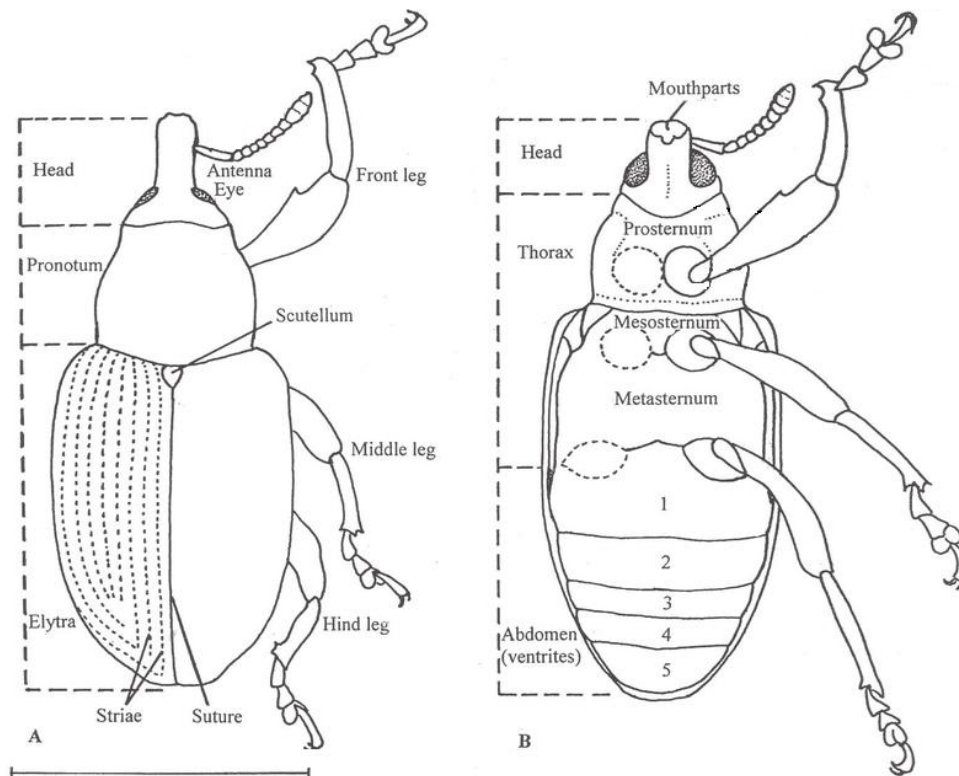


Figure 2.19: Morphology of Curculionidae (Order: Coleoptera) showing (A) dorsal view and (B) ventral view. Scale= 1 cm (Marvaldi & Lanteri, 2005).

The head consists of compound eyes, antennae and mouthpart. The antennae are very important to locate food and to identify pheromones as they provide information about touch, smell and taste. The mouthparts of a beetle are of the chewing type and mandibles are well develop. The mandibles are mostly stout and use in gnawing and crushing. The second region is the thorax, which serves as an attachment point for legs and wings. In a dorsal view, only the pronotum and scutellum are visible, and the other segment is visible in ventral view (Proseternum, mesosternum and metasternum). The third region is the abdomen. The number of visible abdomen sterna varies in different groups and used in key species identification. The abdomen holds the beetle digestive system and reproductive organs. There are series of spiracles on the abdomen where the air passes and circulate throughout the body (Krinsky, 2019).

The coleopterans undergo a complete metamorphosis which consists of eggs, larvae (grub), pupae and adult beetle (Figure 2.20). The life cycle duration for beetles are varies from four generations a year, or one generation in several years. However, most species have one generation a year. (Borror et al., 1992). Once mated, the female beetles will find a suitable place where they are abundant of food source for their offspring before the eggs were deposited. For example, the flower beetles (Family: Scarabaediae) will lay their eggs on decaying leaf matter and stag beetles (Family: Lucanidae) will burry in search of rotten woods. They will chew a tiny hole on a rotten wood, and deposit one single egg and packing the holes again with small fragments of wood. Then, the females will move on and repeat the process all over again. The time length for the eggs to hatch are varies among species and when the eggs hatched, it will move to the second stage of the life cycle which is the larvae (Nelson, 2022).

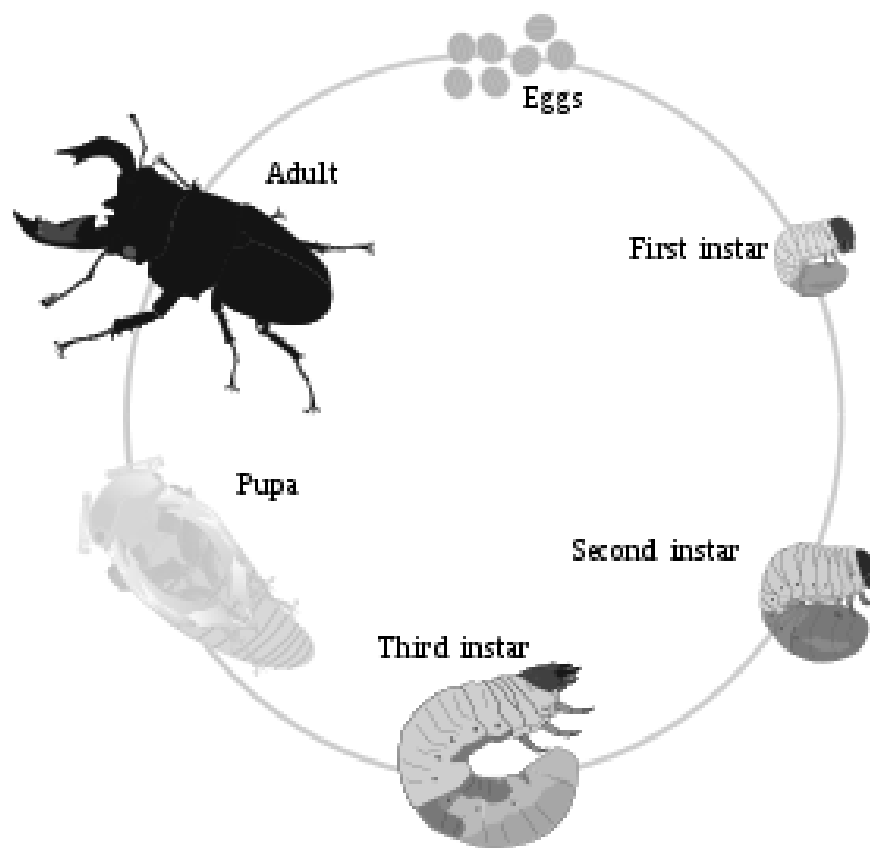


Figure 2.20: The life cycle of stag beetle (Family: Lucanidae) which consists of eggs, larvae, pupa and adult beetle (Silitep, 2016).

The hatched larva is in the first stage which also known as first instar. The larvae moult three times. As they develop, they will shed their skin in order to grow bigger into second instar and finally the third instar. The duration also varies from months to several years depending on the species. During the final instar, the larvae will consume more substrate in order to gains more weight, and become larger and stronger when they eventually develop into adult beetle. The larvae feed on decayed plants or animals, burrow into plant body and some are predatory. The larvae can undergo reverse development or early sexual maturity as a tolerance during poor food environments (Nelson, 2022).

After certain times, the larvae will shed their skin for the last time and develop into a pupa. During this time, they will construct a strong cocoon made of soil in which they pupate. During this stage, the larvae break down and transform into several parts of the adult beetle. Depending on the species, this process will take several weeks to months. Once the metamorphosis is complete, the adult emerged from the pupae. The front wing will be hardened which may take a few days. Some species have an inactive stage, where the adult sits in their chamber until the surrounding are more favourable to find food and mate. Then, the life cycle starts all over again (Nelson, 2022).

There are several species that are forensically importance and plays an important role as a decomposers and tools for estimation minPMI. There are several families that are of forensically importance which include; Silphidae, Histeridae, Staphylinidae, Dermestidae, Cleridae, Trogidae and Scarabaeidae (Villet, 2011).

2.4.2.1 Family Scarabaeidae (Scarab/dung beetles)

The Family Scarabaeidae is one of the largest family in order Coleoptera which comprise of about 2,000 known genera and over 31,000 described species. The beetles belonging to this family are varied in terms of size, colour and habits. Scarab beetles can be recognized by their oval or elongate shaped and convex body. The legs consist of 5-segmented tarsi and 8-11 segmented and lamellate antennae. The last three antennal segments have a form of plate-like structure which form a compact terminal club. The front tibiae are dilated with toothed and scalloped outer edge (Figure 2.21a) (Borror et al., 1992; Ratcliffe & Cave, 2010).

Many of scarab beetles feed on dung or decaying materials, both vegetation and animals, in the nest of other insects (ants and termites), or nests of rodents and birds. The

larvae usually have a C-shaped and referred to as “grubs” (Figure 2.21b). Dung beetles, in some species laid their eggs in a ball of dung collected by the adults. The larvae are white in colour with a brown head and can be found in the soil underneath the body (Woodruff & Beck, 1973).

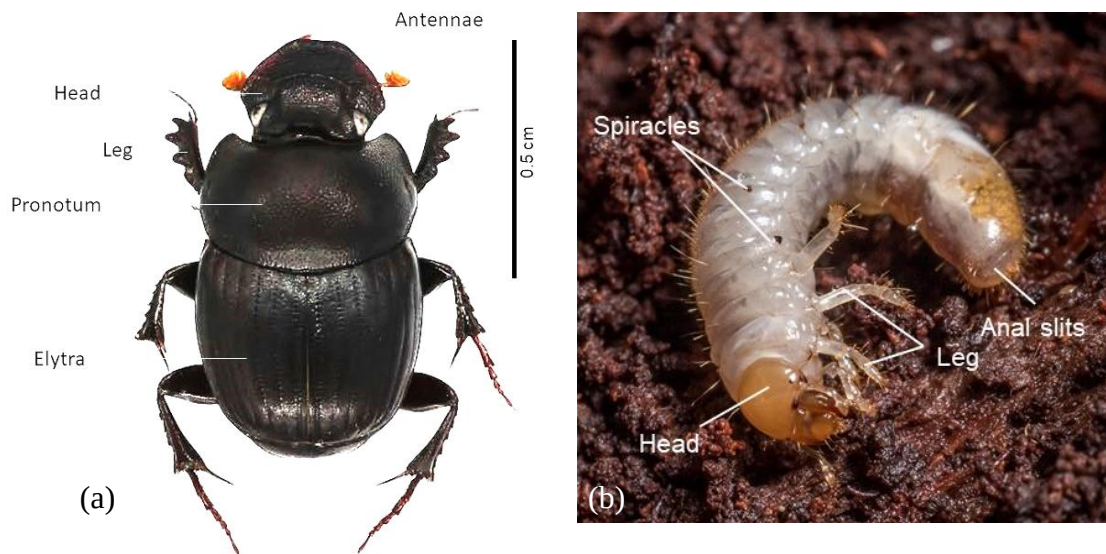


Figure 2.21: The general structure of (a) adult and (b) larvae (grub) of scarab beetle (Family Scarabaeidae) (Cheung, 2018).

2.4.2.2 Family Staphylinidae (Rove beetle)

Family Staphylinidae is a huge family belonging to superfamily Staphylinoidae alongside four other families, Hydraenidae, Ptiliidae, Agyrtidae, Leiodidae and Silphidae (Frank & Thomas, 2010). The forensically important beetles are belonging to family Staphylinidae and Silphidae (Villet, 2011). The rove beetles have a slender and elongate body, ranged about 1-40 mm in length (Frank & Thomas, 2010). Many of these beetles are black or brown in colour. They usually identified by its short elytra, which usually shorter than they combined width. There are six or seven abdomens exposed beyond their apices (Borror et al., 1992) (Figure 2.22a). The abdominal segments are musculature which makes

them flexible, enable them to enter narrow crevices. However, the exposed abdomen also exposed them to desiccation and thus, they are dependent upon humid habitats. At rest, the hind wings are folded under the short elytra.

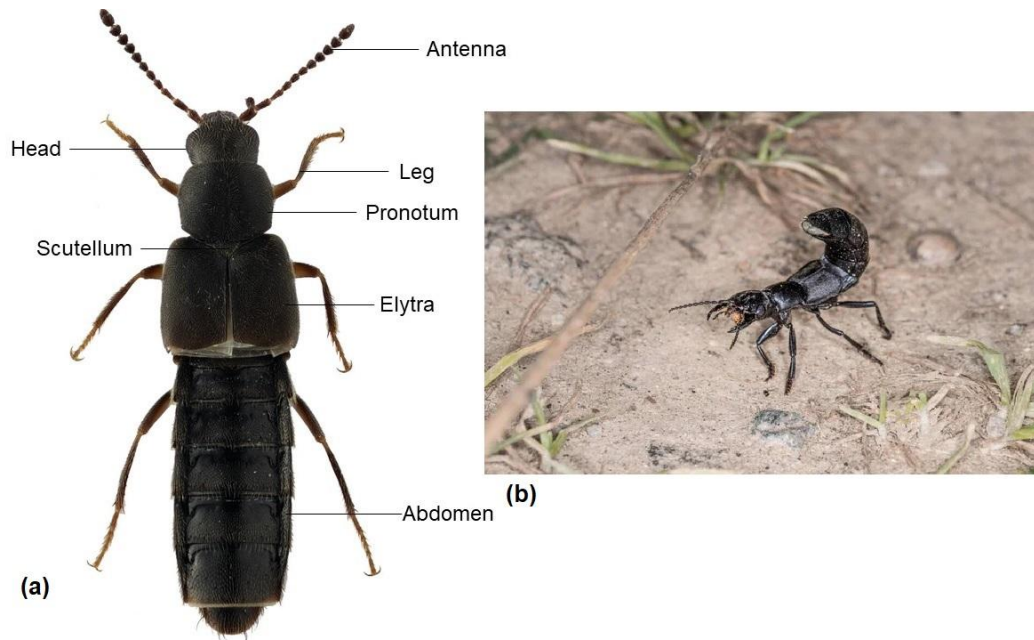


Figure 2.22: Figure showing (a) the general structure of rove beetle (Family: Staphylinidae) and (b) a rove beetle raising the tip of its abdomen when disturbed (Yoo & Ahn, 2011).

They are active insects which run and fly rapidly. They frequently raised the tip of their abdomen when running, disturbed or provoked as scorpions do (Borror et al., 1992) (Figure 2.22b). Staphylinidae should not be confused with the order Dermaptera. The ninth abdominal segment and the radial wing-folding pattern of Dermaptera are unlike that of Staphylinidae (Frank & Thomas, 2010). Rove beetles usually presence in various habitat, but mostly in decaying material particularly dung and carrion. Most species are predaceous. They fed on fly eggs and larvae that presence in the habitat. Some species also eat the fly adults (Frank & Thomas, 2010).

2.4.2.3 Other forensically important beetles

a. Family Silphidae (Carrion beetles)

Beetles belonging to family Silphidae are considered small as it represented by about 200 species worldwide (Ratcliffe & Jameson, 1992; Sikes et al., 2008). The carrion beetles are bright colour insects and majority are feed on bodies of dead animals. Some genera for example; *Ablattaria* Reitter, *Dendroxena* Motschlsky and *Phosphuga* Leach are predators of small invertebrates (snails, caterpillars and slugs), while others are phytophagous for example, genus: *Aclypea* Reitter) (Sikes et al., 2008). The body of carrion beetles are soft and flat, while the antennae are clubbed (clavate or capitate) and the tarsi are five-segmented. The size of the carrion beetles is medium to large, about 3 to 35 mm in length, but majority are over 10 mm. There are two common genera in this family which are *Silpha* and *Nicrophorous* (Borror et al., 1992) (Figure 2.23).

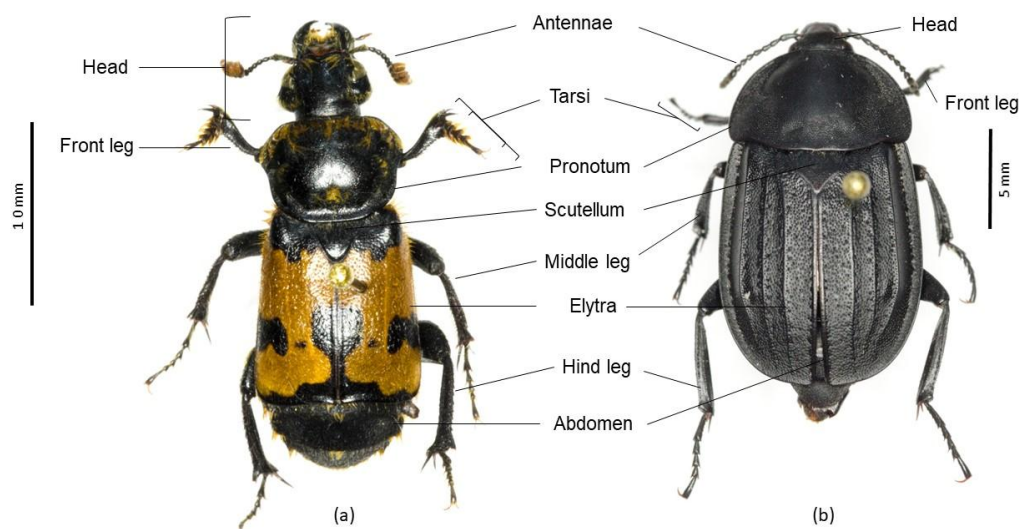


Figure 2.23: The general structure of carrion beetles (Coleopteran: Silphidae) of two common genera; (a) *Nicrophorous* and (b) *Silpha* (Ciftci et al., 2018).

b. Family Histeridae (Hister beetles)

Hister beetles are predacious beetles with a small and shiny black in colour. The beetles belong to 391 genera, 76 subgenera, 4252 species and 34 subspecies worldwide (Mazur, 2011). Figure 2.24 shows the general morphology of hister beetles. The size ranged from 0.5-20.0 mm in length, oval or round shaped in dorsal view. One or two apical abdominal segments are visible as the elytra are cut off square at the apex (Borror et al., 1992; Mazur, 2011). The elytra are truncate often striate or punctate and occasionally with tubercles. All species have geniculate antennae with a compact, three segmented club (Neculiseanu, 2020). The legs are short and retracted. The tibiae of hister beetles are dilate with toothed and spined at anterior. The legs are short and retracted. The tibiae of hister beetles are dilate with toothed and spined at anterior.

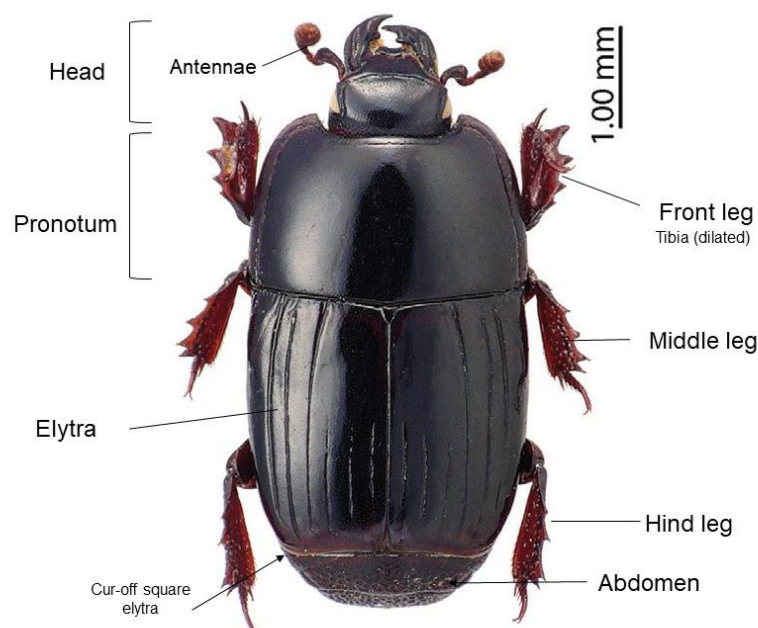


Figure 2.24: The general morphology of hister beetles, *Platylister (Popinus) algricus* (Lucas, 1846) (Shayya & Lackner, 2020).

When disturbed, this species will draw in their legs and antennae and become motionless. The appendages fit so tightly into shallow grooves on the ventral side of the body, which makes it difficult to see them (Borror et al., 1992). This group of beetles can be found worldwide in various habitat, near decaying animal and plant, under tree bark, in dung (Caneparo et al., 2017; Szelecz et al., 2018), inside mammal burrows, inside ants'/termites' colonies (Szelecz et al., 2018) and more, feeding on the soft bodied larvae or eggs and small insects living inside these materials (Kryzhanovskij & Reichhardt, 1976; Borror et al., 1992). Some species are considered cannibalistic beetles as they feed on other beetle species of the same family Histeridae (Arnett & Thomas, 2000). The predaceous behaviour of hister beetles makes them useful in pest-control in poultry and stored food products (Kryzhanovskij, 1965).

c. Family Trogidae (Hide beetles)

Hide beetles are small family of order Coleoptera which about 300 species worldwide (Pittino, 2006; Zidek, 2013). The beetles belonging in this family are identified by their overall warty, dark colour (brown, gray and black), and their flat abdomen. Sometimes, this group is considered as subfamily of a larger family, Scarabaeidae. Figure 2.25 shows the general morphology of hide beetles. The size of hide beetles is about 2.5-20.0 mm in length, with an oblong or oval shape. The back of the body usually rough and covered with ridges (Byrd & Castner, 2001). The beetles have 10-segmented antennae with 3-segmented opposable club. Pronotum is short, broad and convex and sculptured with tubercles. The scutellum is exposed (Figure 2.26) (Jameson, 2005).

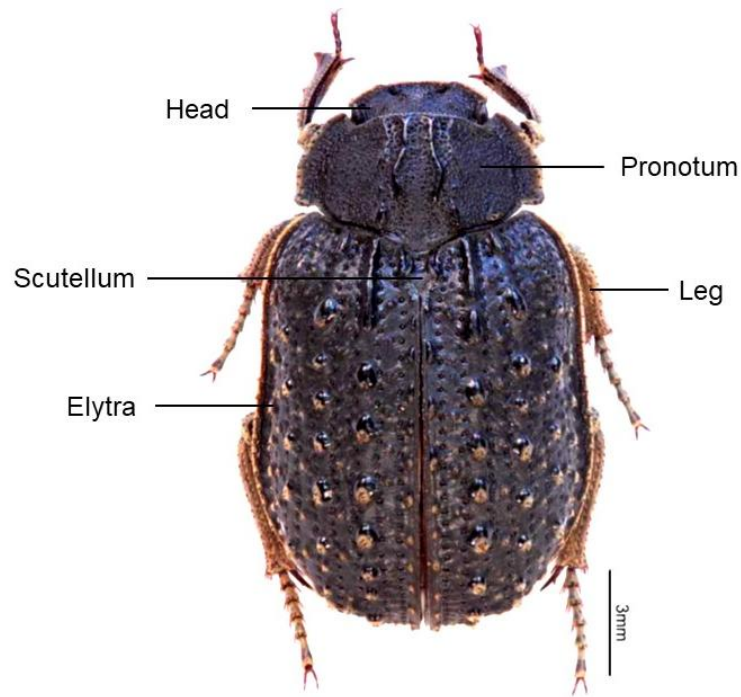


Figure 2.25: The figure showing hide beetle (Coleoptera: Trogidae) and its general structure (Strumpher et al., 2014).

d. Family Dermestidae (Skin beetles)

Dermestid beetles have been given several common names based on their food preference which are, hide beetles, carpet beetles and larder beetles and skin beetles. Approximately, over 500 species of beetles worldwide are of dermestid beetles. They are small in size, which ranged from 2-12 mm in length. Similar with most of beetles, this species has a rounded to oval in shape. The distinctive characteristic of dermestids is they have scales cover their body which form a unique and colourful pattern, while the larvae covered with long and dense hair, range about 5-15 mm in length (Figure 2.26) (Byrd & Castner, 2001).

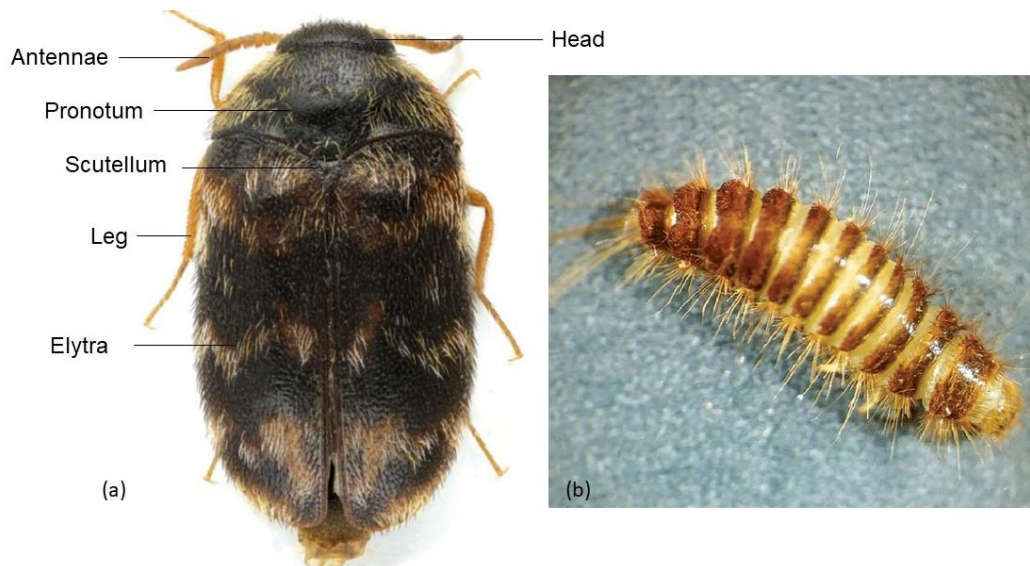


Figure 2.26: Figure showing dermestid beetle, *Trogoderma variabile* (a) adult showing unique pattern on the body and (b) larval stage of the species (Nardi & Vomero, 2017).

2.4.3 Other insects

Some insects that categorized as scavengers or predators are not specialized in feeding on carrion; however, they were opportunistically utilised the carrion as a food source when it is available. For example, the presence of wasps (family Vespidae) on carrion were seen tearing off some tissue and fly away with it. The small portion of tissue may not affect the decomposition rate or insect succession, however, the artefact left by that action may need further interpretation (Byrd & Castner, 2001).

Other examples would be the presence of ants (family Formicidae) that usually have a foraging habit, that leaves marks on the body post-mortem. The marks resemble burn marks which can be misinterpreted into premortem burns. Besides, the ants also predate on fly eggs, which can delay the initial colonisation by 2-3 days (Byrd & Castner, 2001).

2.5 Entomotoxicology

Entomotoxicology is a subspecialty of forensic entomology that examines the toxins present in arthropods, primarily flies and beetles that consume carrion. Investigators can identify the presence of poisons in a body at the moment of death by using the arthropods at the crime scene (Introna et al., 2001). In forensic entomotoxicology, the effects of medications on the growth rate of carrion-eating insects are studied, and these insects are utilised as an alternative sample when other tissues are not available (Goff & Lord, 1994b).

Forensic toxicologist qualitatively and quantitatively identifies drugs and poisons which may be relevant to the cause of death. The biological samples usually obtain during autopsy. However, if the body is badly decomposed, the alternatively, the larvae of the insects are collected and analysed. The use of arthropods as the toxicological samples are well documented globally. The literature has cited the use of arthropods to identify drugs or toxins since 1980 (Beyer et al., 1980).

Insects are important in forensics. They eat the dead bodily tissues, which serve as their food source. The xenobiotics, including as medications and poisons, that are present in the tissues will be passed to the larval insects' metabolic system when they are actively feeding on the tissues. Furthermore, the insects' larvae serve as a suitable toxicological sample since the puparial case remain unchanged for a long period of time (Campobasso et al., 2001; Bourel et al., 2001a).

Death due to pesticides, drugs and other toxins are common around the world. In 2015, it was reported about 450 thousand people died around the world as a result of drug abuse, and it was estimated about 800 thousand people die every year due to suicide, in which around 20% died as a result of pesticide ingestion (Bachmann, 2018). In several cases,

the body was recovered after a long period of time. This is where the entomotoxicology can be applied in solving poisoning cases (Kintz et al., 1990).

2.5.1 Detection of chemical substance in insects

The first article on the use of insects as the sample for toxicological analysis was started in 1980. The drug detected was phenobarbital. The 22-year-old victim was found 14 days after she was last seen, and the body was found in advanced stage of decomposition (Beyer et al., 1980). Since then, many drugs, to name a few, Amitriptyline, Propoxyphene, Benzodiazepines, Barbiturates, Cocaine, insecticides and pesticides and Mercury have been detected in larval tissues and insects. Based on the analysis conducted by Chopi et al. (2019), detection of opiates and opioids in insects are the most study conducted, followed by anti-depressants drugs and stimulants (Figure 2.27).

As we all know, a complete life cycle of flies occurs within and surround the corpse/carrion, from eggs, larvae, pupae until they emerge into adult. Once they become adult, they leave behind puparia cases on the ground, close proximity to the body. Usually, empty puparia case used as the last resort in toxicological analysis when the body recovered in skeletonized stage. It was reported that the drugs and toxins accumulate inside the cuticle of the larvae during development and these in turn transformed into sclerotized puparium during pupariation (Bourel et al., 2001a).

The insects' organic materials that can be collected and use as a sample for toxicological analysis are larvae, pupae, adult insects, puparial cases, exuviae (cast beetle skins), beetle faecal material (frass), fly predators and scavengers (Candela & Aventaggiato, 2001).

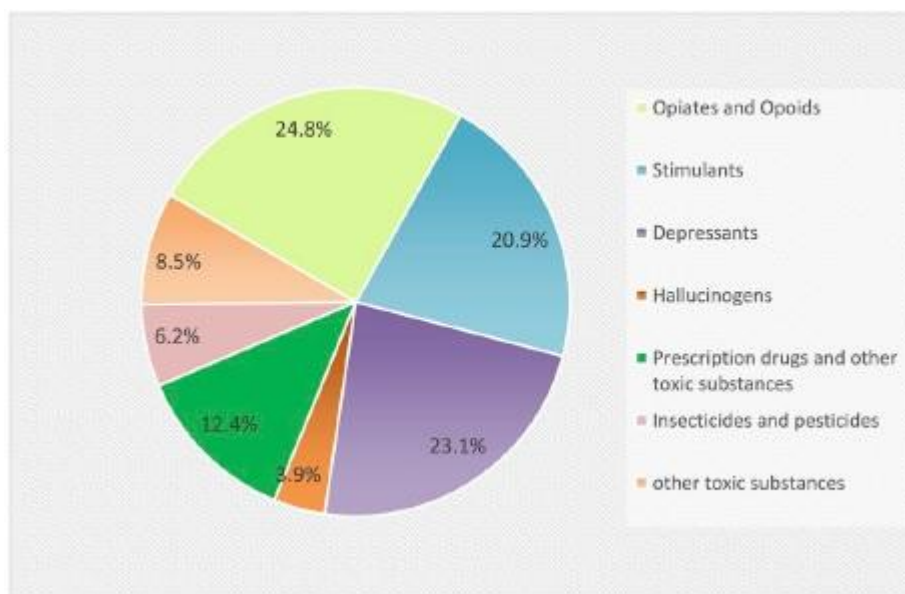


Figure 2.27: Various drugs and toxins substances reported in forensic entomotoxicology field from year 1980 to 2018 (Chophi et al., 2019).

The insects that usually used as toxicological samples are, true flies (order: Diptera) and beetles (order: Coleoptera). The advantages of using maggots instead of body tissues can be seen in chromatography. Chromatogram obtains from larval extracts present less endogenous contaminant peaks than chromatograms from human body extracts (Kintz et al., 1990). This is beneficial if the corpse biological material decomposed and the insects are rather easily collected and reared in laboratory. However, if the body in advanced decay stage, necrophagous and some predator coleopteran that feed on colonising insects can be used as the toxicological samples.

A study on beetles, *Dermestes frischeri* Kugelann (Family: Dermestidae) and *Thanatophilus sinuatus* Fabricus (Family: Silphidae) which were reared on food containing morphine, has successfully detect the presence of drug in all developmental stages of *D. frisdchi* and in the larval stage of *T. sinuatus* (Bourel et al., 2001b).

Insects can be collected around or under the body or body discovery site. However, when the body badly decomposed, it must be aware that the insects collected can originate from a source other than deceased. It is important to collect the insects at different body sites as there are high variation of drug or toxins concentration (Sadler et al., 1995; Gagliano-Candela & Aventaggiato, 2001). It was reported that the best sampling sites for drug detection in insects are the internal organs (e.g., liver) and the head area or muscles (Sankhla et al., 2017). Although the guidelines for insect sampling in forensic entomotoxicology have been published (Amendt et al., 2007; Carvahlo, 2009), other sample criteria such as, the number of specimens and the developmental stages of insects is not specified (Tracqui et al., 2004).

The concentration of drugs or poisons in insect samples are directly comparative to the concentration of the drugs in the substrate. However, this may not be true for all cases. In previous study, the drug concentration in insects shows fluctuation when treated with three different doses (Bushby et al., 2012). The drug concentration decreased especially during post feeding stage as their no uptake of xenobiotics from food (Bushby et al., 2012). The larvae also effectively eliminate drugs or toxins from its body, which contribute to much lesser concentration drugs when the larvae mature into adult (Liu et al., 2009). For example, according to Alves et al. (2008), diethylpropion (Inibex®) was not detected in larvae of *Ch. megacephala* and *Chrysomya putoria* which later suggests that this is due to the rapid excretion of the drug from the larvae's body.

Most of the previous study showed successful results in detecting the chemical substance either qualitative or quantitative analysis. The analysis was carried out using homogenised insect samples through techniques such as immunoassay, high performance

liquid chromatography (HPLC), gas chromatography (GC), liquid chromatography–mass spectrometry (LC-MS), liquid chromatography with tandem mass spectrometry (LC-MS/MS) and gas chromatography–mass spectrometry (GC-MS).

Immunoassay has been utilised in forensic entomotoxicology to identify morphine from a variety of substrates and specimens (Hedouin et al., 1999; Bourel et al., 1999). According to a study utilising the HPLC technique, 76% of the morphine consumed by insects was actively digested, while the remaining 24% was excreted as unaltered morphine (Parry et al., 2011). This technique has also been useful in detecting other drugs and pesticides. For example, Wolf et al. (2004) analysed insect feeding on rabbit carcass sacrificed using parathion. As the result of the analysis, parathion can be detected from various species of insects. Chromatography in combination with mass spectrometry is regarded as the "gold standard" technology for confirmatory testing of compounds in samples because it has great selectivity, sensitivity, accuracy, reproducibility, and the ability to measure tiny amounts of substances (Wallace, 2017). This method has been used in forensic drug misuse analysis to find various drugs in varied environments (Valentine & Middleton, 2000).

2.5.2 Effect of chemical substance on PMI time

PMI time estimation is done by analysing the insect succession pattern and insect developmental stage found on the body (Byrd et al., 2009). However, it was found that the toxins presence in the insects' tissue can affect the growth of the insect. It could either speed up or slow down the insect development (O'Brien & Turner, 2004). Drugs like cocaine, heroin, morphine, methamphetamine, methylene dioxymetamphetamine, triazolam,

oxazepam, chloripriamine, barbiturates, malathion, and paracetamol were frequently involved in cases in forensic entomotoxicology (Sankhla et al., 2017).

Although the application of insects in toxicological analysis has been carried out since 1980, the pharmacokinetic of drug and other toxic substance is not fully understood yet (Magni et al., 2014). The species, developmental stage and their feeding activity are known to affect the pharmacokinetics in insects (George et al., 2009). The result of analysis also could be influenced by the stability of the substance, temperature and humidity (Tracqui et al., 2004). It was suggested that standard protocol should be established which include model organism, standard matrix for feeding substrate, sample size and deploying sophisticated analytical methods. By doing so, comparison of results between research facilities can be made (da Silva et al., 2017).

2.5.2.1 The effect of pesticides on the estimation of PMI time.

Pesticides are important and widely used in Malaysia since agriculture is the backbone to Malaysian economy. As of 2018, about 3.5 thousand metric tons of pesticides were used in Malaysia (Statista Research Department, 2022). In 1985, a study on the survey of pesticide used among agricultural workers, smallholders and estate workers in Malaysia has reported that about 98% of paraquat was used and at the same time, 64% of them also used organophosphorous insecticides (Ramasamy & Nursiah, 1988). Between the year of 1986 to 1996, 700 poisoning cases are due to paraquat and 73% are suicide cases (Majid, 1997).

According to estimation made by the World Health Organization (WHO) in 2016, 60–90% of suicides that occurred in China, Malaysia, Sri Lanka, and Trinidad and Tobago over the course of the previous ten years involved intentional pesticide poisoning (Bertolote

et al., 2006), which is estimated to account for about 300,000 deaths worldwide each year. As a result, research has been done on the preventative benefit of limiting access to pesticides, which are now available and stored in homes without any safeguards (Gunnell & Eddleston, 2003).

The estimation of PMI time is contributed by calculating the insect succession pattern and their development, along with the stage of decomposition the body (Wells & Lamotte, 2001). Therefore, many studies on the effects of insecticide to the rate of decomposition, insect succession pattern and carrion-feeding arthropods development conducted around the world (Rashid et al., 2008; Liu et al., 2009; Yan-Wei et al., 2010 & Abd El-Bar & Sawaby, 2011) as it can interfere with the calculation.

The early detection of organophosphate in maggots was conducted in 1989 where malathion was detected in larvae of *Ch. megacephala* (Fabricius) and *Ch. rufifacies* (Macquart) in a suicidal poisoning case through GC analysis (Gunatilake & Goff, 1989). In agricultural areas, especially when using the pesticide and acaricide parathion, there is a high incidence of intoxication from improper application, inadequate protective gear, and suicides by ingesting extensively used in agriculture and readily absorbed by the soil is parathion (Carvalho, 2010).

Wolf et al. (2004), in their research employed HPLC to detect the presence of parathion in arthropods collected from decomposing parathion-treated rabbit carcasses. The pesticide was detected in different developmental stages of 10 arthropods species which are from the order Diptera, Coleoptera, Hymenoptera, Isopoda and Acari. They were collected throughout the decomposing phases. In terms of insect succession pattern on the carcasses, although there is no alteration on the pattern reported, the insecticidal activity occurs at the

mouth of the treated rabbit carcasses. This is in contrast to results from similar experiment using malathion (Gunatilake & Goff, 1989).

In West Malaysia, a study on the effects of malathion on oviposition and development of blowflies in Kelantan has reported that oviposition of blowflies (*Ch. megacephala* and *Ch. rufifacies*) was delayed for 1-2 days and the pupation period was prolonged to 2-3 days (Mahat et al., 2009). This study was in agreement with a previous study conducted in Kuala Lumpur, where the development of *Ch. megacephala* was longer when fed with rat's liver tissue of malathion-fed rat. The presence of malathion was detected in flies' larvae tissue using GC (Rashid et al., 2008).

Besides insecticide, studies on the effect of herbicide also conducted in Malaysia. From previous study, the presence of herbicide, paraquat hydrochloride has been detected in blowfly larvae tissues. The blowfly larvae that were infesting paraquat-treated rabbit carcasses were collected and subjected to toxicological analysis (Lawai et al., 2015 & Mahmood et al., 2015). The World Health Organisation (WHO) has categorized paraquat as Class II pesticides which are moderately toxic, however, it was extremely toxic to human and animals (Mahmood et al., 2015). Further research needs to be conducted in order to understand the various effect of insecticides over carrion-feeding entomofauna (Carvalho, 2010).

2.6 Pesticides

Pesticides are chemical substances that are used to eradicate undesired organisms such as insects, rodents, fungi, and plants (weeds) (WHO, 2004). The usage of pesticides in Malaysia are regulated and managed by Pesticides Control Division under the jurisdiction of the Ministry of Agriculture by enforcing the Pesticides Act 1974. All pesticides and other

chemicals used in agriculture are managed under the Act. The Pesticides Board in this division, which is made up of several heads of government agencies, approved the registration of pesticides (Jiang, 2017).

In Malaysia pesticide products are evaluate according to their biological effectiveness, compatibility for the needs of the local market, use guidelines, limitations on the dangers to human health and the environment, and legality of use (proven by registration or rejection of registration). The Pesticide Board needs to register certain pesticides before the pesticides distributed in Malaysia. Various research and evaluation were conducted before the pesticide products are ready for sale (Department of Agriculture Malaysia, 2016).

According to Malaysian Department of Agriculture (2016), the ongoing research on pesticides and their effect to public health in Malaysia need to be conducted as multiple exposures and multiple characteristics of pesticides have caused difficulty to diagnose certain diseases attributed to the effect of pesticides. In this thesis, three types of pesticides were tested which are insecticide (chlorpyrifos), herbicide (glyphosate) and rodenticide (coumarin). The information on these pesticides were discussed in next section.

2.6.1 Herbicide: Glyphosate

Glyphosate (*N*-(phosphonomethyl)glycine) is the active ingredient in several commercial herbicides for nonselective weed control. In Malaysia, glyphosate is useful in crop production to increase the yield and decrease the labour cost (Poramacom, 2001). This is important as Malaysia is an agricultural country that exports rice, cassava and rubber to the world market (Dardak, 2022).

Herbicides are successfully used in developed nations, and they have a high penetration rate into numerous crops like maize, sugarcane, and rice. (Finney, 1998). Glyphosate is widely used around the world and it has been certified for use in over 130 countries (Dill et al., 2010). Both annual and perennial weeds can be effectively controlled with glyphosate. It was initially used to suppress perennial weeds in ditches and barren fields because of these qualities. Applications on crops are limited since they can cause crop death. Yet glyphosate is well-liked because it can successfully eradicate weeds on uncultivated land and in conventional tillage techniques prior to planting (Suvittawat, 2014). Worldwide, the number one glyphosate-based herbicide is Roundup®. Other trade names include Roundup Ultra®, Roundup Pro®, Accord®, Honcho®, Pondmaster®, Protocol®, Rascal®, Expedite®, Ranger®, Bronco®, Campaign®, Landmaster®, Fallow Master® and more (Zhou et al., 2004; Tu & Randall, 2001).

Glyphosate is a phosphonic acid resulting from the formal oxidative coupling of the methyl group of methylphosphonic acid with the amino group of glycine. The molecular formula is $\text{HOOCCH}_2\text{NHCH}_2\text{PO}(\text{OH})_2$ (Figure 2.28) and with molecular weight of 169.07 g/mol. Glyphosate has low solubility in water (12 g/L at 25 °C, and 60 g/L at 100 °C), but it is insoluble in other solvents (Mensah et al., 2014).

Glyphosate is a systemic herbicide which disrupts a number of biological processes, such as protein synthesis, photosynthesis, respiration, and the production of nucleic acids. In plants, it readily translocated to all plant's parts through the phloem. The molecules of glyphosate are taken up by plant cells from the leaf surface, where they go to the meristems of developing plants (Satchivi et al., 2000).

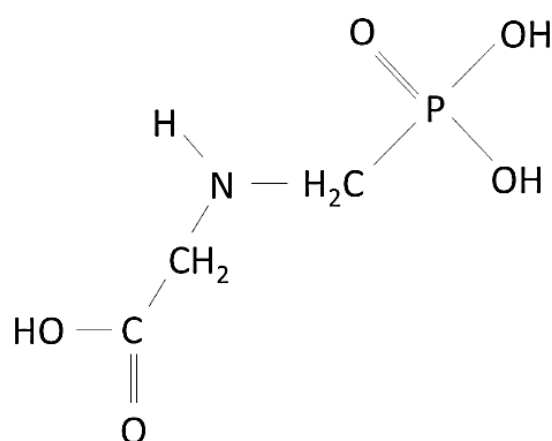


Figure 2.28: The chemical structure of *N*-(phosphonomethyl) glycine (Mensah et al., 2014).

Plant that poisoned with glyphosate will exhibit several phytotoxic signs that include progress from progressive wilting and chlorosis to complete browning, entire degradation and finally death (Perez et al., 2011). Acute effects on animals were observed following intraperitoneal administration of high doses of glyphosate. It was suggested that glyphosate altered the mitochondrial activity possibly due to uncoupling of oxidative phosphorylation during cellular respiration (Pereira et al., 2018).

Glyphosate has limited movement in the environment due to its capacity to strongly adsorbed in the soil. The half-life of this herbicide in soil is around two months but can range from two to ten weeks. It degrades slower in water than soil because there are fewer microorganisms in water than in soils (Tu et al., 2001). Glyphosate degrade to produce aminomethylphosphonic acid (AMPA) and carbon dioxide and the pH reduced when dissolved in water (Meyer et al., 2009). Glyphosate dissolves through degradation, dilution, adsorption on organic substances, inorganic clays and sediments in freshwater ecosystem (Tu & Randall, 2001).

Due to its high solubility in water and widespread usage as herbicide, glyphosate's toxicity towards non-target aquatic creatures has come under scrutiny. Regarding this, a surfactant called polyoxyethylene amine (POEA) has been linked to the reason for the comparatively high toxicity of Roundup® to a number of freshwater invertebrates and fish. Technical grade glyphosate is slightly to very slightly toxic with reported LC50 values greater than 55 mg/L (Giesy et al., 2000; Tsui & Chu, 2003). Although it was generally known that glyphosate has a limited potential for polluting surface waters, nonetheless, glyphosate has been discovered in surface waters decades after it had been used to manage aquatic weeds (Glusczak et al., 2007; Mensah et al., 2014). Although this herbicide is intended to kill plants, numerous studies have demonstrated that it also negatively affects non-target animals (El-Shebly et al., 2008; Giesy et al., 2000; Tsui & Chu, 2003).

US EPA has classified glyphosate as “Group E Carcinogen” which implies that it is non carcinogenic substance to the public (US EPA, 2022). This is the fact that glyphosate interrupts shikimate pathway, which only present in plant and microorganism (Solomon et al., 2007). However, recent studies have proposed glyphosate has genotoxicity, cytotoxicity and reproductive toxicity in wildlife and humans (EC, 2002; WHO, 2004). Studies with rats suggest that the respiratory and hepatic systems, as well as reproductive functions can be altered by exposure to Roundup®, for example, testicular dysgenesis, congenital malformations such as cryptorchidism and (Toppari et al., 2010; Carlsen et al., 1992).

The occupational exposure of herbicide to humans has been associated with increased cancer risk (Bolognesi et al., 2009; Eriksson et al., 2008). Humans may come into contact with herbicide through their skin or through breathing it in during manufacture. Many studies indicate that glyphosate can change other pathways, despite the fact that it is

well recognised that people and animals do not have the shikimate pathway that glyphosate disrupts. Despite the lack of conclusive data, the cytochrome P450 (CYP) pathways and mitochondria are potential candidate sites of action (Mensah et al., 2015). Detoxification of xenobiotics is one of the crucial functions of CYP enzymes. Thus, glyphosate increases the harmful effects of other food-borne chemical residues and environmental pollutants by interfering with the CYP pathways (Samsel & Seneff, 2013).

Over time, although the glyphosate effects are slow and subtle, it will damage the cellular systems throughout the body. This will eventually lead to illnesses like digestive problems, obesity, diabetes, heart disease, depression, autism, infertility, cancer, and Alzheimer's disease which also associated with diet. The glyphosate is an example of “exogenous semiotic entropy” which can be defined as homeostasis disruption caused by environmental toxins (Samsel & Seneff, 2013).

From previous studies and investigations, it was clear that exposure to glyphosate can affects other organisms besides plants. The use of glyphosate needs to be reviewed as the glyphosate-contaminated plant products as the plants can be part of dietary supplements. This can be the sources of genotoxicity, cytotoxicity and reproductive toxicity in animals and humans which may caused cancer in humans (Mensah et al., 2015).

2.6.2 Insecticide: Chlorpyrifos

Chlorpyrifos (CPF) (IUPAC: O, O-diethyl O-3, 5, 6-trichloropyridin-2-yl phosphorothioate) is an organophosphate insecticide, acaricide, and miticide principally used to control foliar and soil-borne pests on a variety of food and feed crops. Pure chlorpyrifos are white or colorless crystal. It has slightly skunky odour like rotten eggs or garlic (Christensen et al., 2009). The products contained chlorpyrifos often sold as liquids,

granules, water dispersible granules, wettable powders and water-soluble packets. It is used on crops and non-agricultural uses which includes, golf courses, turf, green houses and non-structural wood treatments such as utility poles and fence posts (Environmental Protection Agency, 2017).

CPF has been in the world market since 1965 and uses trade names such as Brodan, Detmol UA, Dowco 1970, Dursban, Empire, Eradex, Lorsban, Paqean, Piridane, Scout, Stipend and other. Despite the restriction on home use in certain countries, it remains popular pesticide throughout the world. About 100 countries worldwide, including CPF Ukraine, U.S.A., Canada, the United Kingdom, Spain, France, Italy, Japan, Australia New Zealand and the majority of other developed countries, have approved the use of CPF (Salyha, 2010).

CPF has chemical formula $C_9H_{11}Cl_3NO_3PS$ (Figure 2.29) with molecular weight 350.62 g/mol. Water solubility is 2 mg/L in room temperature, and it also soluble in benzene, acetone, chloroform, carbon disulphide, diethyl ether, xylene, methylene chloride, ethanol and methanol. The melting point of CPF is 41.5-44 °C with vapour pressure 2.5 mPa in room temperature (Salyha, 2010).

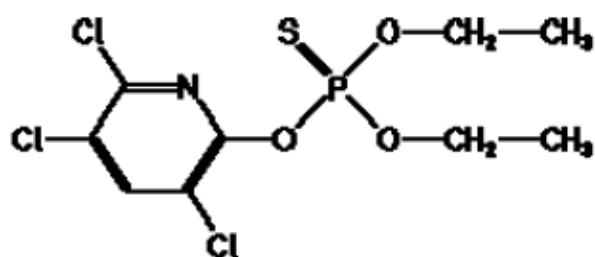


Figure 2.29: Chemical structure of chlorpyrifos (Salyha, 2010).

Chemical's toxicity is a measurement of its ability to poison people, whereas poisoning is the result of an interaction between a toxin and a biological system that causes

harm to a living thing (CCOHS, 2020). Test animals are given varying dosages of the active ingredient in a pesticide as well as each of its formulated products in order to determine the toxicity of that particular pesticide. The chemical element of the pesticide that kills the pest is known as the active ingredient (NPIC, 2019).

CPF is exposed to the environment due to its application as insecticide. According to Barr (2006), the insecticide is moderately persistent in soils. The half-life is usually between 60 to 120 days and can range from two weeks to one year depends on the soil type, climate and other conditions. Since CPF is not soluble in water, it is unlikely for it to contaminate groundwater (Barr, 2006). Research suggest that this compound is unstable in water. In water, pH7 and at 25 °C, it had half-life of 35 to 78 days (Aldridge et al., 2003; Shemer et al., 2005; Slotkin & Seidler, 2007).

As mentioned earlier, CPF is used to kill broad range of insects and mites, but it is also toxic to humans and living organisms. The acute oral LD50 values indicate that CPF is toxic to small mammals. For example, the oral LD50 of CPF in rat is 95 to 270 mg/kg, 60 mg/kg in mice, 1000 mg/kg in rabbits, 32 mg/kg in chickens, 504 mg/kg in guine pigs and 800 mg/kg in sheep (Aldrige et al., 2003; Vidair, 2004; Ricceri et al., 2006). CPF is also very highly toxic to freshwater fish, aquatic invertebrates and estuarine and marine organisms (Colborn et al., 2006; Eskenzi et al., 2008). CPF can bioaccumulate in fish and other aquatic organisms and enter the aquatic food web. It was stated that, due to its high toxicity in water and its persistent in sediments, CPF can represent a hazard to aquatic organisms (Salyha, 2010).

CPF also has high systemic toxicity in neonates compared to adults (Pope et al., 1991; Pope & Chakraborti, 1992; Meyer et al., 2003). Although CPF known to be moderately toxic

to humans, it was reported that this organophosphate pesticide has causes thousands of deaths per year worldwide (Garcia et al., 2001; Slotkin, 2004; Eyer et al., 2009). It can absorb to bloodstream through gastrointestinal tract via ingestion, through respiratory system and dermal exposure. In humans, CPF and its metabolites can be eliminated rapidly. After a single oral dose, it was reported that the CPF half –life in the blood is about one day. Besides an irritant to skin and eye, the CPF poisoning may affect the central nervous system, cardiovascular system and respiratory system (Dam et al., 2000; Garcia et al., 2002; Garcia et al., 2003).

Numbness, tingling sensations, incoordination, headache, dizziness, tremor, nausea, abdominal cramps, sweating, blurred vision, difficulty breathing, and slow heartbeat are the symptoms of CPF poisoning. Similar symptoms can be observed when children accidentally put CPF contaminated food or objects into their mouth. High doses of CPF may cause unconsciousness, incontinence, convulsions and eventually death. In addition, similar symptoms can be experienced after prolonged and repeated CPF exposure. Other symptoms that can be experienced after CPF exposure include; impaired memory and concentration, disorientation, severe depressions, irritability, confusion, headache, speech difficulty, nightmares and insomnia (Mense et al., 2006; Ricceri et al., 2006; Bjørling-Poulsen et al., 2008).

2.6.3 Anticoagulant rodenticides: Coumarin

Rodenticides are products used to control rodents such as rats and mice. Anticoagulant rodenticides (AR) are the most widely used rodenticides and also referred as Anti-Vitamin K's (AVKs). The AR inhibits the metabolic cycle of vitamin K, which causes

the interferences with biosynthesis of vitamin K-dependent coagulant factors in the liver which are important in coagulation process (NPIC, 2016).

The control and management of rodents are important for public health and hygiene, as rodents are able to carry diseases, caused food contamination and also damage to buildings. The use of rodenticide is one of popular choice in managing rodents' infestation besides using biological control. However, there is a potential risk to people and animals from primary and secondary poisoning and as well to the environment (NPIC, 2016).

Besides harming the buildings and food, rats also known to destroy agricultural crops. Crops such as cereals (rice, wheat, maize, millet, barley and sorghum), roots, fruit, legumes and vegetables are particularly susceptible to rodent attack. Besides that, industrial scale plantation such as sugarcane, coconut, cocoa and oil palm also suffered from the rodent infestation. This caused significant loses to the farmers (Buckle, 1999).

Coumarin derivatives can be grouped into two generations. First generation are warfarin and coumachlor and the second generation include, brodifacoum and flocoumafen which characterised by their long period of biological half-lives (100-200) days (van Sittert & Tuinman, 1994). Coumarin is water insoluble, however, 4-hydroxy substitution confers weakly acidic properties to the molecule that make it water soluble under slightly alkaline conditions (Desai et al., 2000). The coumarin derivatives include; coumarin, 4-hydroxycoumarin, warfarin and dicoumarol (Figure 2.30). Warfarin is marketed as the sodium salt. However, the commercial warfarin is a racemic mixture.

Coumarins are competitive inhibitors of vitamin K in the biosynthesis of prothrombin. The coagulation cascade which involves the conversion of prothrombin to

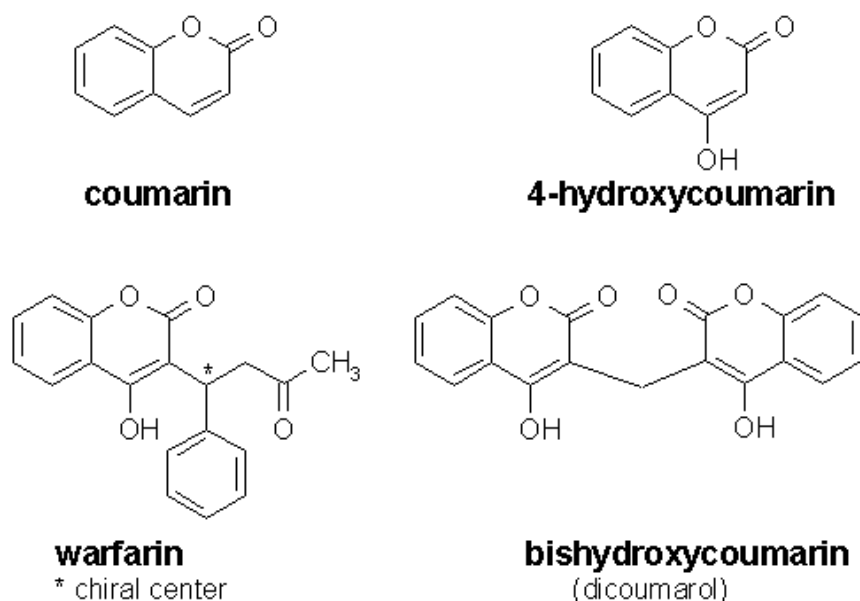


Figure 2.30: Chemical structure of coumarin and its derivatives (Desai et al., 2000).

thrombin in a very important step (Figure 2.31). However, this conversion depends on the presence of 10 γ - carboxyglutamic acid (Gla) residues in the N-terminus of prothrombin. Several Gla residues come together to form a binding site for Ca^{+2} .

Several Gla residues come together to form a binding site for Ca^{+2} . Prothrombin's glutamic acid (Glu) residues are typically changed to Gla residues through a post-translational process. Vitamin K epoxide reductase and vitamin K reductase are the enzymes that catalyze this post-translational modification. Co-factors in this conversion process include vitamin K. As a result, it alternates between an epoxide form and a reduced form. Coumarins are believed to bind to vitamin K reductase and vitamin K epoxide reductase without aiding in the conversion of prothrombin's Glu residues to Gla because of their structural resemblance to vitamin K. Factor Xa cannot therefore act on prothrombin (Desai et al., 2000).

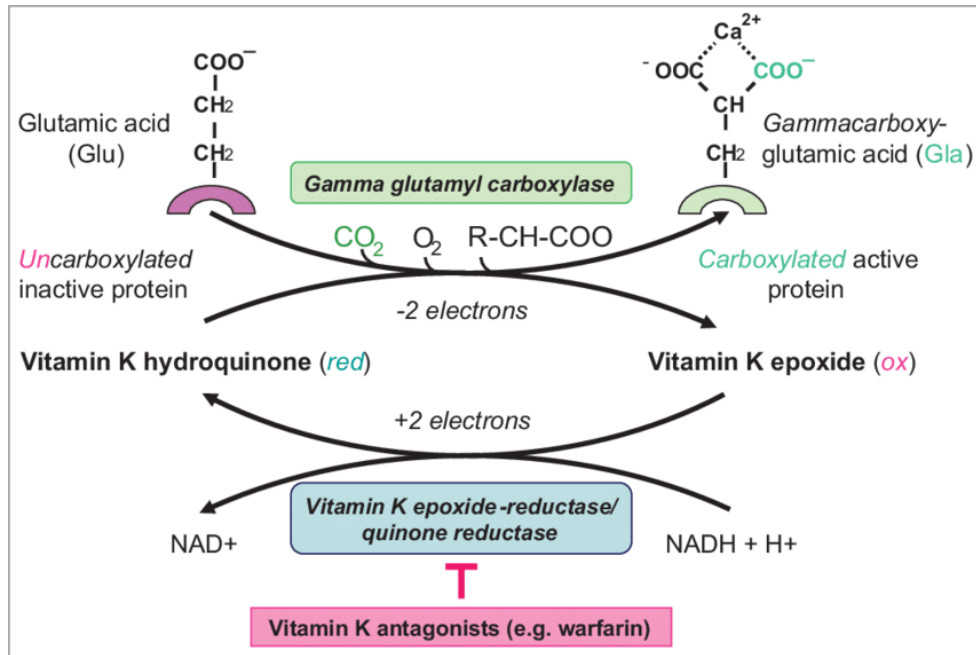


Figure 2.31: Coagulation cascade interrupted by vitamin K antagonist (Gröber et al., 2014).

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Study area

For the experiment to study the effect of pesticides on fly development, the blowfly collection was conducted in Kuching, Sarawak which explained in detailed in Chapter 4 (section 4.3.1, pg 86). Meanwhile, field experiment involving decomposing pesticide-treated carcasses and insect activities was carried out at Kg Sri Kandong, Kuching, Sarawak. The details on the location were described in Chapter 5 (section 5.2.1, pg 105).

3.2 Insect collection and identification

3.2.1 Adult insect collection and identification

The insects in adult stage and immature stage at the study site were monitored and collected for preservation and identification. The adult insects that were attracted to the carcasses were caught using a sweep net and hanging net, while the insects that were crawling on the ground were collected using forcep or hands. The adult insects were then killed in a killing jar. Killing jar consists of round, glass jar containing cotton balls that have been soaked with ethyl acetate (Sigma, USA). Ethyl acetate was used as it can retain the morphology of the insects.

The killed insects were then pin using an insect pin (BioQuip, USA) and stored into the insect box for species identification later. The species were identified based on key identification by Clearwater (1981), Nazni et al. (2011), Creedy & Mann (2012) and Nemes & Price (2015). The identified insects were labelled accordingly following the labelling standard. A pinning block is used to adjust the specimens and labels on pin.

3.2.2 Larvae collection and preservation

The larvae infesting the carcass body were collected by handpick and forceps. The larvae collected were divided into three groups. The first group was killed in hot water (80 °C) and then put into universal bottle containing 70% ethanol for preservation. Meanwhile the second group was reared into adult for species confirmation and the third group of larvae was killed in hot water, then kept frozen in -40°C freezer for subsequent toxicological analysis.

3.2.2.1 Species identification of preserved insect larvae

The larvae that were preserved in 70% ethanol underwent a series of preservation and mounting process for species identification. The procedure was following the method by Heo et al. (2015). The process was started by removing the internal organs of individual larvae which then left with its outer skin. The larvae were then soaked with 10% of potassium hydroxide (KOH) (Merck, USA) for 24 to 48 hours until all the tissues left inside the skin fully digested by KOH. Then, the larvae were washed with distilled water before they were soaked with 10% of acetic acid for 30 minutes. The larvae were subsequently washed again with distilled water before soaking in a series of ethanol (HmbG, Malaysia) concentrations i.e., 30%, 50%, 70% and 90% for 30 minutes respectively, in ascending order. Then, the larvae were soaked in 100% ethanol for one hour with two changes of the ethanol.

After one hour, the ethanol was removed and clove oil (Fisher chemical, UK) was added until the larvae submerged. The larvae were soaked into the clove oil for 30 minutes. Then, each larva was put on a clean glass slide. The larva was washed with xylene (Sigma, USA), and the excess xylene was blotted with filter paper. Two drops of Canada balsam

(Sigma Aldrich, USA) were added onto slide until covering the whole larva and covered with a cover slip.

Then, the prepared slide was let air dried at room temperature. When the slide has dried, the larvae were observed under a compound microscope (Optika, Italy). The characteristics of the larvae were identified and photographed using a smartphone camera (Leica optics). Identification of larvae were done morphologically based on Szpila (2009) and Akbarzadeh et al. (2015) and were recorded. The process of larvae preservation is shown in Figure 3.1.

3.2.2.2 Larvae reared for species identification

Another group of larvae collected were put into a one-liter beaker based on their physical appearance and site of collection. The larvae were fed with minced meat and the container was added with sawdust to replace soil. The sawdust was used as a medium for the larvae to pupate. The larvae development was monitored until it emerged into adult. The emerged adult fly was pinned and identify for their species.

3.2.2.3 Larvae for toxicological analysis

The sample preparation procedure for toxicological analysis was described in detail in Chapter 6, section 6.2.3.3 (pg. 151) and section 6.2.4.1 (pg. 154).

3.3 Pesticides preparation

There are three types of pesticides used in this study which are, chlorpyrifos (KENSBAN 400®, registration number: LRMP.R1/4644), glyphosate (Asset®, registration number: LRMP.R1/3106) and rodenticide coumarin. The pesticides for each category were chose as it is the most commonly used pesticides and can be purchased at a local agriculture shop except for coumarin.

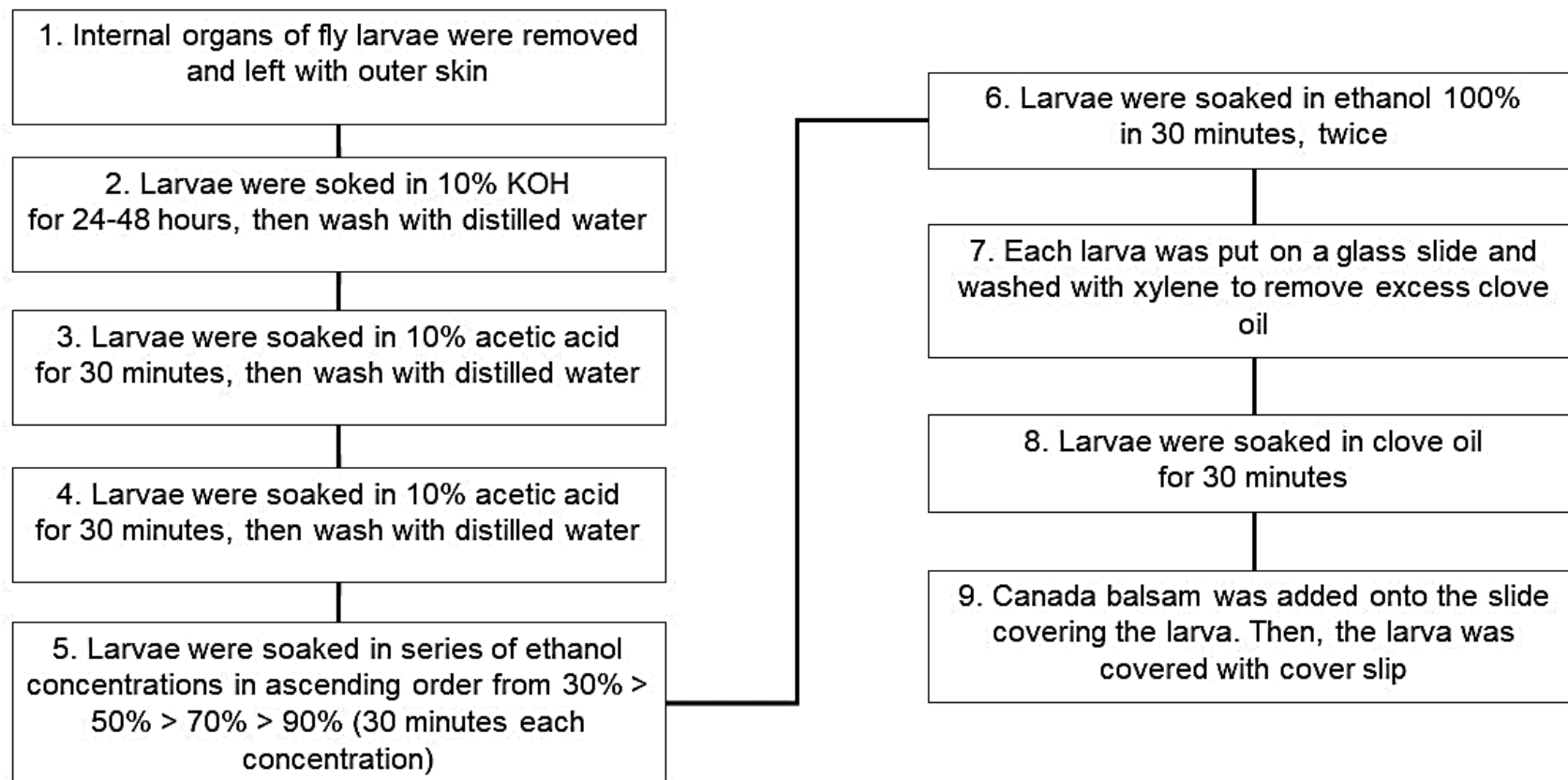


Figure 3.1: Larvae preservation and mounting procedure.

The coumarin used in this study is a laboratory grade coumarin ($\geq 99\%$ purity), purchased from Sigma-Aldrich (China). The pesticides were diluted accordingly with distilled water for subsequent experiment which was explained in detail in chapter 4 (section 4.3.2, pg. 86) and chapter 5 (section 5.2.2, pg. 106).

3.4 Ambient temperature, relative humidity and rainfall data collection

The ambient temperature, relative humidity and rainfall at the study site were provided by Malaysia Meteorology Department (MET) throughout the study period.

3.5 Data analysis

All statistical testing was carried out using Statistical Package for the Social Sciences (SPSS) software ver. 24. The comparison of more than two independent variable were analysed using the analysis of variance (One-way ANOVA) and post hoc test. The entomological data were tabulated in a table using Microsoft Office ver. 2020. Data analyses methods were described in detail in Chapter 4 (section 4.2.4., pg. 86) and Chapter 5 (section 5.2.6., pg. 109).

CHAPTER 4

EFFECT OF PESTICIDES ON THE DEVELOPMENT OF *Chrysomya megacephala*

4.1 Introduction

Blowflies are the most important flies to estimate the post-mortem interval (PMI) time as they are among the earliest fly that locate the dead body remains (Byrd & Tomberlin, 2019). The estimation of PMI is largely based on the state of decomposition of the corpse at the time of discovery. In forensic entomology, utilising the insects to estimate PMI is based on two methods. The first one is by the analysis of succession pattern of the colonising insects and other arthropods, and the second method based on the development of the immatures that are deposited onto the corpse. The use of these approach is greatly influenced by external factors such as the weather, location, and the treatment of the corpse (Verma & Paul, 2013).

Entomotoxicology is a relatively new field utilising insects in toxicological analysis to detect poisons or drugs in decomposing tissue (Gautam et al., 2013). The insects as they feed on the decomposed tissue, they also accumulate drugs and metabolites from the corpses into their own body. The use of insects in toxicological analysis is an alternative when conventional samples such as blood, urine or tissues are unavailable (Gautam et al., 2013). The research scope of entomotoxicology not only limited to the study of analytic procedures for detecting certain chemical substances; the effects of these substances on the insects' development and arrival on corpses are also among the crucial studies that have been conducted and reported worldwide (Mahat et al., 2009; George et al., 2009; Abd El-Bar et al., 2011; Jales et al., 2020; Abd Al Galil et al., 2021).

The presence of toxins or chemical contaminants in carrion has been demonstrated to influence the arrival and development rate of the insects, especially flies as their immatures are directly feeding on the decay tissues, in which is crucial for PMI estimation (Verma & Paul, 2013). In 1992, Leclecq and Vaillant reported that the presence of lead arsenate has changed the colonisation of necrophagous flies and thus altered the PMI estimation. Earlier in the 80s, the presence of gas and paint can also disrupt the arrival and larval development of blowflies (Marchenko, 1993). Besides that, the household products were proven to delay the flies' appearance on carrion as the products repelled adult flies (Charabidze et al., 2009) and delay the development time of the blowflies (Aubernon et al., 2015b). Hence, the toxic substances on a carrion are a potential factor in disturbing both fly arrival and development.

Chrysomya megacephala is a widely distributed blowfly (Diptera: Calliphoridae) and has a significant importance in public health, food industries and forensic entomology. Large populations of *Ch. megacephala* inhabit human settlements (Monzon et al., 1991). The adults can be seen visits the food waste and decaying carrion and faeces where they usually breed abundantly (Badenhorst & Villet, 2018). The larvae were then feed on the decaying materials which also include animal and human corpses. Therefore, this species is beneficial and have been used in medico-legal entomology field. *Chrysomya megacephala* also considers the predominant blowflies in Thailand and Malaysia (Sukontason et al., 2007; Abdullah & Zuha, 2017).

The *Ch. megacephala* undergoes a complete metamorphosis which consist of egg, larva, pupa and adult stages (Gerhardt & Hribar, 2019). Upon locating a suitable substrate for oviposition (e.g., animal carrion or corpse), the female *Ch. megacephala* deposit eggs

onto the substrate and then the larvae feed on and colonising the decaying tissue (Anderson & VanLaerhoven, 1996). During the feeding stage, the larvae develop into three stages known as the 1st, 2nd, and 3rd instar until they eventually cease to feed when a maximum larval size is obtained. Then, they crawling away from the remains searching a suitable place for them to pupate. Later, the adult flies were emerged from the pupae after several days (Gerhardt & Hribar, 2019).

The study of certain chemical substances on the development of blowflies have been carried out worldwide to provide a baseline data on the effect of a commonly used pesticides or drugs on the development of forensically important blowflies (Charabidze et al., 2009; Aubernon et al., 2015b; Ekrakene & Odo, 2017; Essarrass et al., 2018; Denis et al., 2018; Maddheshiya & Singh, 2021). A study by Liu et al. (2009), feeding on *Ch. megacephala* larvae treated with malathion has decreased its growth rate. The size of the larvae was smaller than the average size. It was also reported that, larvae collected from liver and muscle alter the PMI time by 36 hours and 28 hours respectively. On the other hand, other drug, metamphetamine has shown to speed up the larvae development and change the PMI time by 18 hours (Goff et al., 1992). There are more studies showed the effect of toxins and drugs on flies' growth, hence altered the estimated PMI time (Kharbouche et al., 2008; Oliver et al., 2009). Therefore, the presence of drugs and toxins in insects' samples should be considered when estimating the PMI time. Therefore, this study aims to determine the effects of three pesticides; glyphosate, chlorpyrifos and coumarin on *Ch. megacephala* development in-vitro and to verify the alterations occurred that could lead to the error in PMI estimation time where the pesticides involvement is suspected. Based on previous study, it was expected that there is a significant difference in developmental rate and duration of *Ch. megacephala* treated with pesticides compared to the control group.

4.2 Materials and methods

4.2.1 Flies' collection

The blowfly was collected at Kg. Semerah Padi, Petra Jaya, Kuching (1°35'12.1" N, 110°19'07.6" E) (Figure 4.1). The blowfly adult was trapped following method by Jales et al. (2020) with modifications. The rotten minced meat (beef) was used as a bait to attract adult *Ch. megacephala*. The minced beef was purchased from a local groceries store.

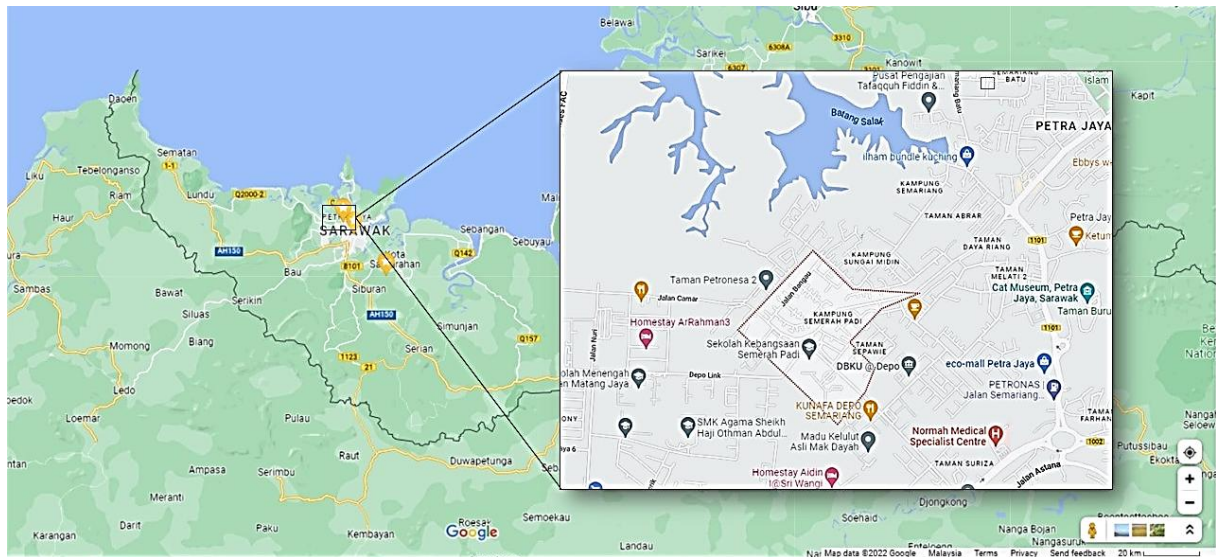


Figure 4.1: Map showing fly trapping site at Kampung Semerah Padi, Kuching Sarawak (1°35'12.1" N, 110°19'07.6" E). Map courtesy of Google Map 2022.

The rotten minced meat was put inside a container and then, the container was placed at a shaded area exposed to the outside environment. Then, after 30 minutes, the *Ch. megacephala* flies on the rotten meat were trapped with another container upside down, and then the container was covered with lid immediately. The lid was punctured with small holes for flies' respiration (Figure 4.2). The fly trapping was carried out for at least five times in 30-60 minutes' interval. Then, the trapped flies were brought to the lab and put into insect cages (30 x 30 x 30 cm) for subsequent experiment.

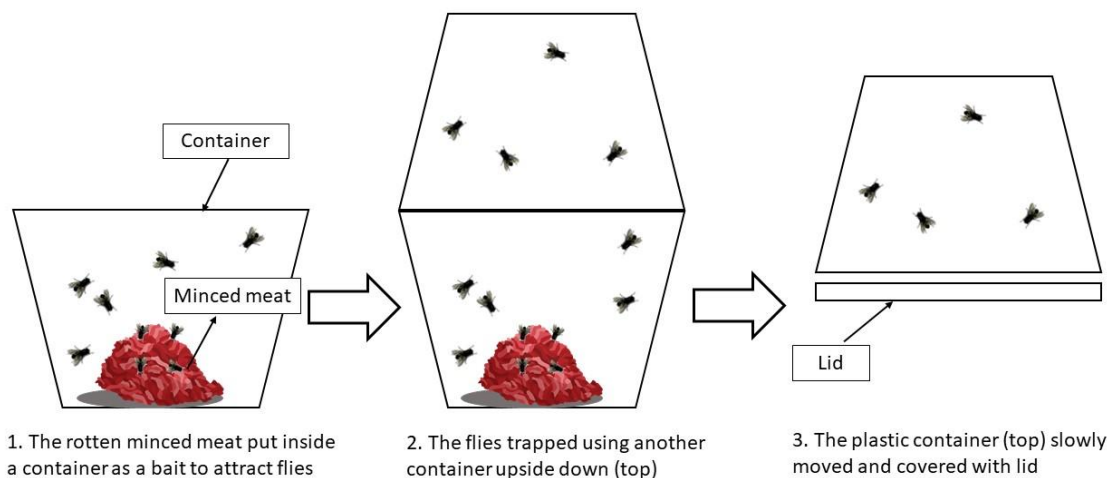


Figure 4.2: The method for trapping blowflies. Minced meat was used as a bait.

4.2.2 Preparation of pesticides and meat substrate

Three types of pesticides, glyphosate, chlorpyrifos and coumarin (as described in Chapter 3, section 3.2, pg. 77) was diluted into three concentrations with distilled water. The pesticides concentration was determined based on preliminary experiments that were conducted previously. Three pesticide concentrations (T1 [low], T2 [medium] and T3 [high]) were chosen as shown in Table 4.1. Then, 5 ml of diluted pesticides were mixed with 50 g of rotten minced meat and mixed thoroughly by hand manipulation for five minutes (Mahat et al., 2014).

Table 4.1: Concentration of each pesticides mixed with minced meat.

Type of pesticides	Concentration of pesticides			Minced meat (g)
	T1	T2	T3	
Glyphosate	4 $\mu\text{l/ml}$	40 $\mu\text{l/ml}$	400 $\mu\text{l/ml}$	50
Chlorpyrifos	0.001 $\mu\text{l/ml}$	0.005 $\mu\text{l/ml}$	0.01 $\mu\text{l/ml}$	50
Coumarin	1 mg/ml	10 mg/ml	100 mg/ml	50

4.2.3 Experimental design

The trapped adults *Ch. megacephala* were put into four insect cages. Each cage contained at least a pair of male and female adults and provided with 10% sugar solution soaked into a cotton to sustain their life. Then, a mixture of minced meat and pesticide solutions was put into a petri and into each insect cage to initiate oviposition. After 24 hours, the treated meat inside the cages were checked for the presence of eggs. Once the eggs were observed on the petri dish, the eggs were transferred to a beaker and hatched larvae were reared until adult (as described in Chapter 3, section 3.2.2.2, pg. 76).

The immatures were checked for every eight hours for their development. During each observation, three larvae were taken in each pesticide concentration and killed to observe for their larvae developmental stages. The larvae developmental stages were determined by the number of slits on their posterior spiracles and also by measuring the length of the larvae. Beside the developmental stages, the duration of each stage to complete were noted.

The abnormality in terms of morphology of the insects and its ability to emerge into adult was also observed in this experiment. These procedures were following procedures described by Abd Al Galil et al. (2021) with modifications and is summarised in Figure 4.3. The temperature and relative humidity in the laboratory were recorded using data logger (Extech Instrument, China). Each experiment was repeated in three trials of experiments.

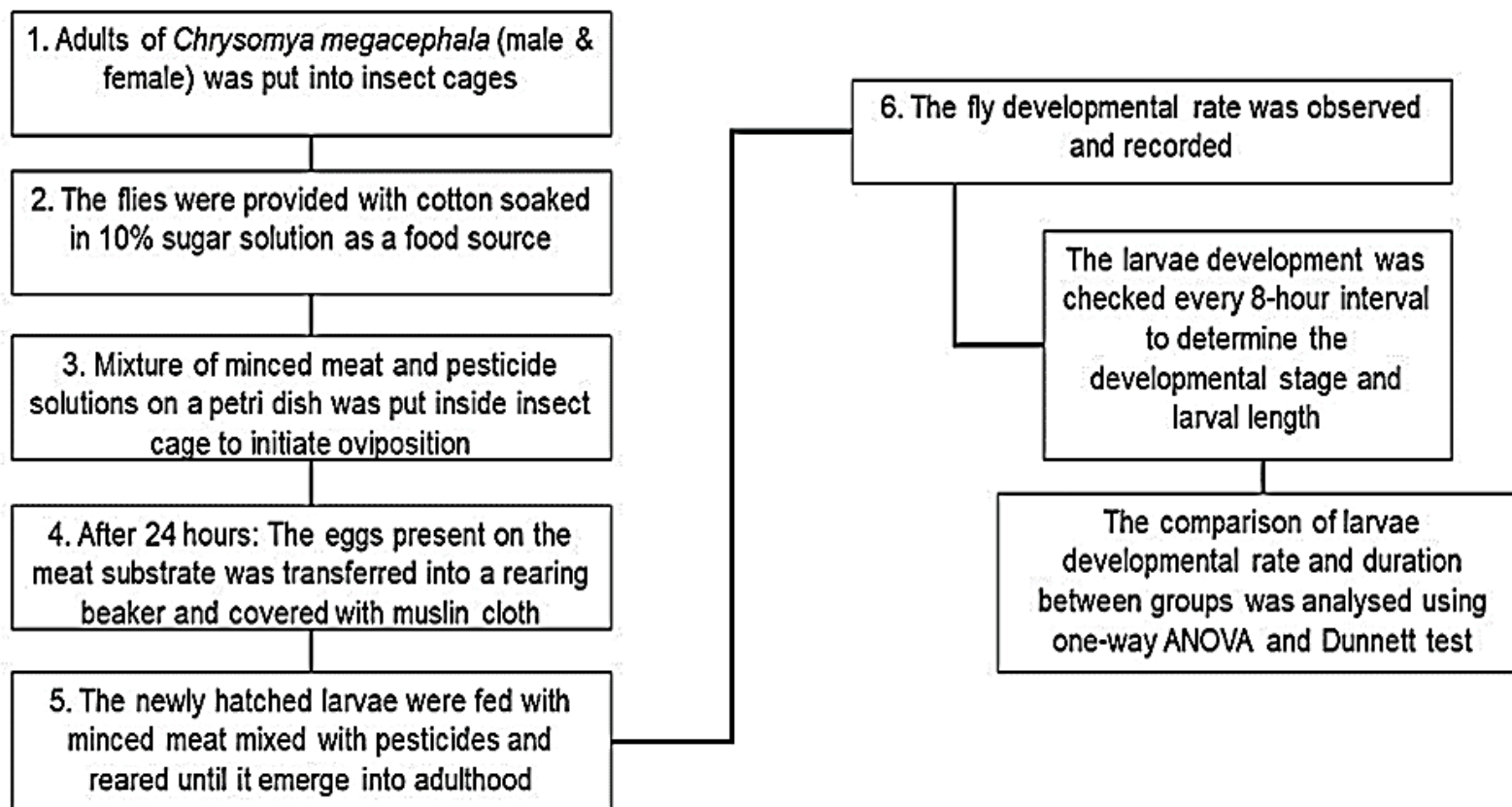


Figure 4.3: Flow chart showing the procedure to study the effect of pesticides on *Chrysomya megacephala* development.

4.2.4 Data analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software ver. 24. One-way ANOVA analysis was performed to compare the difference of larvae growth and development duration between control and pesticide-treated groups with significant level 0.05. Multiple comparison analysis (post-hoc: Dunnet test) was conducted to determine which treatment group that was significantly different from control group. The data was also tabulated into tables and growth curve was plotted using Microsoft office 2022.

4.3 Results

4.3.1 Indoor environment parameters

The collection of flies was conducted in the morning from 7 to 10 am. The mean ambient temperature was 27.6 ± 0.6 °C the mean relative humidity was $76.5 \pm 3.8\%$. The environmental data was provided by MET Malaysia.

Fly development study was carried out indoor. The experiment room was located inside an insectarium provided with air conditioner that set on 25 °C from 8 am to 5 pm every day. The mean indoor temperature inside the experiment room was 24.04 ± 0.46 °C (Mean $\pm$ S.D.), ranged from 22.3 to 25.1 °C. The mean relative humidity was 73.94 ± 0.81 %, and ranged from 55.6 to 94.6%. Figure 4.4 shows the trend of the temperatures and relative humidity in 100 days of the experiments were conducted.

4.3.2 Morphology of *Chrysomya megacephala*

The eggs were deposited in groups of about ~100 eggs. The egg was elongated, sausage-like shaped with a tapered end and has a creamy white colour. The eggs were measured 1.36 ± 0.06 mm. The eggs hatched in 24 hours into 1st instar larvae.

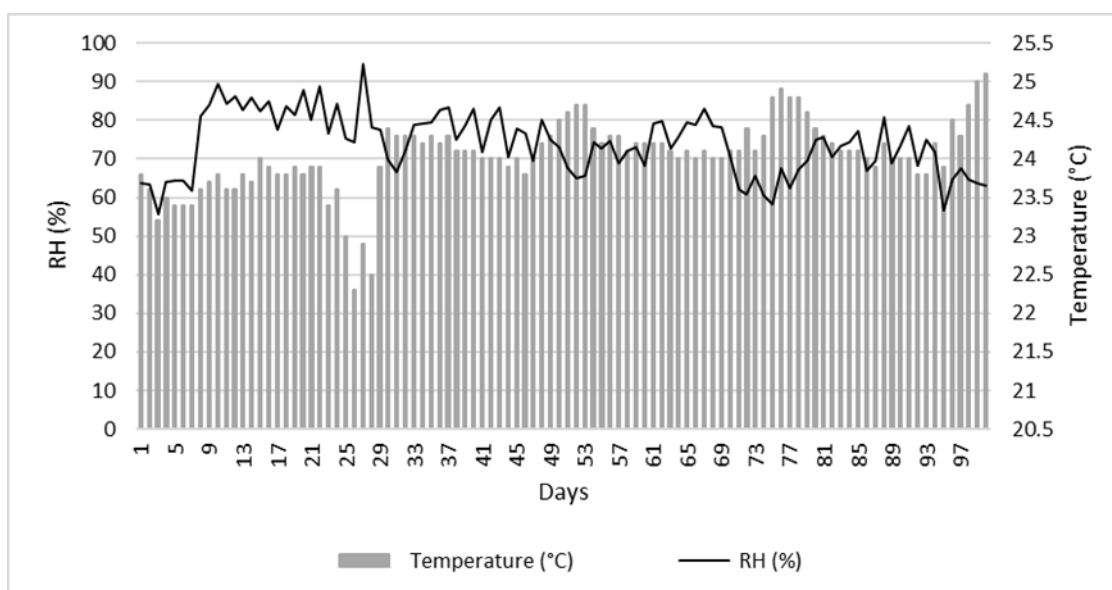


Figure 4.4: Indoor temperatures and relative humidity recorded in the laboratory over study period.

The 1st instar larvae have a whitish colour and the body is transparent, where the internal organs are apparent. The larvae have a single slit at the posterior spiracle. Then, the 1st instar larvae moulted into 2nd and 3rd instar which determined by the number of slits on their posterior spiracle (Figure 4.5). After 2-3 days, the 3rd instar larvae body contract rapidly just before pupariation. The puparium formed from the exoskeleton of the third instar larva. The mouth hooks adhering inside of the enclosed puparium. The colour of the pupae was creamy in colour during the first day of pupation and turning reddish brown and darker in several days (Figure 4.6). After 5-6 days, the adult *Ch. megacephala* emerged from the pupa casing. The adult is metallic blue-green colour and has a body length ranged from 8-11 mm. At the head area, the gena and post gena are orange-yellow in colour. The eye facet has a large upper eye facet (2/3) and small lower eye facet (1/3). The eyes of adult male are closed together, while far apart for female adult (Figure 4.7).

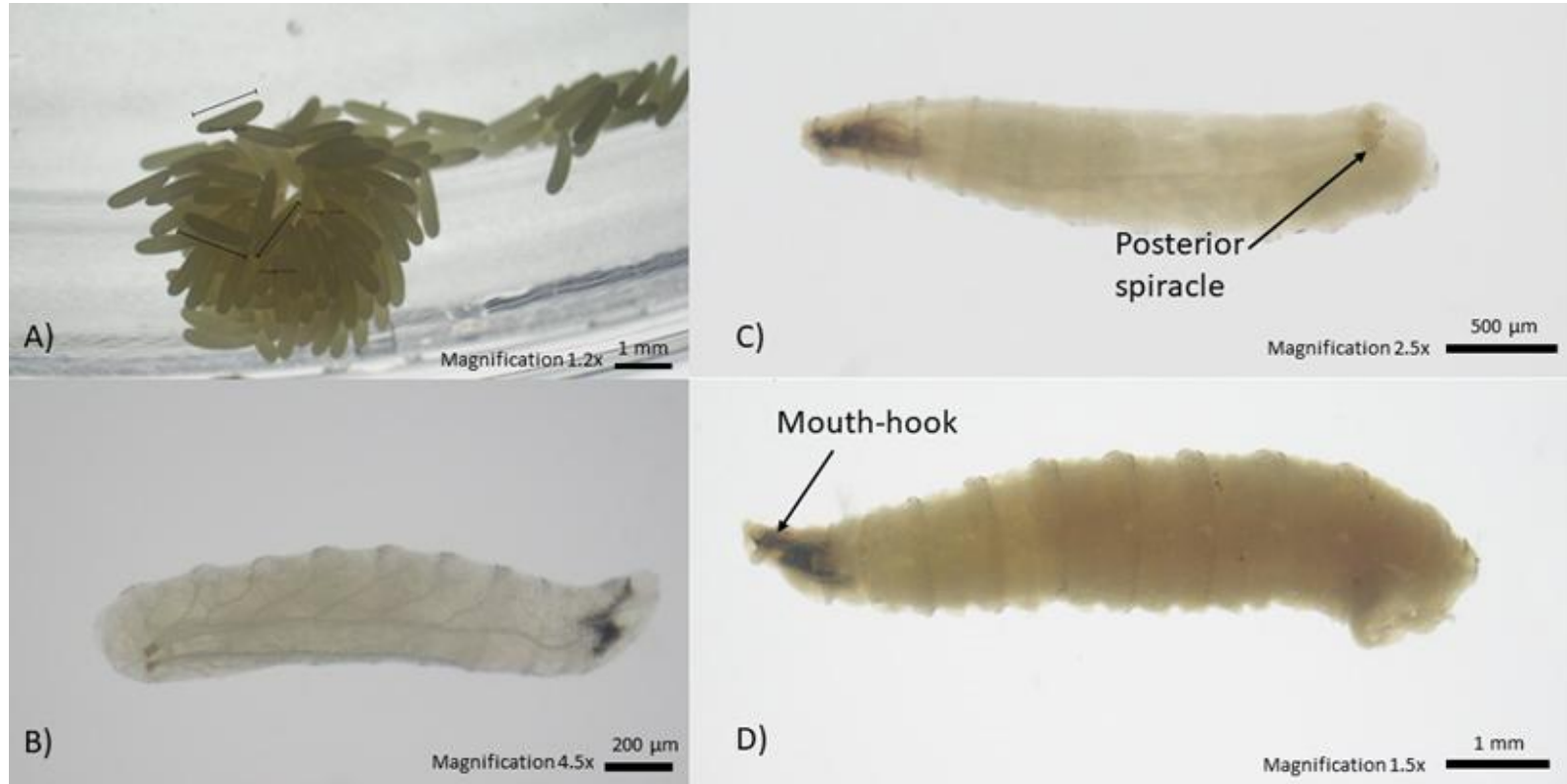


Figure 4.5: Immature stages of *Chrysomya megacephala*. (A) Eggs, (B) 1st instar larva, (C) 2nd instar larva, and (D) 3rd instar larva.



Figure 4.6: Pupae of *Chrysomya megacephala* showing the colour became darker towards the end of the pupation time (Scale bar= 1 mm).

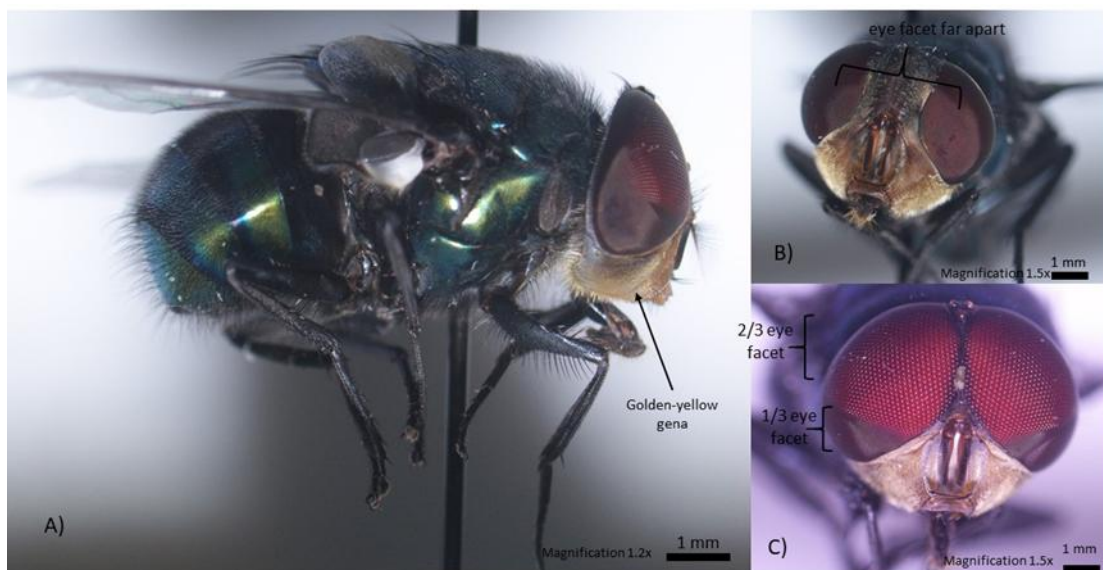


Figure 4.7: Figure shows the (A) lateral view of adult of *Chrysomya megacephala* which characterised by golden-yellow gena. Male and female of *Ch. megacephala* was differentiated by the characteristic of eye facet, where in (B) female the eye facet was far apart, meanwhile in (C) male, the eye facet was closed together. Figure (C) also shows differentiated sizes of upper and lower facets of the male's eyes (Scale bar= 1 mm).

4.3.3 Effect of glyphosate on *Chrysomya megacephala* development

4.3.3.1 Effect of glyphosate on development duration

The blowfly, *Ch. megacephala* oviposit 24 hours after the meat substrate introduced into the cage. The eggs took about 24 hours of incubation to hatch the 1st instar larvae. After the hatching, the larvae undergo three stages of development which are the first stage (L1), second stage (L2) and third stage larvae (L3). In this experiment, the average duration for the L1 larvae to moult into L2 stage was constant at 16 hours for control and T1 group. Longer time needed for L1 larvae fed with higher concentration (group T2 and T3) of glyphosate to develop into L2 stage which took 21.3 ± 5.3 and 48.0 ± 0.0 hours respectively.

The L2 larvae then develop into L3 stage in 24.0 hours for control group, and 16 hours for T1 and T2 larvae. The third stage larvae in glyphosate-treated group took longer time (61.3 ± 4.6 hours) to develop into pupal stage compared to the control group (53.3 ± 14.1 hours). The duration for the adult to emerge from the pupa stage was similar which took about 5 days in average. The total development duration of *Ch. megacephala* since the eggs were laid was 245.3 ± 14.8 hours for control group and longer for treated group. The longest time was in group T2 which took about 264.0 ± 24.4 hours (Table 4.2).

One-way ANOVA analysis performed shows there is a significant difference of the *Ch. megacephala* total development duration between control, and glyphosate-treated groups [$F(3,8) = 155.15$, $P < 0.05$] (Appendix A). Multiple comparison analysis, Dunnett t test shows there is a significant difference in development duration of T2 and T3 larvae compared to the control group.

Table 4.2: Average development duration in each stages of *Ch. megacephala* in control and glyphosate-treated groups from three trials of experiment.

Developmental stages	Development duration (Hours $\pm$ S.D.)			
	Control	T1	T2	T3
Eggs	24.0 $\pm$ 0.0	24.0 $\pm$ 0.0	24.0 $\pm$ 0.0	24.0 $\pm$ 0.0
L1	16.0 $\pm$ 0.0	16.0 $\pm$ 0.0	21.3 $\pm$ 5.3	48.0 $\pm$ 0.0
Larvae L2	24.0 $\pm$ 0.0	16.0 $\pm$ 0.0	16.0 $\pm$ 0.0	-
L3	53.3 $\pm$ 14.1	61.3 $\pm$ 4.6	74.7 $\pm$ 11.6	-
Pupa	128.0 $\pm$ 8.0	136.0 $\pm$ 13.9	128.0 $\pm$ 8.0	-
Total duration (hours)	245.3 $\pm$ 14.8	253.3 $\pm$ 10.7	264.0 $\pm$ 24.4	72.0 $\pm$ 0.0

4.3.3.2 Effect of glyphosate on the larvae rate of development

The larvae rate of development was studied by measuring their length (mm) during the feeding stage in eight hours' interval. The growth curve of *Ch. megacephala* larval stage showed a sigmoid form (Figure 4.8). Based on the growth curve, the larval length in control group was developed from 2.30 ± 0.09 to 15.99 ± 0.70 mm. Meanwhile, the larvae in glyphosate treated groups have recorded a lower larval length measurement in which the maximum larval length recorded was 12.71 ± 0.25 and 14.10 ± 0.97 mm for T1 and T2 group respectively. One-way ANOVA analysis performed on the larvae measurement for all groups (except T3) shows there is no significant difference in the length of the larvae feeding the glyphosate-treated substrate and the control group [$F(2,32) = 0.304$, $P > 0.05$] (Appendix B).

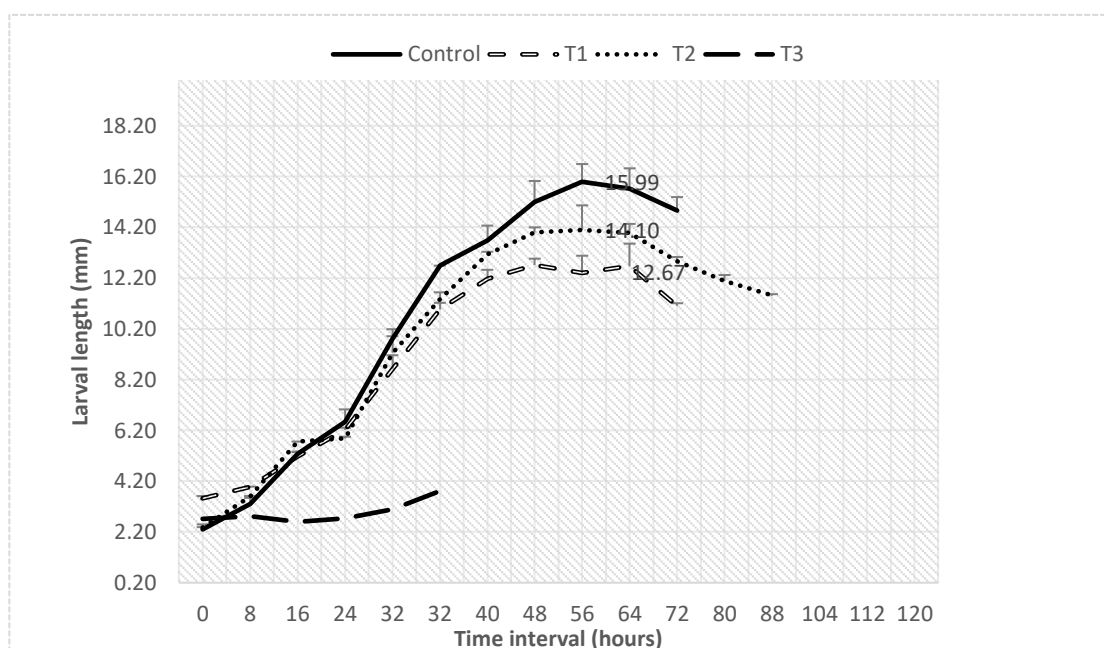


Figure 4.8: Figure shows the larval length in eight-hour interval. A rapid growth of larvae was observed where delayed larvae development was observed in glyphosate-treated larvae.

4.3.4 Effect of chlorpyrifos on the development of *Chrysomya megacephala*

4.3.4.1 Effect of chlorpyrifos on development duration

The eggs and the first instar larvae of *Chrysomya megacephala* did not survive when placed on meat substrate that was mixed with chlorpyrifos. The eggs did not hatch, and the first stage larvae died after 24 hours of observation. The experiment was repeated again by placing the second instar larvae onto the meat substrate mixed with chlorpyrifos.

The result shows that at a lower concentration, T1 (0.001 μ l) and T2 (0.005 μ l), the larvae were able to develop into third stage larvae, pupae and emerge into adult. However, at higher concentration of chlorpyrifos, the larvae were only surviving during the third stage and died after four to five days, and unable to pupate. Based on the result in Table 4.3, it was shown that the average development duration from the L2 to L3 larvae was 16.0 ± 0.0 hours for chlorpyrifos-treated larvae, meanwhile it took 10.67 ± 4.6 control group.

The development duration of L3 larvae was the shortest in control and T1 (48.0 ± 0.0 hours) and the longest in group T3 which took about 112 ± 0.0 hours before they died and unable to pupate. The adult flies successfully emerged from puparial stage from control, T1 and T2 group after 5 to 7 days. The one-way ANOVA analysis to compare the development duration between control and chlorpyrifos treated groups showed there is a statistically difference between development duration between chlorpyrifos-treated group compared to the control group [$F(3,8) = 144.8, P < 0.05$] (Appendix C). The multiple comparison Dunnett t test shows larvae in T3 group have shorter development duration which was caused by incomplete life cycle.

Table 4.3: Average development duration in each stages of *Ch. megacephala* in control and chlorpyrifos-treated groups from three trials of experiment.

Developmental stages		Development duration (Hours $\pm$ S.D.)			
		Control	T1	T2	T3
Larvae	L2	10.7 ± 4.6	16.0 ± 0.0	16.0 ± 0.0	16.0 ± 0.0
	L3	48.0 ± 0.0	48.0 ± 0.0	56.0 ± 0.0	112.0 ± 0.0
Pupa		168.0 ± 0.0	176.0 ± 13.9	144.0 ± 0.0	N/A
Total duration (hours)		226.7 ± 4.6	240.0 ± 13.9	216.0 ± 0.0	128.0 ± 0.0

4.3.4.2 Effect of chlorpyrifos on the larvae rate of development

The larvae development over time was studied by measuring their length (mm) during the feeding stage in eight hours' interval. The sigmoid curve was observed in Figure 4.9. The larvae were exposed to the insecticide at 16-hours. From the line graph, it can be observed that shorter length of larvae was observed in higher chlorpyrifos concentration (T3), where the maximum of larval length recorded in L3 larvae was 13.84 ± 0.43 , compared

with maximum L3 larvae length was 15.45 ± 1.41 in control group. The third stage of larvae development in T3 group was delayed and failed to develop to the next stage. The size of larvae in control, T1 and T2 group was similar. Statistical analysis has shown that there is no significant difference between the mean larval length from control and treated groups [$F(3,47) = 0.233, P > 0.05$] (Appendix D).

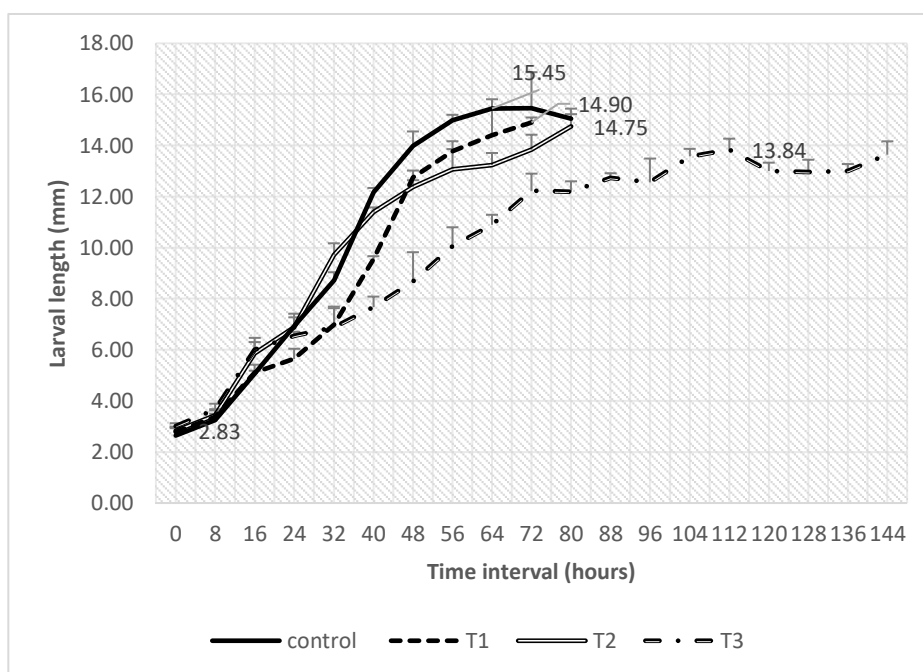


Figure 4.9: Figure shows the larval length in eight-hour interval. A rapid growth of larvae was observed in control group. Meanwhile, delayed larvae development and shorter larvae length was observed in chlorpyrifos-treated larvae.

4.3.5 Effect of coumarin on the development of *Chrysomya megacephala*

4.3.5.1 Effect of coumarin on development duration

The blowfly, *Ch. megacephala* oviposit 24 hours after the meat substrate introduced into the cage. The eggs took about 24 hours of incubation to hatch the 1st instar larvae. After the hatching, the larvae undergo three stages of development which are the first stage (L1), second stage (L2) and third stage larvae (L3). In this experiment, the average duration for the L1 larvae to develop into L3 stage was 69 to 72 hours for control, T1 and T2 group. On

the other hand, group T3 larvae took a longer time to develop especially from L2 to L3 stage, which took about 109.3 ± 4.6 hours.

The L3 stage larvae took about 48 ± 0.0 to 53.3 ± 4.6 hours to develop into pupa stage in all groups except the T3 group larvae which took about 170.67 ± 4.6 to pupate. On average, it took five to six days for the adult fly to emerge (control and T1), however, the pupae in T3 group of coumarin shows no sign of adult emergence until the end of the study (~ 30 days) (Table 4.4).

One-way ANOVA analysis performed shows there is a significant difference of the *Ch. megacephala* total development duration between control, and coumarin-treated groups [F (3,8) = 134.14, P<0.05] (Appendix E). Multiple comparison analysis, Dunnet test shows there is significant difference on the development duration of T2 and T3 larvae group as compared with the control group.

Table 4.4: Average development duration in each stages of *Ch. megacephala* in control and coumarin-treated groups from three trials of experiment.

Developmental stages		Development duration (Hours $\pm$ S.D.)			
		Control	T1	T2	T3
Eggs		24.0 ± 0.0	24.0 ± 0.0	24.0 ± 0.0	24.0 ± 0.0
	L1	16.0 ± 0.0	16.0 ± 0.0	13.3 ± 4.6	16.0 ± 0.0
Larvae	L2	32.0 ± 0.0	32.0 ± 0.0	32.0 ± 0.0	109.0 ± 4.6
	L3	53.3 ± 4.6	50.7 ± 4.6	48.0 ± 0.0	170.7 ± 4.6
Pupa		136.0 ± 13.9	160.0 ± 13.9	N/A	N/A
Total duration (PMI)		261.3 ± 18.5	282.7 ± 16.7	117.3 ± 4.6	320.0 ± 8.0

4.3.5.2 Effect of coumarin on the larvae length during feeding stage

The larvae development over time was studied by measuring their length (mm) during the feeding stage in eight hours' interval. The growth curve of *Ch. megacephala* larval stage showed a sigmoid form except for larvae growth in T3 (Figure 4.10). The slow progress in the development of T3 larvae can be observed through its growth curve whereby a little or no change of larval length observed over time until 144 hours.

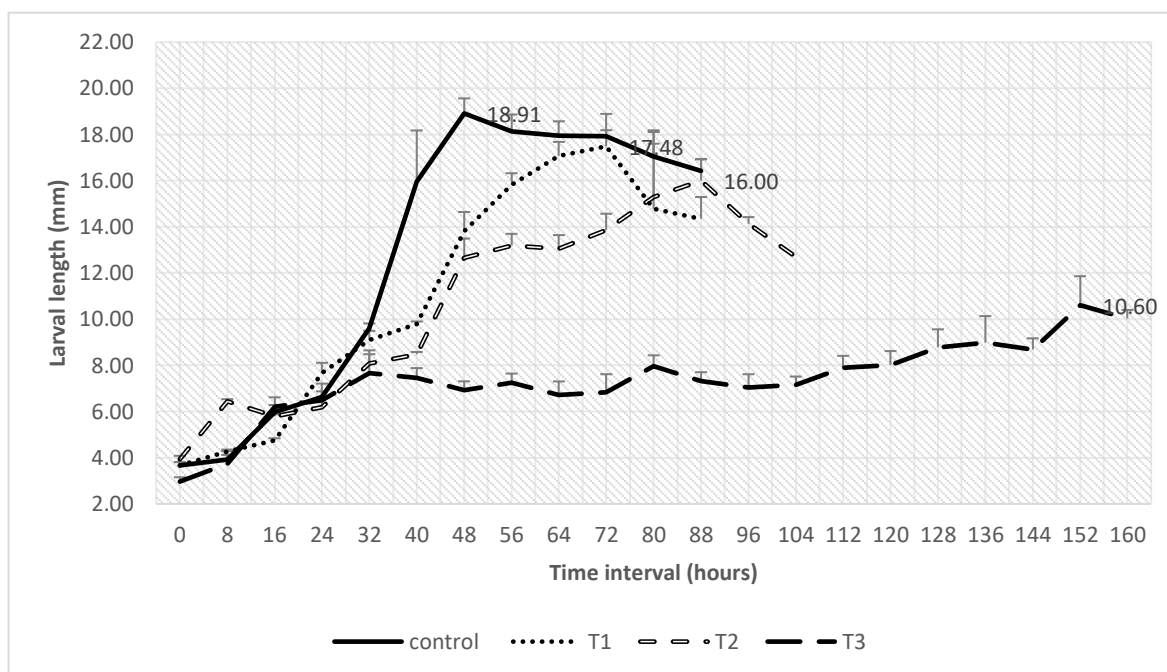


Figure 4.10: Figure shows the sigmoid growth curve of *Ch. megacephala* in control and coumarin treated groups in several concentration except for T3 larvae. The growth of larvae fed on the highest concentration of coumarin (T3) was decreased in size and the development was delayed significantly.

Based on the growth curve, the larval length in control group developed from 3.67 ± 0.14 to 18.91 ± 0.65 mm. Meanwhile, the larvae in coumarin treated groups have recorded a lower larval length measurement in which the maximum L3 stage larval length recorded was 17.48 ± 0.70 , 16.00 ± 0.94 and 10.60 ± 1.26 mm for T1, T2 and T3 group respectively.

One-way ANOVA analysis performed on the larvae measurement for all groups shows there is a significance difference of the larvae length between control and treated group during larvae development [$F(3,55) = 4.717$, $P < 0.05$] (Appendix F). Multiple comparison analysis, Dunnett test shows, the mean larvae length of T3 larvae is significantly different compared to larvae length of other groups. *Ch. Megacephala* development rate in all treated groups was summarised in Figure 4.11.

4.4 Discussion

The presence of pesticides in the meat substrate have altered the development rates of *Chrysomya megacephala* during the larvae stage until adulthood. Three concentrations of pesticides (glyphosate, chlorpyrifos and coumarin) were tested during the larvae stage where the larvae were actively feeding on the meat substrate. The experiments showed that there was a significant delay in the larvae development especially in higher concentration of pesticides. This is in agreement with the study conducted by Rashid et al. (2008) and Liu et al. (2009), where the *Ch. megacephala* development into adulthood was delayed compared with the control group by 8 to 72 hours when exposed to malathion.

In this study, the first instar larvae fed on substrate spiked with higher concentration of pesticides did not survive, especially in chlorpyrifos and glyphosate. This is in contrast with finding reported by Meijer et al. (2021), where the *Hermetia illucens*' larvae feeding on substrate spiked with chlorpyrifos did not show any significant difference in terms of growth rate and size compared to the larvae in control group. This is may be due to the very low concentration of chlorpyrifos used in their experiment (0.5 mg/kg). They were also suggested that the said insecticide did not bioaccumulate in the larvae, in which as a result there is no significant changes in larvae development and survival.

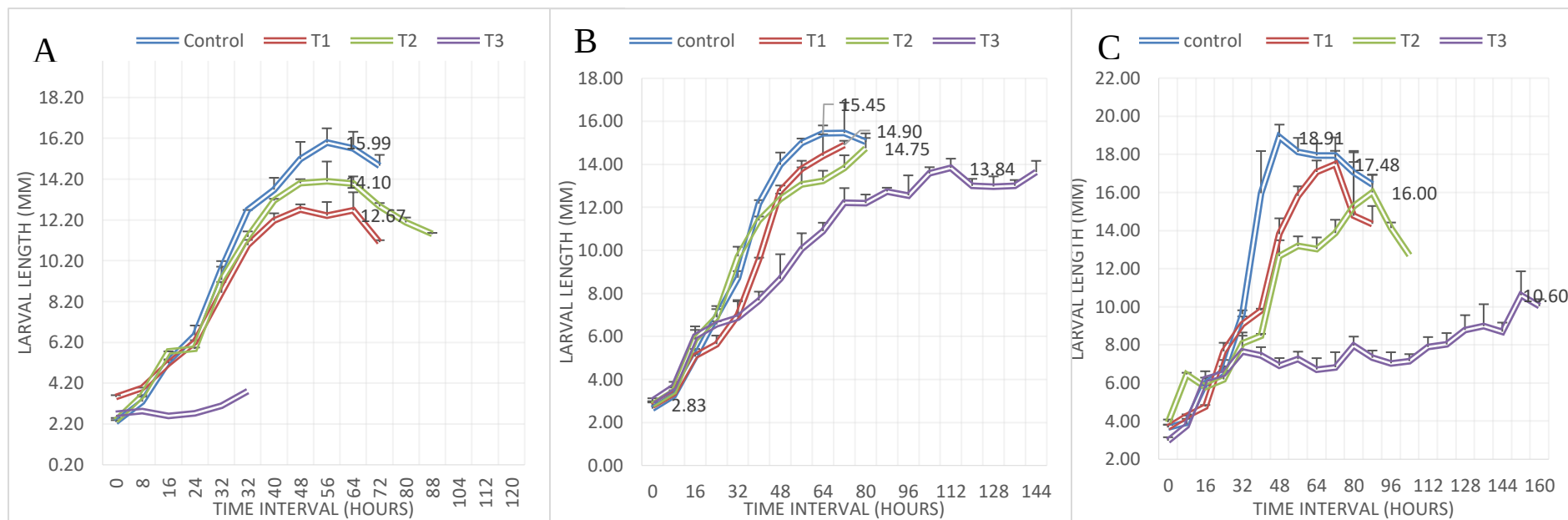


Figure 4.11: Larvae developmental curve of *Ch. megacephala* in (A) glyphosate-treated group, (B) chlorpyrifos-treated group and (C) coumarin-treated group.

Besides, according to Meijer et al. (2021), different species have various responses to different toxins. Therefore, it was assumed that similar variations exist in other insect species when exposed to chlorpyrifos. Two other insecticides (spinosad and cypermethrin) that were also tested in their experiment were negatively affecting the survival of the larvae at higher concentration (2.0 and 0.3 mg/kg respectively) (Meijer et al., 2021).

In comparison, the third stage larvae in control group developed more quickly than the larvae in higher (T3) concentration of chlorpyrifos and coumarin-treated groups. This observation has indicated that high concentration of pesticides bioaccumulate in the larval tissues and have a significant effect on their development. This observation was also observed by Rashid et al. (2008) in their report where they concluded that the changes in the rate of *Ch. megacephala* development was as a result of toxins bioaccumulation in the larvae tissue throughout their development. In addition, other study using dimethoate insecticide also had shown delayed in the development of four blowflies' species; *Ch. megacephala*, *Chrysomya rufifacies*, *Chrysomya saffrana* and *Chrysomya Indiana* (Abd Al Galil et al., 2021). Carvalho et al. (2001) have also reported that the larvae feeding on substrate laced with chemical will either bioaccumulate or excrete the substance and its metabolites. In this experiment, it was suggested that the rate of chemical absorption was exceeds the rate of elimination, as supported by Introna et al. (2001). Therefore, as a result, the third stage larvae developed more slowly than the control group.

Besides pesticides, the household products were proven to have a significant effect on the growth rate of calliphorid larvae. Low concentrations were proven to decrease the survival rate and alters the growth rate and size, while high concentration was proven to be

lethal (Aubernon et al., 2015a; Aubernon et al., 2015b) which was similar to the findings observed in this study.

From this experiment, chlorpyrifos insecticide was lethal towards the early stages of *Ch. megacephala* development. The fly eggs that were exposed to a very low concentration of chlorpyrifos (0.002 µl/ml), was unable to hatch, and the first instar larvae that were exposed to the same concentration also did not survive and died before they reach the second stage of development. This may be due to the mode of action of the chlorpyrifos where it exhibits nonsystemic anticholinesterase activity (Testai et al., 2010) in which negatively affects immatures organism than adults (Koshlukova & Reed, 2014).

Glyphosate is a nonselective herbicide and is known to be toxic to non target organism. In this study, the glyphosate was lethal to *Ch. megacephala* larvae at higher concentration (>400 µl/ml). The glyphosate affects the shikimate pathway which only present in plants and some bacteria. The shikimate pathway does not present in insects; therefore, does not directly affect the insects (Kiefer et al., 2021). Nonetheless, there are numerous studies showed the compound's indirect effects on insects. Previous literatures have shown that the insects often rely on bacteria to produce amino acids. For example, grain beetles *Oryzaephilus surinamensis* live in symbiosis with bacteria to supply them with vital amino acid known as tyrosine. According to a study by Kiefer et al. (2021), the presence of glyphosate disrupted this beetle species' interaction with bacteria, leaving the beetle defenceless against stress, drought, or predator assault. Similarly, previous study also showed that glyphosate has harmful effect on two insect species i.e., *Galleria mellonella* (Lepidoptera: Pyralidae) and *Anopheles gambiae* (Diptera: Culicidae) by inhibit the

phenoloxidase activity which hampered the melanin production upon wounding or infection in *G. mellonella* (Smith et al., 2021).

Meanwhile, meat substrate laced with coumarin has shown a significant change in larvae development. Previous researches have reported the insecticidal activity of coumarin, where high mortality in insects' eggs and larvae of potato tuber moth, *Phthorimaea operculella* was observed (Sharma et al., 2006). Coumarin can be found naturally in plants and are part of the plants' defense mechanism against pest, in which the coumarin production was three to five times higher in damage plants (Hussain et al., 2019). This is not new, since research on the bioactive chemical coumarin against the growth of *Drosophila melanogaster* has been conducted since the 1980s. It was discovered that when exposed to the compound during the egg stage, coumarin inhibits the larval growth of the fruit fly (Nakajima & Kawazu, 1980). Besides insecticidal properties, the coumarin and its metabolites were used in medicine where coumarin anticoagulant used to treat and prevent thromboembolism (Verhoef et al., 2014) and also used as anticoagulant rodenticide which have been used widely to control agriculture and urban rodent population (Watt et al., 2005).

4.5 Conclusion

The delayed in developmental rates of blowflies are significant enough to change the post-mortem interval (PMI) estimation. In this present study, all three pesticides glyphosate, chlorpyrifos and coumarin have a significant effect on the development of *Ch. megacephala* and found to be lethal at higher concentration. There is no significant difference in the development and duration rate of *Ch. megacephala* larvae treated with glyphosate compared to the control group. However, at higher concentration (>400 µl/ml), the larvae did not survive, and only developed until the second stage of larva development. Meanwhile for

chlorpyrifos and coumarin, at lower concentration, there is no significant difference compared to larvae in control group. However, at high concentrations, the larvae development rate was delayed. The larvae treated with coumarin (100 mg/ml) able to reach puparial stage despite smaller size of the larvae. However, the adult flies did not emerge and continue to be in puparial stage until the end of the study period (> 3 months). Meanwhile, the third stage larvae treated with chlorpyrifos have insignificantly smaller size compared to the other groups of larvae. However, after ~4 days, all the larvae died and unable to reach puparial stage. The larvae development is important in entomotoxicology, as the larvae were actively feeding and analyte absorption occurred during this stage. The changes in larval development rate and duration showed there was bioaccumulation of pesticides inside the larvae tissue, and this may have caused under-determination of larvae age during minimum post-mortem interval (minPMI) estimation. Therefore, it is important to consider the presence of toxins and other chemicals when estimating minPMI.

CHAPTER 5

DECOMPOSITION AND INSECT SUCCESSION PATTERN OF PESTICIDES INTOXICATED - RAT CARCASSES

5.1 Introduction

Studies on decomposition process is crucial because of its important effect on forensic investigation. The decomposition process has been studied using animal carcasses which represent a biological model in entomology and forensic biology research (Byrd & Castner, 2001; Benecke, 2001; de Moretti et al., 2006).

Besides bacteria and fungus, insects are the most important decomposers of carrion (Villet, 2010). Therefore, the application of insects in estimating minimum post-mortem interval ($_{\min}\text{PMI}$) is basically means the rough estimation of the larvae age (Matuszewski, 2021). In forensic entomology, several factors were taking into account in estimating the $_{\min}\text{PMI}$ which include the decomposition stages, insect succession pattern and insect developmental stages (Catts & Goff, 1992; Oliviera-Costa & Mello-Patiu, 2003). These factors are closely related as the decomposition process is heavily influenced by the organisms that feed on the body.

In general, the decomposition process comprises of five stages which are fresh, bloated, active decay, advanced decay and dry remains (Gunn, 2006). During each stage of decomposition, the varying in biological, chemical and physical changes that occur throughout the decay process will attract different species of insects, for example blowflies, flesh flies and carrion beetles (Anderson & VanLaerhoven, 1996; Grassberger & Frank, 2003). The decomposing body present as a temporary habitat to carrion insects with

abundant of food source that are suitable for oviposition and the growth of their immature (Voss et al., 2009; Matuszewski et al., 2010).

Therefore, the colonisation time, development stages and departure time of the carrion insects inhabiting and infesting on the decomposing remains are important to predict and estimate the minPMI in legal investigation (Fuller, 1934). For example, the age of the oldest immature insect specimen found on the decomposing body can provide information on the minimum time of the remain was exposed to the environment which available for insect colonisation, hence the minPMI (Catts & Goff, 1992; Matuszewski et al., 2010; Nassu et al., 2014)

However, the circumstances surrounding the death can strongly affect the time of insects' arrival on the carrion hence may alter the decomposition process, for example in the presence of drugs or poisons in the body will affect the estimation of minPMI (Goff & Lord, 1994). There are several studies investigate the effects of toxic substances such as organophosphate (Abd El-bar & Sawaby, 2011; Jales et al., 2020), ethanol (Tabor et al., 2005), gasoline (Rumiza et al., 2010), morphine (Bourel et al., 1999) and many more. From their study, the chemical or toxic substances not only affecting the insects' arrival time and colonisation time, but it was found that the chemical also interfere with the development of insects that infesting on the body (Goff et al., 1997; Carvalho et al., 2001; Wolff et al., 2004; Mahat et al., 2009).

In developing country, the pesticides are easily available for agricultural purposes. This makes them one of the most important causes of poisoning by accidental exposure, suicide and sometimes homicide (Sunger & Guven, 2001). In Malaysia, a study by National Poison Centre (NPC) of Malaysia from 2006 to 2015 had shown an increasing trend in

pesticides poisoning incidents over the 10-year duration with the highest number of poisonings was due to herbicides (44%) followed by agriculture insecticides (34%) rodenticides (9.9%) and others (10%). Sixty percent of poisoning cases are intentional, which mostly caused by suicide attempts (93.6%). Meanwhile, 37.8% are unintentional poisoning, where 91.8% incidents were reported as accidental (Kamaruzaman et al., 2010). The increase of pesticides poisoning incidents required further investigation to understand the effect of pesticides related death of rat carcasses which may eventually interfered with post-mortem interval estimation. Therefore, this study was aims to determine the effect of pesticides-intoxicated carcasses on decomposition rate and insect activities of the carcasses in secondary forest. The findings from this study can be used to establish the baseline data for fauna succession populating the decomposing rat carcasses in a secondary forest Kuching, Sarawak.

5.2 Materials and methods

5.2.1 Study area

The study was conducted in a secondary forest at Kampung Sri Kandong, Jalan Taman Negara Kubah, Kuching, Sarawak (1°38'03"N, 110°09'35"E), 24 m above sea level (Figure 5.1). The location is 29 km from Kuching town and about 45 km from University Malaysia Sarawak (UNIMAS) main campus. The study site was a secondary forest, consist of small trees and dense ground vegetation.

Based on the environmental data provided by Meteorological Department of Malaysia (MET Malaysia), the annual ambient temperature in Kuching was ranged from 23 to 33 °C (annual mean: 27 °C). Kuching receives a lot of rain even in the driest month, where the average rainfall accumulated at 2435.1 mm yearly. The experiment was repeated in three

trials of experiment at the same location to maintain the same environment condition of decomposing pesticide-treated carcasses.

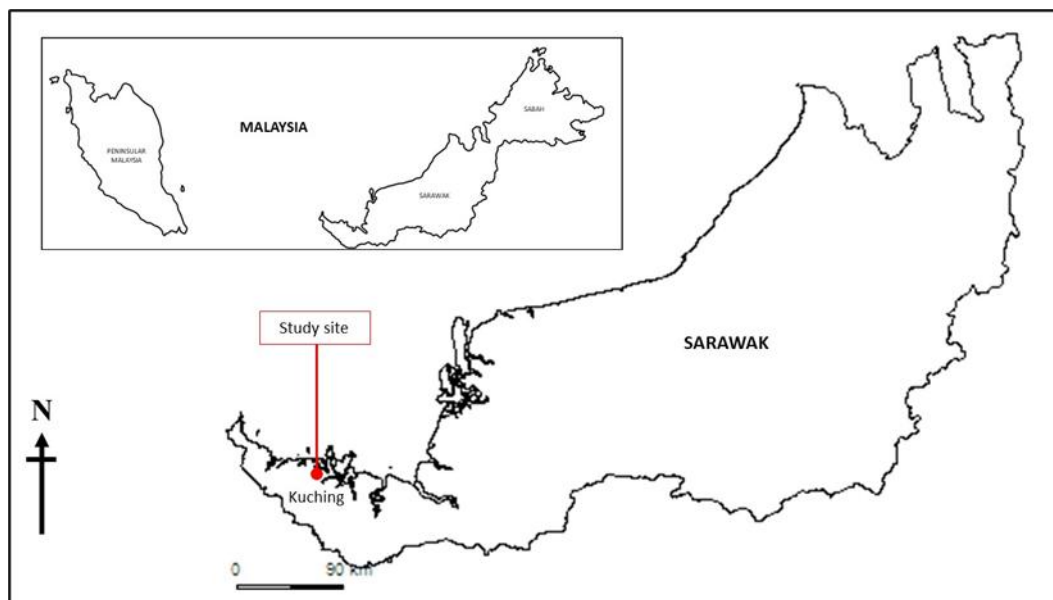


Figure 5.1: Map of the study site at Kampung Sri Kandong, Jalan Taman Negara Kubah, Kuching, Sarawak.

5.2.2 Pesticide preparation

The type of pesticides used in this experiment was glyphosate, chlorpyrifos and coumarin as described in Chapter 3 (section 3.3; pg 76). The pesticides were prepared according to oral LD50 dosage for rats as shown in Table 5.1 below:

Table 5.1: Pesticides LD50 dosage for rats (oral) (NPIC, 2019).

Pesticides	LD50 dosage (g/kg)
Glyphosate	7.2
Chlopyrifos	0.229
Coumarin	0.293

5.2.3 Animal model preparation

The rat used as animal model for this study were albino rats (*Rattus norvegicus*), each weighing approximately 180-200 g. The rats were purchased from a local rodent farm in Kuching, Sarawak. The rats were put inside a cage individually and were given rats' food and water. However, prior to the pesticide administration, the rats were fasted overnight to increase bioavailability and absorption of the pesticides inside the gastrointestinal system (Smith, 2009).

All the rats were intoxicated with the pesticides according to designated groups respectively, and the pesticides were allowed to absorb in the rats' body for at least one hour before the rats were euthanized by cervical dislocation. These procedures were performed by a trained medical laboratory technologist of Faculty of Medicine and Health Sciences, UNIMAS. This research has obtained animal ethic approval by Animal Ethic Committee UNIMAS (UNIMAS/AEC/F07/051).

Rat carcasses were then immediately transported to the study locations. The distance between each rat in a group is at least one meter, and the distance between groups is greater than 20 meters (Figure 5.2). The visits to the carcasses were made twice daily roughly at the same times. Environmental parameters were observed and recorded. The carcasses were covered with a slotted plastic basket after each visit to prevent them from being eaten by other predators and to give the insects access to the carcasses. The experiment was repeated in three trials of experiment.

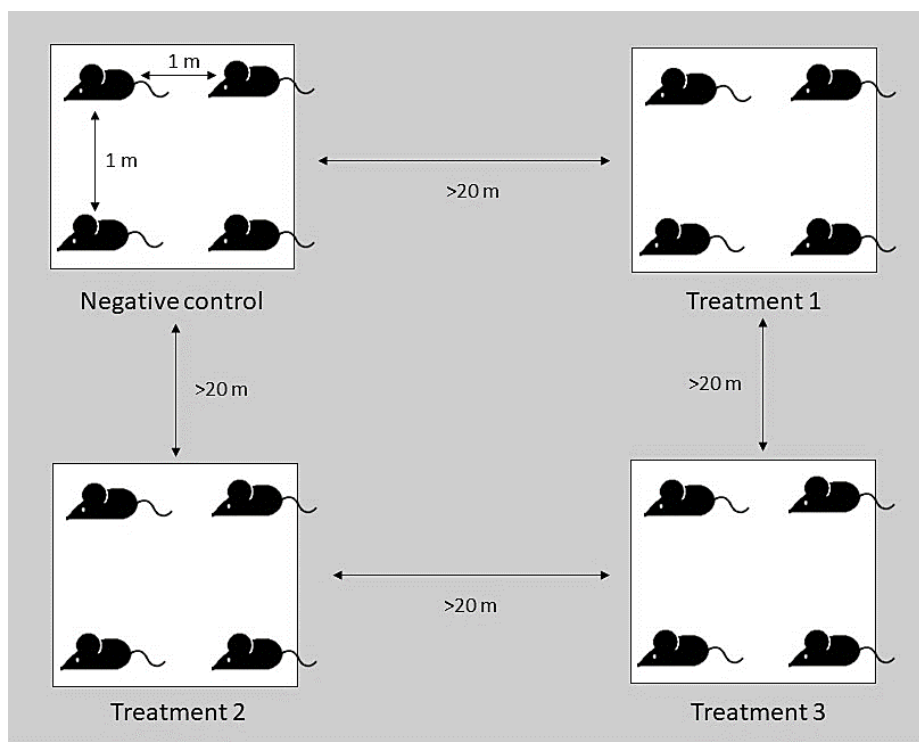


Figure 5.2: Experimental design for field experiment following method by Feugang et al. (2012).

5.2.4 Insect collection

All insects arrived on the carcasses were observed and collected. The details on insect collection, rearing and preservation were described in detail in Chapter 3 (section 3.2; pg 74).

5.2.5 Environmental parameters

The environmental parameters; daily surface temperatures, relative humidity and rainfall were obtained from Malaysian Meteorological Department (MET).

5.2.6 Data analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software ver. 24. The comparison of environmental parameters in between trials were analysed using one-way ANOVA and Tukey HSD test. The entomological data was tabulated in a table using Microsoft Office 2020. The insect relative abundance was calculated by using formula below:

$$\text{Insect relative abundance} = \frac{\text{Total number of individual species of insects caught}}{\text{Total number of insect species caught}} \times 100$$

The comparison of insect composition between control, T1, T2 and T3 was carried out using one-way ANOVA analysis.

5.3 Results

5.3.1 Environmental parameters at the study site.

The environment parameters that were provided by Malaysia Meteorological Department (MET) were mean daily surface temperatures, mean relative humidity and rainfall data. Throughout the three intervals of study period, the average daily surface temperatures were ranged from 26.4 ± 1.2 to 28.2 ± 1.2 °C.

The highest accumulated rain was recorded during the first trial of experiment which was on January to February of 2021 (total=542.6 mm), which reflected by a significantly lower average temperatures recorded during that period. The least accumulated rain was recorded during third trial (September-October) (total=65.4 mm), in which significantly lower average relative humidity was recorded during that period of time ($82.8 \pm 5.5\%$). The summary of environmental data was presented in Table 5.2.

One-way ANOVA analysis was conducted to compare the environmental parameters between trials. The analysis showed there is a significant difference of daily surface temperatures [$F(2,48) = 11.21, P = 0.000$] (Appendix G) and relative humidity [$F(2,48) = 10.29, P = 0.000$] (Appendix H). Multiple comparison analysis, Tukey post-hoc test showed the environmental parameters during the third trial (September-October) was significantly different than the average temperatures and humidity during January until March.

Table 5.2: Environmental data throughout the study period provided by (MET).

Intervals	Mean daily surface temperatures (°C) (Mean±S.E.)			Average relative humidity (%)	Accumulated rain (mm)
	Maximum	Minimum	Average		
21st Jan–15 th Feb	29.1 ± 0.5	23.7 ± 0.2	26.4 ± 1.2	89.7 ± 4.1	542.6
3 rd –20 th March	31.2 ± 0.4	23.2 ± 0.2	27.3 ± 1.0	88.1 ± 4.0	283.5
*22 nd Sept– 11 th Oct	32.5 ± 0.6	24.0 ± 0.2	28.2 ± 1.2	82.8 ± 5.5	65.4

*Statistical significance $P < 0.05$

5.3.2 Composition of forensically important insect

There was a total of 1,579 of insects associated with decomposing rat carcasses belongs to four orders and eight families were collected over the study period. There were 455, 407, 178 and 539 insects collected in control, T1, T2 and T3 group of rat carcasses respectively (Table 5.3). Although T2 group shows the least number of insects, statistical analysis (ANOVA) showed there is no significance difference of insects between all groups [$F(3,52) = 0.248, P = >0.500$] (Appendix I).

Among 1,579 insects collected, 71.5% were blow flies with *Chrysomya rufifacies* (Macquart, 1843) was the most abundant followed by *Chrysomya megacephala* (Fabricius,

1794) and *Hemipyrellia ligurriens* (Wiedemann, 1830) which total up to 136 and 54 of individual flies respectively. *H. ligurriens* was present in all groups of rat carcasses except for Group T2 carcasses.

Other blow flies, such as *Lucilia porphyrina* (Walker, 1857) and *Hypopygiopsis violacea* (Macquart, 1835) were the least number of blow flies collected (≥ 2 flies each species). Besides blow flies, *Sarcophaga* sp. (Family: Sarcophagidae) and house flies (Family: Muscidae) were also recovered on the carcasses which accounts for only 2.0% and 5.5% respectively from the total of insects collected.

The *Sarcophaga* sp. was the highest observed and collected in group T1 carcasses (Total=23) if compared to the other groups. Meanwhile, *Ophyra chalcogaster* (Wiedemann, 1830) (Total=44) and *Atherigona orientalis* (Schiner) (Total=30) were the most common collected muscid flies. Among coleopterans, *Onthophagus* sp. (family Scarabaeidae) was the most abundant species recovered from rat carcasses. Approximately, higher number of scarab beetles recorded in all intoxicated group of rat carcasses, with the highest scarab beetles recorded was in group T2 (Total=47). Other beetles collected was from family Staphylinidae and Hydrophilidae. The family Staphylinidae was represented by *Aleochara* sp. (Gravenhorst, 1802) while family Hydrophilidae represented by *Sphaeridium lunatum* (Fabricius, 1792) which were observed in all group of carcasses. Few other species that classify as scavenger and predator of other insects feeding on carcasses were also observed and identified. They were belonging to the order of Dermaptera, Hymenoptera and Hemiptera which mostly targeting the larvae present on the carcasses during decay stage. The *Vespa affinis* (Order: Hymenoptera) was observed on carcasses but not collected (Table 5.3). All insects collected were presented in Appendix M and N.

Table 5.3: Number of insect recovered from decomposing rat carcasses in all groups over study period.

Order	Family	Genus/Species	Total number of individual species in each group				Total (ΣNsi)	Relative abundance (%)
			Control	T1	T2	T3		
Diptera	Calliphoridae	<i>Chrysomya rufifacies</i>	366	337	50	376	1129	71.5
		<i>Chrysomya megacephala</i>	16	12	37	71	136	8.6
		<i>Hemipyrellia ligurriens</i>	48	1	0	5	54	3.4
		<i>Lucilia porphyrina</i>	2	0	0	0	2	0.1
		<i>Hypopygiopsis violacea</i>	1	0	0	0	1	0.01
	Sarcophagidae	<i>Sarcophaga</i> sp.	1	23	3	5	32	2.0
	Muscidae	<i>Ophyra chalcogaster</i>	9	2	10	23	44	2.8
		<i>Musca domestica</i>	0	2	10	0	12	0.8
		<i>Atherigona orientalis</i>	7	5	7	11	30	1.9
	Scarabaeidae	<i>Onthophagus</i> sp.	3	19	47	36	105	6.6
Coleoptera	Hydrophilidae	<i>Sphaeridium lunatum</i>	1	2	8	3	14	0.9
	Staphylinidae	<i>Aleochara</i> sp.	1	3	1	7	12	0.8
Dermaptera	Anisolabididae	<i>Euborellia annulipes</i>	0	1	5	1	7	0.4
Hemiptera	Rhyparochromidae	Not identified	0	0	0	1	1	0.01
Hymenoptera	Vespidae	<i>Vespa affinis</i>			Not collected			-
Total			455	407	178	539	1579	100

5.3.3 Insect associated with the decomposition stages of rat carcasses in several treatments

5.3.3.1 Insect associated with the decomposition stages of control group rat carcasses

The decomposition process of rat carcasses was consisting of five stages which are fresh, bloated, active decay, advanced decay and dry remain stage. The decomposition period of control group carcasses in average, took seven to nine days to complete (Table 5.4).

Table 5.4: Decomposition period of rat carcasses in control group in three trials of experiment.

Decomposition stage	Decomposition duration (Day)		
	Trial 1	Trial 2	Trial 3
Fresh	2	1	1
Bloated	2	1	1
Active decay	2	3	2
Advanced decay	1	1	2
Dry remain	1	1	1
Total days	8	7	7

The fresh stage starts as soon as the rats were euthanised. During fresh stage of decomposition, the body undergo pathological changes such as *algor mortis* where the carcass' body temperature decreases and *rigor mortis* where the muscle and limbs became stiff, 30 to 60 minutes' post-mortem (Figure 5.3A). However, after the carcasses laid on the ground at the study site, the body temperatures increased to the environmental temperature at the time. There were fire ants seen (Order: Hymenoptera) and few adult blowflies attracted to the carcasses.

After 24 hours, the carcasses were in a bloated stage. In this stage, physical changes can be observed on the carcasses. All the carcasses have inflated abdomen (Figure 5.3B) and released foul smell which then attracted the adult flies which were *Ch. megacephala*, *Ch. rufifacies*, *Hy. violacea* (Diptera: Calliphoridae) and *Sarcophaga* sp. (Diptera: Sarcophagidae). Besides blowflies and flesh fly, a few muscid flies also seen attracted to the decompose body fluid which later identified as *O. chalcogaster* and *A. orientalis* (Diptera: Muscidae). The fire ants were seen taking the rats' fur besides predate on the adult flies. The bloated stage lasted for 24 to 36 hours as observed in all five trials of experiments.



Figure 5.3: Decomposition stages of rat carcass consist of (A) fresh, (B) bloated, (C-D) active decay, (E) advanced decay and (F) dry remains.

The bloated stage ends when the abdomen of the carcasses was no longer inflated, and the decomposed body fluid can be seen surrounding the carcass due to the active tissue penetration by the fly larvae. In this stage, a pool of blowflies' larvae, which were dominated by *Ch. rufifacies* followed by *Ch. megacephala* were seen actively infesting the carcasses especially at the natural orifices such at the mouth and eyes and also at the rear area (Figure 5.3C-D). The rats body tissues and organs were liquefied.

The rotten smell from the decompose body fluid released through orifices attracts more adult flies to come and deposit their eggs on the carcasses. The active decay stage lasted for two days. The larvae infesting the tissues molted from L1 larvae to L3 larvae and eventually leaving the carcasses to pupate. Besides immature flies, small number of beetles, *Onthophagous* sp. (Coleoptera: Scarabaeoidea) and *A. lata* (Coleoptera: Staphylinidae) also seen hiding under the carcasses.

The active decay stage ends when the larvae leaving the carcasses to a drier ground to pupate. This also marked the fourth stage of decomposition process which are the advanced decay stage (Figure 5.3E). During this stage, large portion of the carcasses' initial mass has gone and left with skin, some portion of fur and bones. The carcasses' body dries out and attracts more dung beetles and muscid flies. The fire ants still surrounding the carcasses to predate on the migrating larvae. This stage only lasted for 24-48 hours. As the carcasses continue to decay, the body dried and all the furs are gone leaving only the bones on the ground (Figure 5.3F). The insect succession pattern during decomposition process in three trials of experiments were summarised in Table 5.5.

Table 5.5: Insects succession pattern during decomposition process of control group rat carcasses.

Order	Family	Genus/Species	Decomposition stages				
			Fresh	Bloated	Decay	Advanced Decay	Dry remains
Diptera	Calliphoridae	<i>Chrysomya rufifacies</i>					
		<i>Chrysomya megacephala</i>					
		<i>Hemipyrellia ligurriens</i>					
		<i>Lucilia porphyrina</i>					
		<i>Hypopygiopsis violacea</i>					
	Sarcophagidae	<i>Sarcophaga sp.</i>					
	Muscidae	<i>Ophyra chalcogaster</i>					
		<i>Musca domestica</i>					
		<i>Atherigona orientalis</i>					
Coleoptera	Scarabaeidae	<i>Onthophagus sp.</i>					
	Hydrophilidae	<i>Sphaeridium lunatum</i>					
	Staphylinidae	<i>Aleochara lata</i>					
Dermaptera	Anisolabididae	<i>Euborellia annulipes</i>					
Hymenoptera	Vespidae	<i>Vespa affinis</i>					
Hemiptera	Rhyparochromidae	Not identified					

 Adult
  Immatures

5.3.3.2 Insects associated with the decomposition of T1 group rat carcasses

The decomposition process of rat carcasses took seven to eight days for it to complete (Table 5.6). There are five stages of decomposition of carcasses observed in this group namely, fresh, bloated, active decay, advanced decay and dry remain stage. The first insect that came in contact after the carcasses were exposed to the environment was the fire ants, and followed by a group of blowflies, *Ch. rufifacies*, *Ch. megacephala* and *Hy. violacea*.

Table 5.6: Decomposition period of T1 rat carcasses in three trials of experiment.

Decomposition stage	Decomposition duration (Day)		
	Trial 1	Trial 2	Trial 3
Fresh	1	1	2
Bloated	2	1	1
Active decay	2	2	2
Advanced decay	2	2	2
Dry remain	1	1	1
Total days	8	7	8

The carcasses were in the state of rigor mortis when arrived at the study site (Figure 5.4A). Within 2 hours of observation, no fly eggs were observed, only adult blowflies were attracted to the carcasses. After 24 hours, on the next visit, the carcasses' body bloated and producing foul smelling liquid (Figure 5.4B). As a result, more blowflies came and gather mostly around the natural orifices such as ear, nose and mouth where the foul gas released. There were no fly eggs visible on body surface or near the orifices. The fire ants continue to predate on the adult flies and possibly the fly eggs.

On the third day of the experiment, the carcasses were in active decay stage. The adult blowflies continued to burrow inside the carcasses through the mouth area to breed. A pool of L1 larvae can be seen infesting on carcasses at the head area and the torso (Figure 5.4C). The larvae collected were later identified as the immatures of *Ch. rufifacies*, which was the most dominant species infesting the carcasses and followed by *Sarcophaga* sp. larvae. The carcasses' tissues and organs were liquidified and gave out a strong foul smell which attracted not only the blowflies, but also the muscid flies, *O. chalcogaster*.

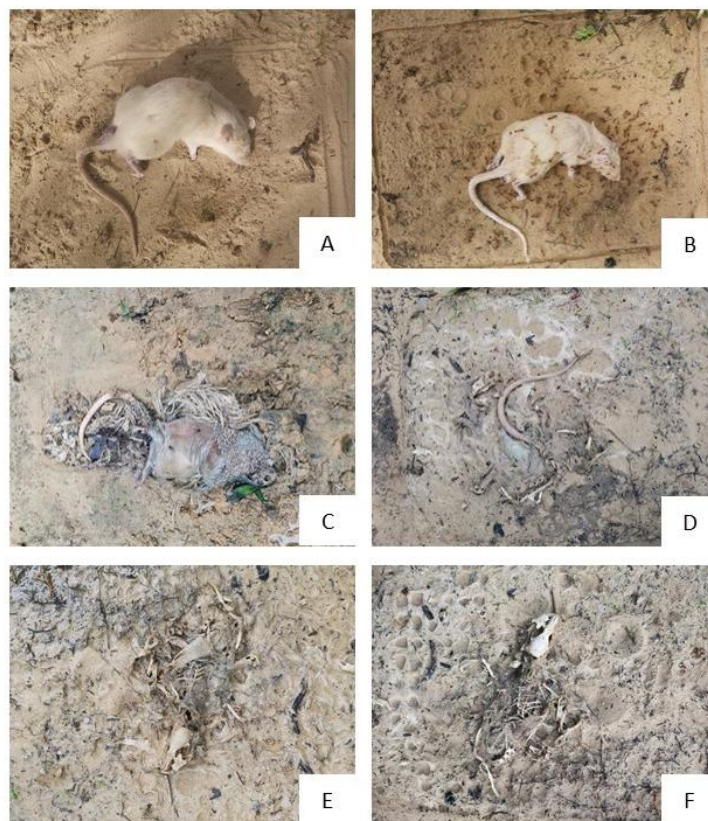


Figure 5.4: Decomposition stages of rat carcass intoxicated with glyphosate consist of (A) fresh, (B) bloated, (C) active decay, (D-E) advanced decay and (F) dry remains.

On the fifth to sixth day, the decomposition progress to advanced decay stage whereby, most of the L3 larvae (*Ch. rufifacies*) were seen leaving the carcasses towards a drier ground to pupate while others (*H. ligurriens* larvae) can be seen infesting on the remnants of tails and skins. The fire ants surrounding the carcasses wait and preying on the migrating larvae. The rat carcasses decrease in mass and left with a portion of skin and bones (Figure 5.4D-E).

The flesh flies were seen visiting even though the carcasses started to dried up. On the seventh to eighth day, the carcasses at a dry remains stage (Figure 5.4F). Few dung beetles can be seen underneath the bones which the only part that left from the carcasses. All trials of experiment exhibit similar pattern of decomposition process. The insect succession pattern during decomposition process of rat carcasses were summarised in Table 5.7.

5.3.3.3 Insects associated with the decomposition of T2 group rat carcasses.

The decomposition process for rat carcasses in Group T2 took about 17-21 days to reach the last stage (Table 5.8). It consists of five stages which are, fresh, bloated, active decay, advanced decay and dry remain. The fresh stage (Figure 5.5A) lasted about 24 hours before the bloated abdomen started to show. The carcasses underwent a slow decay process where the bloated stage lasted for 4 to 5 days (Figure 5.5B). In this stage, there is no insect visiting the carcasses and there is also no prominent decay smell on carcasses.

The active decay and advanced decay stage are hard to differentiate due to slow decomposition process of the carcasses. The active decay stage (Figure 5.5C-D) was indicated with the flattened of the carcasses' abdomen and the decomposed body fluid released from the natural orifices of the carcasses which were at the head and rear area.

Table 5.7: Insect succession pattern of decomposing glyphosate- intoxicated rat carcasses (Group T1).

Order	Family	Genus/Species	Decomposition stages				
			Fresh	Bloated	Decay	Advanced Decay	Dry remains
Diptera	Calliphoridae	<i>Chrysomya rufifacies</i>					
		<i>Chrysomya megacephala</i>					
		<i>Hemipyrellia ligurriens</i>					
		<i>Lucilia porphyrina</i>					
		<i>Hypopygiopsis violacea</i>					
	Sarcophagidae	<i>Sarcophaga</i> sp.					
	Muscidae	<i>Ophyra chalcogaster</i>					
		<i>Musca domestica</i>					
		<i>Atherigona orientalis</i>					
Coleoptera	Scarabaeidae	<i>Onthophagus</i> sp.					
	Hydrophilidae	<i>Sphaeridium lunatum</i>					
	Staphylinidae	<i>Aleochara lata</i>					
Dermaptera	Anisolabididae	<i>Euborellia annulipes</i>					
Hymenoptera	Vespidae	<i>Vespa affinis</i>					
Hemiptera	Rhyparochromidae	Not identified					

 Adult
  Immatures

Table 5.8: Decomposition period of T2 rat carcasses in three trials of experiment.

Decomposition stages	Decomposition period (Day-n)		
	Trial 1	Trial 2	Trial 3
Fresh	1	1	2
Bloated	4	3	2
Decay	8	11	9
Advanced decay	7	5	3
Dry	1	1	1
Total days	21	21	17

The prominent pungent smell from the body fluid eventually attracted several flies' species to the carcasses i.e., *Ch. megacephala*, *Ch. rufifacies*, *Sarcophaga* sp. and *O. chalcogaster*. However, there is no fly eggs and fly larvae observed on carcasses. The dung beetles and rove beetles were also found underneath the carcasses. After two to three days of decay stage, the soft tissues at the head area (eyes, ears, etc) decayed, but the body still intact with skin, tissues and fur. The bodily fluid of the decompose internal tissues and organs of the rat carcasses continue to flush out for several days until it dries out, where the advanced decay stage began (Figure 5.5E-H).

The carcasses which already dried up, has fungus grown on skin that was in contact with the soil. Few adult flies (*Ch. rufifacies*) and rove beetles can be seen around the carcasses. The carcasses became drier, less pungent and flattened (Figure 5.5I). Similar characteristics observed until the last day of the experiment, day 17-21. All three trials of experiment exhibit similar pattern of decomposition process. The insect succession pattern during decomposition process of rat carcasses were summarised in Table 5.9.



Figure 5.5: Decomposition stages of rat carcass intoxicated with chlorpyrifos which consist of five stages, (A) fresh; (B) bloated; (C-D) active decay, (E-H) advanced decay and (I) dry remain stage.

Table 5.9: Insect succession pattern of decomposing chlorpyrifos- intoxicated rat carcasses (T2).

Order	Family	Genus/Species	Decomposition stages				
			Fresh	Bloated	Decay	Advanced Decay	Dry remains
Diptera	Calliphoridae	<i>Chrysomya rufifacies</i>					
		<i>Chrysomya megacephala</i>					
		<i>Hemipyrellia ligurriens</i>					
		<i>Lucilia porphyrina</i>					
		<i>Hypopygiopsis violacea</i>					
	Sarcophagidae	<i>Sarcophaga</i> sp.					
	Muscidae	<i>Ophyra chalcogaster</i>					
		<i>Musca domestica</i>					
		<i>Atherigona orientalis</i>					
Coleoptera	Scarabaeidae	<i>Onthophagus</i> sp.					
	Hydrophilidae	<i>Sphaeridium lunatum</i>					
	Staphylinidae	<i>Aleochara lata</i>					
Dermaptera	Anisolabididae	<i>Euborellia annulipes</i>					
Hymenoptera	Vespidae	<i>Vespa affinis</i>					
Hemiptera	Rhyparochromidae	Not identified					
	Adult						

5.3.3.4 Insects associated with the decomposition of T3 group rat carcasses

There are five stages of decomposition process identified which took about 12 days to complete (Table 5.10). The fresh stage started as soon as the rats euthanized (Figure 5.6A). Then, after 24-48 hours, the rat carcasses were in bloated stage where the carcasses' body inflated. During bloated stage, only few adult *Ch. rufifacies* and ants can be seen visiting the carcasses, and besides that, dung beetle also can be seen underneath the carcasses (Figure 5.6B).

The active decay stage (Figure 5.6C-D) started when the abdomen of the carcasses was flattening, and internal organs and tissues were decayed into decompose body fluid and purged out from the body through the natural orifices. The putrefaction odour was strong, which then attract other adult flies such as *Ch. megacephala*, *Hy. Violacea*, *H. ligurriens*, *Sarcophaga sp.* and *O. chalcogaster*.

Table 5.10: Decomposition period of T3 rat carcasses in three trials of experiment.

Decomposition stages	Decomposition period (Day/s)		
	Trial 1	Trial 2	Trial 3
Fresh	1	1	2
Bloated	2	1	1
Active decay	5	4	3
Advanced decay	3	5	4
Dry	1	1	1
Total days	12	13	11

Besides flies, more beetles can also be seen underneath the carcasses such as dung beetles and rove beetles. Starting from the beginning of active decay stage, a mass of larvae formed around the head area and then move to the abdomen. The larvae actively infesting the tissues and moulted into several larval stages. The active decay stage took about three to four days before the larvae started to leave the carcasses to pupate. This marked the next stage which was the advanced decay stage.

During advanced decay stage (Figure 5.6E), a few *Sarcophaga* sp. larvae and beetles can still be seen on carcasses. The advanced decay stage took about 4 to 8 days. After several days, the carcasses were drier and left with some portion of skin and bones. The carcasses were in dry stage on the 11th-12th day (Figure 5.6F). The insect succession pattern during decomposition process of rat carcasses were summarised in Table 5.11.

Besides flies, more beetles can also be seen underneath the carcasses such as dung beetles and rove beetles. Starting from the beginning of active decay stage, a mass of larvae formed around the head area and then move to the abdomen. The larvae actively infesting the tissues and moulted into several larval stages. The active decay stage took about three to four days before the larvae started to leave the carcasses to pupate. This marked the next stage which was the advanced decay stage.

During advanced decay stage (Figure 5.6E), a few *Sarcophaga* sp. larvae and beetles can still be seen on carcasses. The advanced decay stage took about 4 to 8 days. After several days, the carcasses were drier and left with some portion of skin and bones. The carcasses were in dry stage on the 11th-12th day (Figure 5.6F). The insect succession pattern during decomposition process of rat carcasses were summarised in Table 5.11.

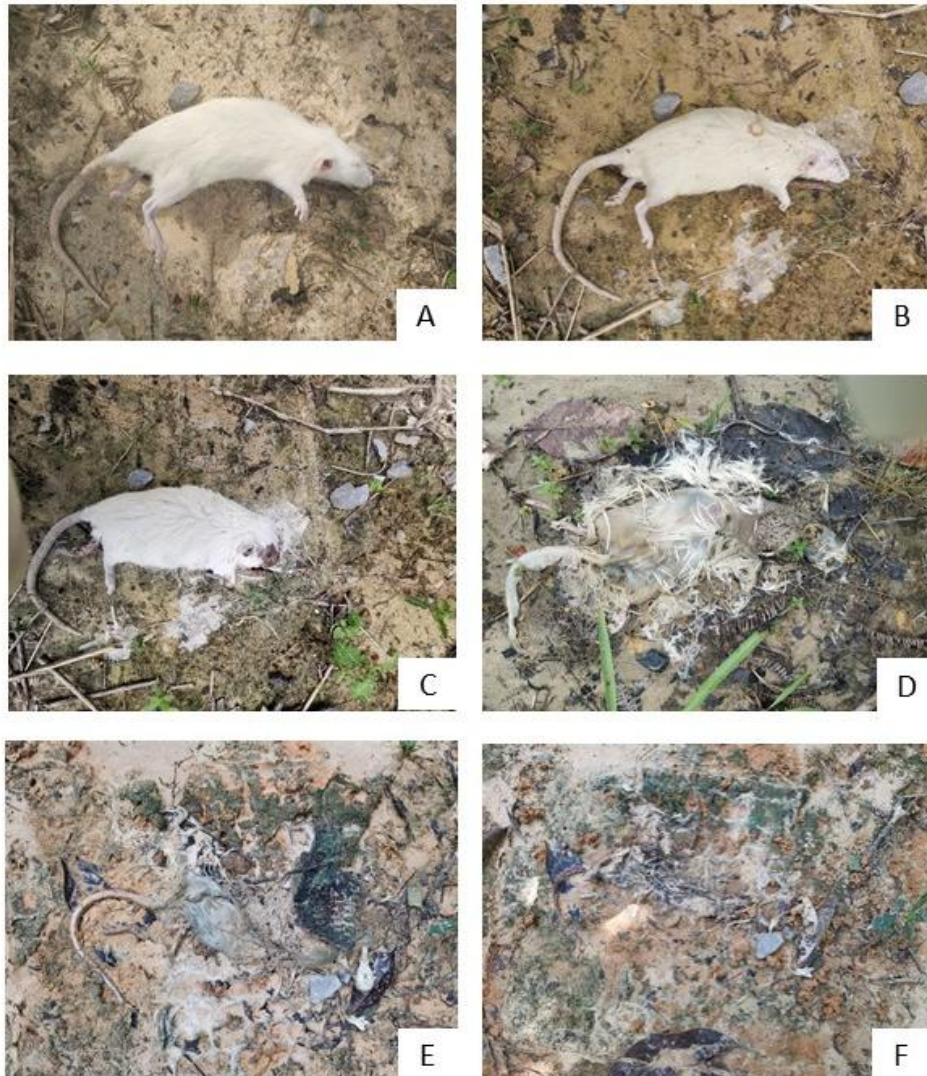


Figure 5.6: Decomposition stages of rat carcass intoxicated with coumarin which consist of (A) fresh; (B) bloated; (C-D) active decay; (E) advanced decay and (F) dry remains stage.

Table 5.11: Insect succession pattern of decomposed coumarin-intoxicated rat carcasses (T3).

Order	Family	Genus/Species	Decomposition stages				
			Fresh	Bloated	Decay	Advanced Decay	Dry remains
Diptera	Calliphoridae	<i>Chrysomya rufifacies</i>					
		<i>Chrysomya megacephala</i>					
		<i>Hemipyrellia ligurriens</i>					
		<i>Lucilia porphyrina</i>					
		<i>Hypopygiopsis violacea</i>					
	Sarcophagidae	<i>Sarcophaga</i> sp.					
	Muscidae	<i>Ophyra chalcogaster</i>					
		<i>Musca domestica</i>					
		<i>Atherigona orientalis</i>					
Coleoptera	Scarabaeidae	<i>Onthophagus</i> sp.					
	Hydrophilidae	<i>Sphaeridium lunatum</i>					
	Staphylinidae	<i>Aleochara lata</i>					
Dermaptera	Anisolabididae	<i>Euborellia annulipes</i>					
Hymenoptera	Vespidae	<i>Vespa affinis</i>					
Hemiptera	Rhyparochromidae	Not identified					

 Adult
  Immatures

5.3.4 Morphology of predominant carrion blowflies

5.3.4.1 *Chrysomya rufifacies* (Macquart, 1984)

Chrysomya rufifacies was the most dominant blow fly collected in this study. From the total population of insects collected, 65.18% belongs to *Ch. rufifacies*. The *Ch. rufifacies* can be identified by its metallic green colour and a whitish or silvery white postgena. The body length ranged from 6.0-9.5 mm with brown antennae and a yellowish palpus. At the thorax area, the mesonotum have a short, simple bristles. The mesothoracic spiracle is white in colour and the thoracic squama is whitish in colour with dark setulae. At the abdominal area, the tergite consist of white hair (Figure 5.7).

The third instar larvae of *Ch. rufifacies* have a muscoid-shaped body, which about 14 mm in length. The larvae were muscoid-shaped and brownish in colour. Microscopically, the abdominal segments have a large, and elongate tubercles (TB) which looks like hairs when observed macroscopically. At the anterior region is tapered and consist of two structures that are important in identification, which are the cephalopharyngeal skeleton and the anterior spiracle.

The cephalopharyngeal skeleton have hook-like mouthparts (HK) and two other structures which are, dorsal cornua (DC) and ventral cornua (VC) which were in similar size. The anterior spiracles (AS) have 9-12 marginal branches (MB). At the posterior region, the body is larger and rounder and consist of a pair of posterior spiracles (PS). The posterior spiracle consists of three spiracular slits and incomplete peritreme (PE) which encircled the spiracular slits (SS) (Figure 5.8)

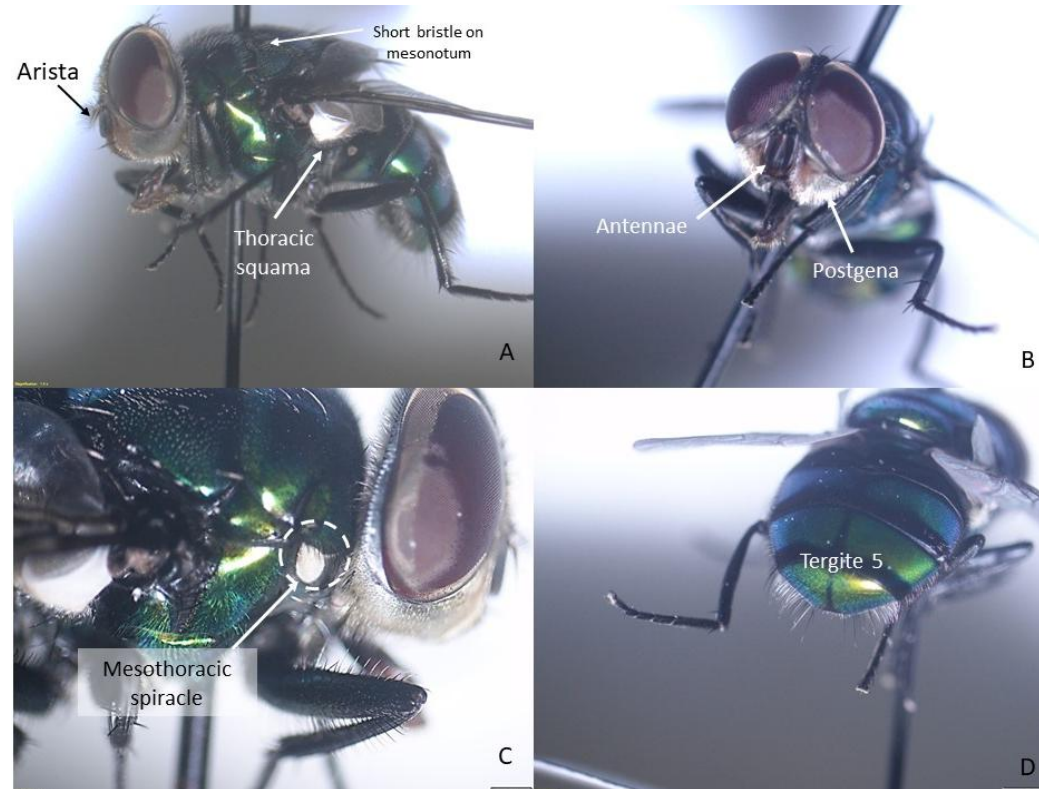


Figure 5.7: Characteristics of adult *Chrysomya rufifacies* collected from study site. (A) The lateral view showing arista on the segmented antennae, a short bristle on mesonotum and whitish thoracic squama. (B) The frontal view showing the holoptic eye of male *C. rufifacies*, postgenal area characterised by whitish/silvery white colour. The antennae is brown in colour. (C) Close up on the thorax showing white mesothoracic spiracle [1 x 1.2 magnification] and (D) tergite 5 with white hair [1 x 1.5 magnification] Scale bar = 1.0 mm.

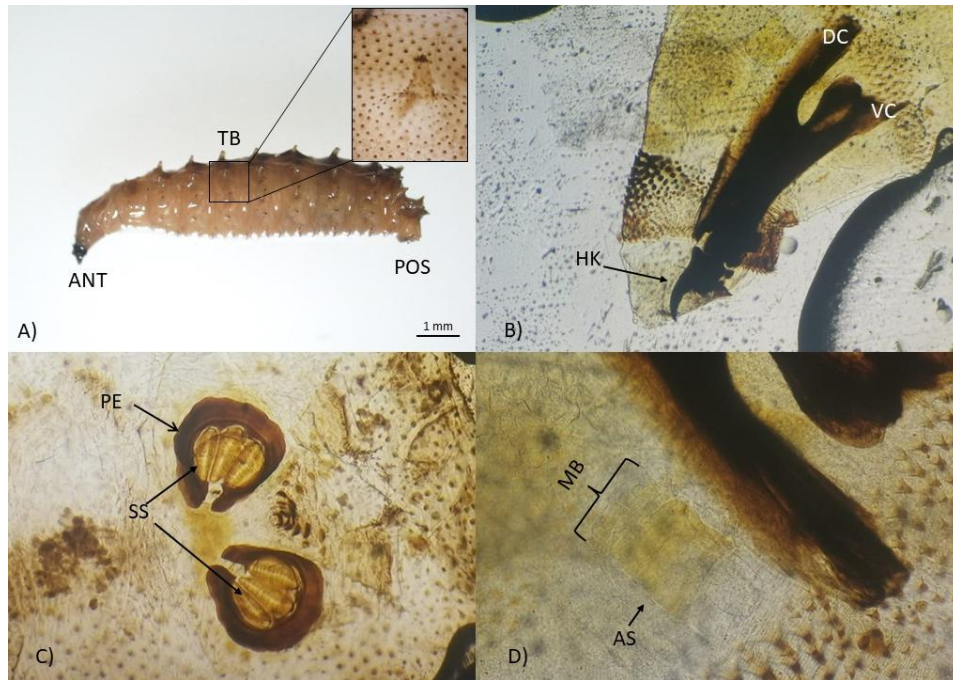


Figure 5.8: Morphology of *Chrysomya rufifacies* larva. **(A)** The morphology of third instar larva showing the projection of tubercles (TB) on the body segment of the larvae. Microscopically (100x), the tubercles are enlarge, elongate and covers with small spines at the tip. **(B)** The hook (HK) mouthpart and the similar length of dorsal cornua (DC) and ventral cornua (VC). **(C)** The posterior spiracle with incomplete peritreme (PE) and spiracular silts (SS). **(D)** The anterior spiracle (AS) with 10 marginal branches (MB). Figure (A) was observed under stereo microscope (1x1.0 magnification), while figure (B-D) were observed under compound microscope (10x10 magnification).

5.3.4.2 *Chrysomya megacephala* (Fabricius, 1794)

Chrysomya megacephala was another dominant adult blow fly collected from this study. Among the insect species collected, 12.60% was *Ch. megacephala*. The adult *Ch. megacephala* can be identified based on its metallic greenish color with orange-yellow gena and postgenal area. The body length of this species is about 8 to 11 mm. Similar with *Ch. rufifacies*, *Ch. megacephala* has short bristles on its mesonotum. The thoracic squama is large and lobulated (Figure 5.9).

The larvae of third instar *Ch. megacephala* is muscoid in shape which tapered at the anterior, and enlarged and rounder towards the posterior region. The cephalopharyngeal skeleton consist of hook-like mouthparts (HK), dorsal cornua (DC) and ventral cornua (VC). The DC is longer than VC in *Ch. megacephala* if compared to that of *Ch. rufifacies*. The spines between the first and second thoracic segment are moderate, with single or multiple spine (SP). At the posterior end, the posterior spiracles consist of spiracular silts (SS) and moderately sclerotized incomplete peritreme (Figure 5.10).

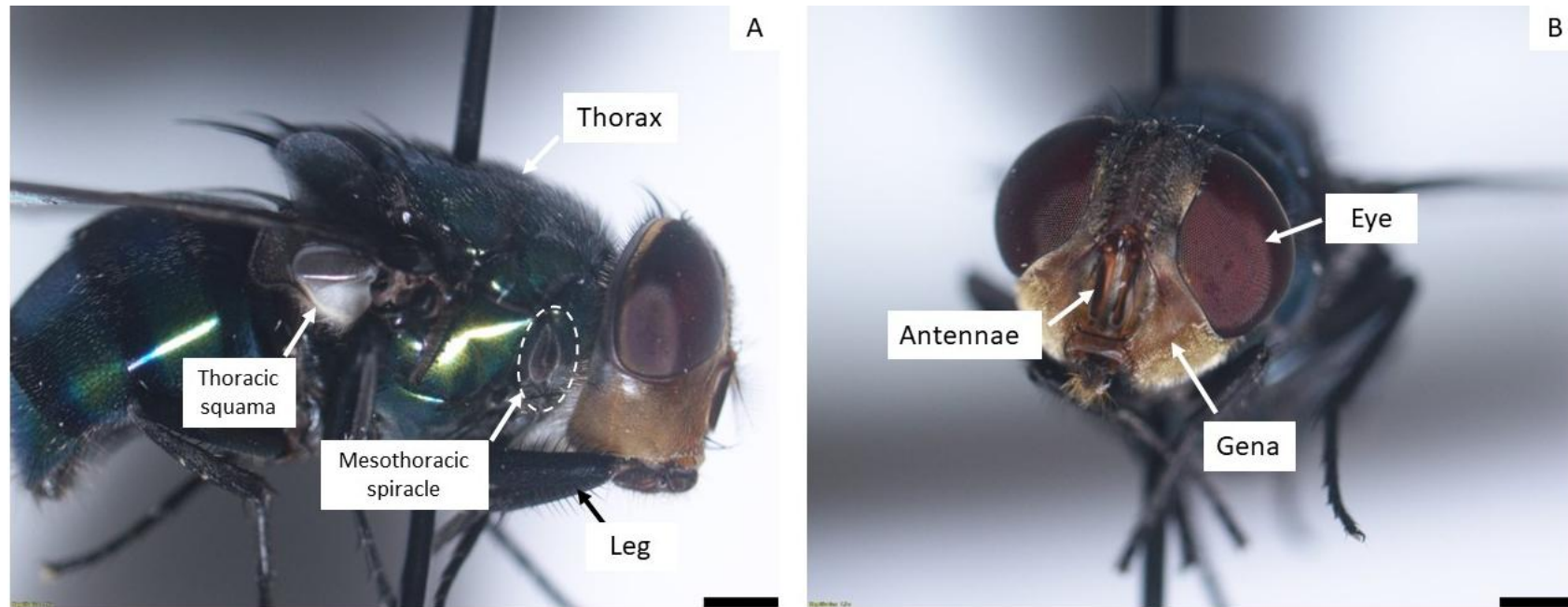


Figure 5.9: Characteristics of adult *Chrysomya megacephala* collected from study site. (A) The lateral view showing a short bristle on mesonotum and whitish thoracic squama and dark and large mesothoracic spiracle . (B) The frontal view showing the eye of a female *Ch. megacephala* with orange-yellow gena and postgena. The gena is covered with pale yellow hairs. The antennae is brown in colour. [1 x 1.5 magnification] Scale bar = 1.0 mm.

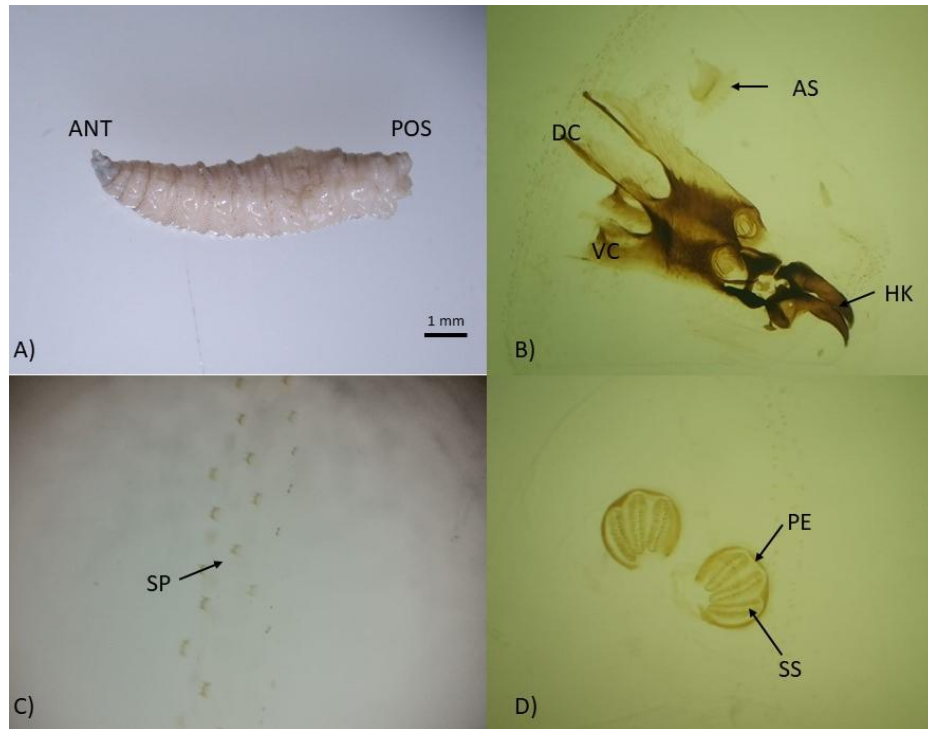


Figure 5.10: Morphology of *Chrysomya megacephala* third instar larva. **(A)** The larvae is muscoid shaped with tapered head at the anterior (ANT) and larger and rounder towards the posterior (POS) end. **(B)** Cephalopharyngeal skeleton showing the hook (HK) mouthpart and the longer length of dorsal cornua (DC) than ventral cornua (VC). The anterior spiracle at the upper region with 10 marginal branches. **(C)** The spines (SP) occur in between the first and two thoracic segment of the larva. **(D)** A pair of posterior spiracles with incomplete peritreme (PE) and the absence of button. The three spiracular silts (SS) on each spiracle indicate the larval stage. Figure (A) was observed under stereo microscope (1x1.0 magnification), while figure (B-D) were observed under compound microscope (10x10 magnification).

5.3.4.3 *Hemipyrellia ligurriens* (Wiedemann, 1830)

H. ligurriens was the third most dominant blowfly collected at the study site (Total= 75). The adult is about 6.5-10.5 mm in length. The fly has a metallic copper green exterior, with a grayish pollinosity facial and the silverish gena was covered with black hair. The antennae have a dark brown or orange ventrally. On the thorax, the thoracic squama was brownish white and mesothoracic spiracles are brownish (Figure 5.11).

Similar to the larvae of *Chrysomya* sp., the *H. ligurriens* larvae are muscoid shape that is pointed in anterior and blunt in posterior. The caudal segment had a pair of posterior spiracles. The cephalopharyngeal skeleton of third instar larvae was well developed, which consist of hook mouthparts (HK) with the presence of parastomal bar (PB) and pharyngeal sclerite. At the posterior end, the posterior spiracles (PS) consist of complete peritreme (PE) which is highly pigmented and have an interslit projection. The third instar consists of three separated slits (SS) (Figure 5.12).

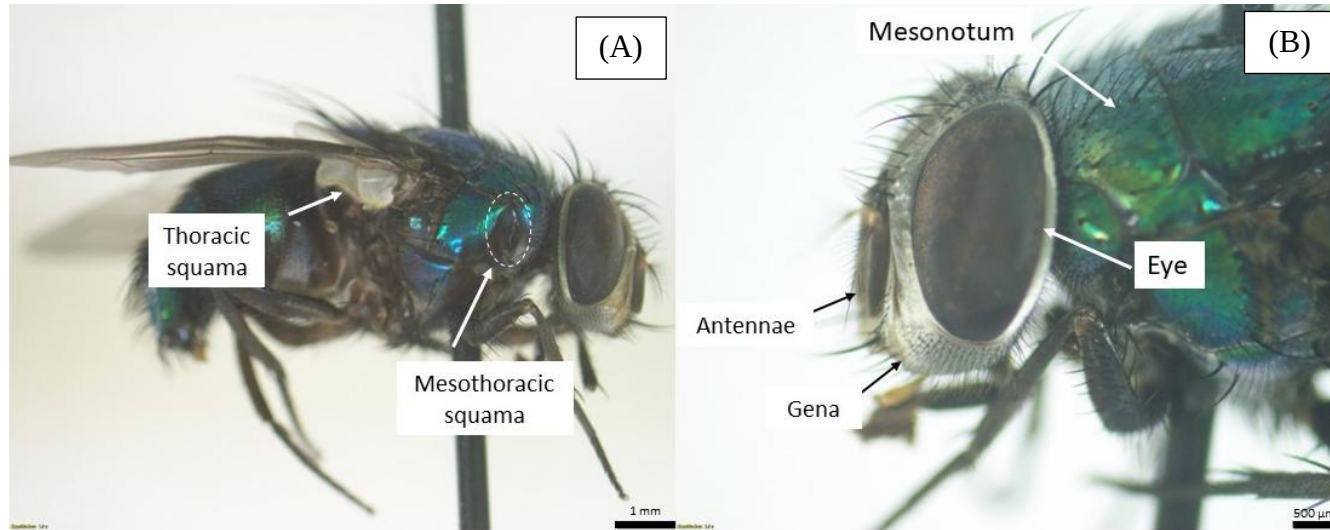


Figure 5.11: The (A) lateral view of adult of *Hemipyrellia ligurriens* which is characterised by metallic copper green colour of the exterior and brownish-white colour of thoracic squama and dark brown of mesothoracic squama at the thorax. (B) the head area of *Hemipyrellia ligurriens* showing dark brown antennae and silverish white gena which is covered with black hair. The mesonotum is covered with strong bristle. Magnification (A) 1 x 1.5, scale bar = 1 mm (B) 1 x 3.5, scale bar = 500 µm.

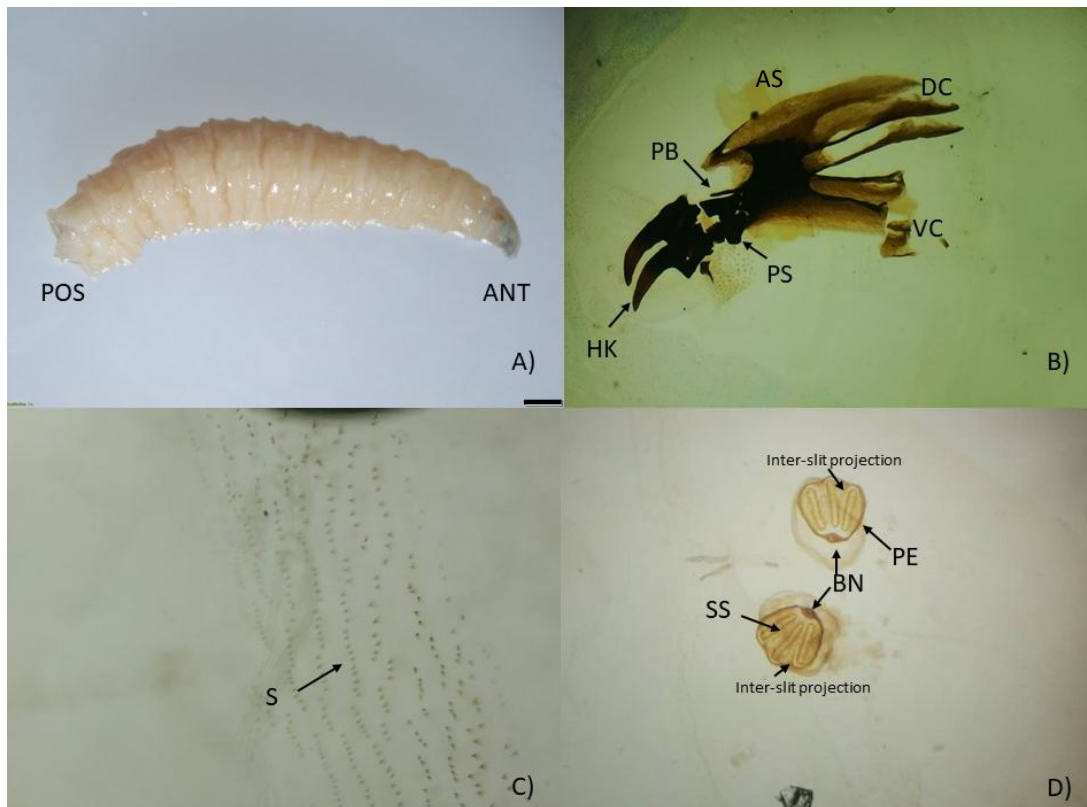


Figure 5.12: Morphology of *H. ligurriens* larva. **(A)** The third instar larva is muscoid-shaped, pointed at the anterior (ANT) and blunt end at the posterior (POS). **(B)** The cephalopharyngeal skeleton consists of hook mouthpart (HK), parastomal bar (PB), pharyngeal sclerite (PS) and dorsal cornua (DC) which is longer than ventral cornua (VC). There anterior spiracle (AS) can also be observed behind the DC. **(C)** The presence of spines between the first and second thoracic segment of the larva. **(D)** At the posterior end, posterior spiracles present with complete peritreme and button (BN) located outside of the peritreme. Three spiracular slits (SS) indicated the larva is in third stage and there is a prominent interslit projection between middle slit and lower slit on both spiracle. Figure (A) observed under stereo microscope (Magnification 1 x 1.5), and Figure B-D were observed under compound microscope (Magnification 10 x 10).

5.3.5 Morphology of predominant beetles associated with rat carcasses

5.3.5.1 *Onthophagus* sp. (Coleoptera: Scarabaeidea)

This species is approximately 5-10 mm in length and has an oval-shaped body and have a dark-greenish metallic color. The head consists of the eyes, mouthpart and a pair of antennae (Figure 5.13). The last three antennal segments are flattened into plate-like structures which are spread apart forming a terminal club (Figure 5.14 B). The pronotum is not covering the head, and the elytra is closed together at apex with not clearly visible scutellum (Figure 5.14 A). The front tibiae are a bit dilated, with toothed outer margin (Figure 5.14 A). The middle and hind tibia are enlarged apically and flattened at the entire length with five-segmented tarsi (Figure 5.14 B).

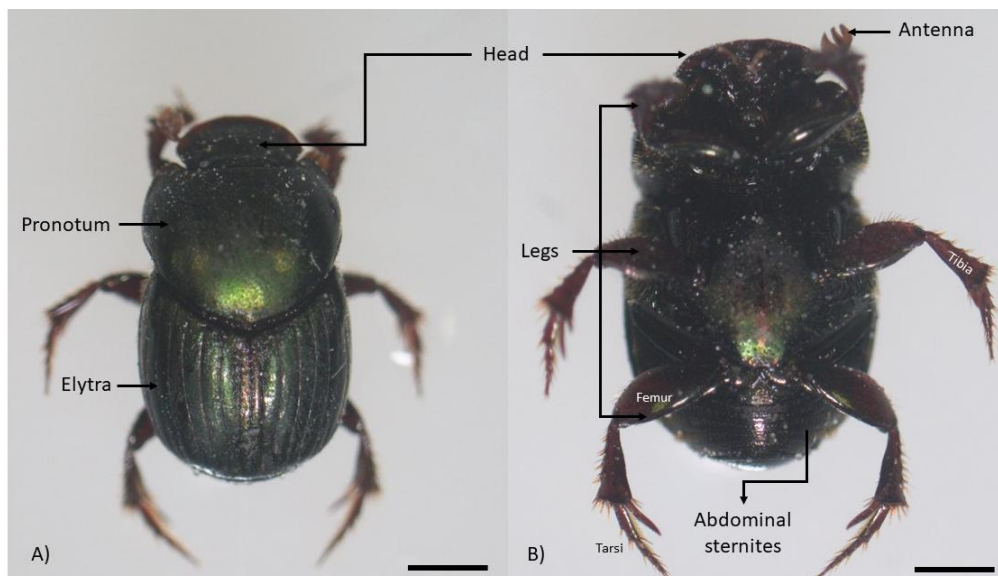


Figure 5.13: The morphology of *Onthophagus* sp. collected at the study site. (A) The dorsal view shows the head, pronotum, foretibia and elytra. (B) The ventral view shows the antennae, separated middle coxa, middle tibia and hind tibia. Abdominal sternites also can be seen at the ventral view. [Magnification = 1.5x, scale bar = 1 mm].

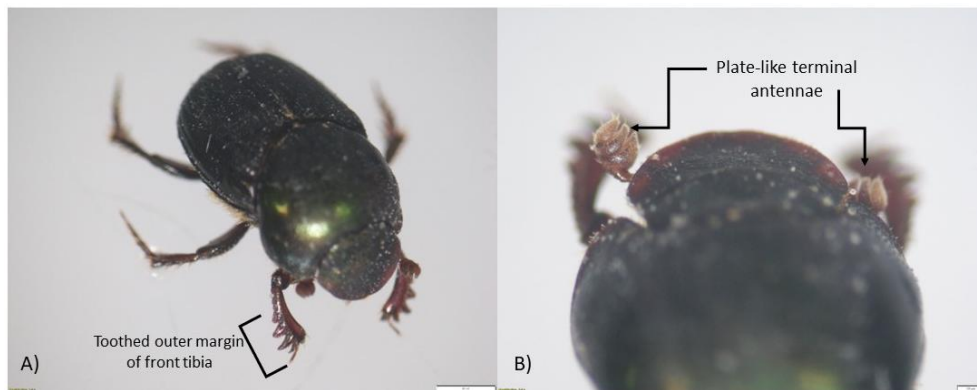


Figure 5.14: The morphology of *Onthophagus* sp. showing (A) front tibiae with toothed edge and (B) the flattened terminal antennae that form a plate-like structure.

5.3.5.2 *Aleochara* sp. (Coleoptera: Staphylinidae)

The length of *Aleochara* sp. was ranged from 5-9 mm. It has transverse antennae with 5-10 segments. The *Aleochara* sp. have a slender and elongate body with black and brown in color. The abdomen of *Aleochara* sp. was well visible about six to seven sterna. The wings are well developed and folded under the short elytra. The antennae and legs are reddish-brown in colour (Figure 5.15).

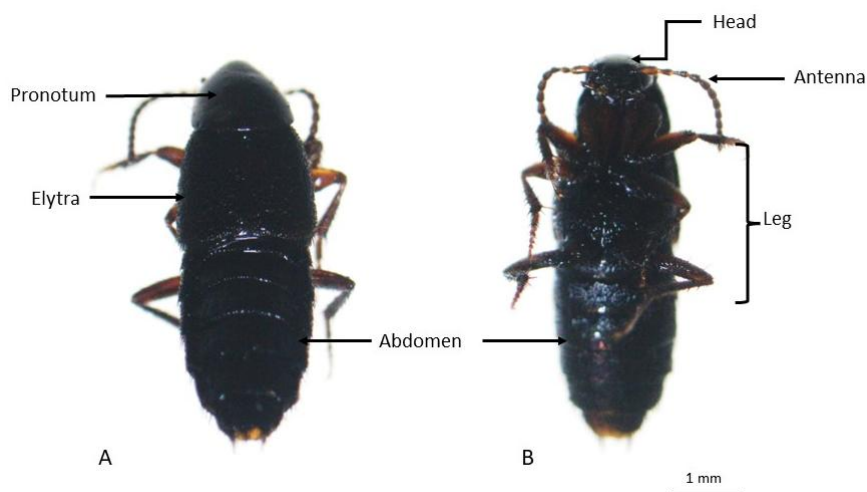


Figure 5.15: The figure showing (A) dorsal view and (B) ventral view of *Aleochara* sp. and its main characteristics.

5.4 Discussion

5.4.1 Influence of environment parameters on carrion decomposition and insect activity

The environment parameters are the primary factor in forensic entomology that can affect the rate of decomposition and insect activity on human and animal carcasses (Mahat et al., 2009). The weather in Malaysia influenced by the winds blowing from the Indian Ocean (Southwest monsoon) around May to September and the South China Sea (North-Eastern monsoon) around November to March (MET, 2022). This study was conducted in three trials of experiments, from January until October of 2021, with at least two weeks-interval between each trial. Although there are significantly lower temperatures and relative humidity during the third trial, the changes in the environment does not influence the rate of decomposition and insect arrival and activity on the carcasses as the duration of decomposition and insect succession are similar in all three trials of experiments.

5.4.2 Insect composition associated with decomposed rat carcasses

In the current study, *Chrysomya* sp. was the fly species most frequently found near decomposing rat carcasses. *Chrysomya* sp. have been reported in several studies where they were among the first visitor and primary carrion decomposers in Malaysia (Chin et al., 2007; Azwandi et al., 2013; Adrus & Rahim, 2018). In a study of fly species composition taking from 34 human remains that were referred to Universiti Kebangsaan Malaysia Medical Center (UKMMC) had shown that the highest number of individual maggot's presence was *Chrysomya megacephala* (70.6%) and followed by *Chrysomya rufifacies* (44.1%) (Syamsa et al., 2010).

Besides human remains, other studies using animal carcasses have reported that both *Ch. rufifacies* and *Ch. megacephala* were the most abundant calliphorid flies visiting and infesting carcasses in peninsular Malaysia (Chin et al., 2007; Azwandi et al., 2013). Similarly, in Sarawak, the occurrence of *Ch. megacephala* and *Ch. Rufifacies* as a dominant species infesting on carcasses was also observed in studies conducted in two different locations which are; mangrove forest in Kuching and peat swamp in Kota Samarahan (Maramat & Nor Aliza, 2015; Adrus & Rahim, 2018). However, other location for example in Mount Kapur cave in Kuching has observed *Hypopygiopsis violacea* as the dominant blowfly infesting rabbit carcasses (Nordin et al., 2020).

Other flies such as flesh fly (Order: Sarcophagidae) and house fly (order: Muscidae) can also be observed on the carcasses in both control and organophosphate intoxicated group. In this present study, the *Sarcophaga* sp. was observed during the bloated stage of decomposition. Previous studies have reported that *Sarcophaga* sp. was found to be the earliest species to colonise a carrion in peninsular Malaysia and Egypt (Zuha et al., 2017; Abd. El-Gawad et al., 2019).

Fewer muscid flies are known to colonise animal carrion and human cadavers if compared to fly species of other families, especially Calliphoridae (Matuszewski et al., 2008). In this study, three species of muscid flies were identified, and *Ophyra chalcogaster* was the most common house fly found on the carcasses. *O. chalcogaster* was known to attract to the carcasses during early stages of decomposition (Carvalho et al., 2008) in both human cadavers and carcasses particularly during active decay stage (Tantawi et al., 1996). Comparably, the presence of *O. chalcogaster* was observed during decay stage for all carcasses in this present study.

Beetles are among the most significant insects in determining PMI time, along with flies (Kulshrestha & Satpathy, 2001). The beetles use corpses during the later stages of decomposition, when the coleopteran insects on the corpses have already been expelled by the fly larvae (Midgley et al., 2009). In this study, a smaller number of beetle species found on carcasses if compared to dipteran flies. The highest number of individual beetles collected was a dung beetle, *Onthophagous* sp. (Family: Scarabaeidea). The beetles were usually observed during decay and advanced decay stage of decomposition. In previous study on dung beetle diversity, similar finding was observed where the occurrence of dung beetles was reported during decay stage of rat carcasses. It was stated that the decaying carcass tend to attract *Onthophagous* sp. (Goh, 2014).

Other insects such as, earwigs can also be seen hiding underneath the carcasses and predate on larvae as they decay. They consume a wide range of insects, preferring soft-bodied larvae, as was previously mentioned (Moerkens et al., 2011). Earwigs are known to be nocturnal insect which hide in crevices, bark and in debris during the day and commonly found in temperate and tropical climates. Earwigs that live in temperate and tropical region are known to be predaceous. Additionally, earwigs also consume dead and decaying organic matter (Moerkens et al., 2011).

5.4.3 Effects of pesticides on carcasses' decomposition and insect succession

The decomposition stages classification is subjective and most widely described are fresh, bloated, active decay, advanced decay, dry and remains (Payne, 1965). However, the final two stages are very difficult to distinguish, and these two stages are usually merged together into dry/remains (Chin et al., 2008; King, 2012). During advanced decay stage, the carcass was left with skin, cartilage and bones and cease of dipteran flies. Thus, allows the

soil dwelling taxa for example, beetles (Goff, 2009). In this present study, the five decomposition stages were determined which are, fresh, bloated, active decay, advanced decay and dry remains.

The presence of chemical substance on carrion can interfere with the decomposition process and insect activity, therefore, alter the post-mortem interval estimation (Goff & Lord, 1994). In this study, three types of pesticides; herbicide, insecticide and rodenticide were intoxicated into rats to study the effect of these pesticides on the decomposition and carrion insects' activity on the carcasses. Each pesticide used are commercially available except for rodenticide. Coumarin is one of the active ingredients in anticoagulant rodenticide. However, the commercially available rodenticides only have $\leq 1\%$ of the active ingredient because this type of rodenticide effectiveness depends entirely on repeated exposure, hence the small doses (NPIC, 2016). Therefore, to induce acute poisoning by oral administration to the rats is difficult due to the small doses. Hence, the analytical grade of coumarin (99%) was used and diluted into oral rat LD50 doses accordingly.

The herbicide and insecticide, especially organophosphate was widely used for agricultural purposes and are easily available which eventually leads to poisoning, whether it was accidental or intentional (Sungur & Güven, 2001). It was reported that 60-90% of suicides in China, Malaysia, Sri Lanka, Trinidad and Tobago were by pesticide ingestion (Bertolote et al., 2006). Therefore, several articles on epidemiological study and management of organophosphate poisoning have been published in those countries for example, Egypt (El-Karadawy et al., 2006), Turkey (Sungur & Güven, 2001), Sri Lanka (Premaratna et al., 2001) and Malaysia (Kamaruzaman et al., 2020).

In this study, the pesticides were orally administered to the rats to mimic the pesticide poisoning that usually occurred by ingestion (WHO, 2016; Mahat et al., 2009). The finding shows that the decomposition of all groups of carcasses consisted of five stages. The duration of carrion decomposition for pesticide-intoxicated carcasses were longer compared to the control group. For glyphosate-intoxicated carcasses, the decomposition was slightly delayed for about ~14 hours. Meanwhile, the coumarin and chlorpyrifos-intoxicated carcasses group, took longer time to decompose, which was about 5 to 10 days longer than control and glyphosate-intoxicated groups of carcasses.

In previous literature, it was reported that there is a distinct delay in decomposition of rabbit carcasses that were poisoned with pirimiphosmethyl (organophosphate) relative to the control (Abd El-Bar & Sawaby, 2011), which was similarly observed in present study. Other studies applying other type of pesticides, for examples; pyrethrins and pyrethroids (de Souza Sandoval & de Ascensão Medeiros, 2013) and carbamate (de Siqueira et al., 2016) were all resulted in similar observation where prolonged decomposition time were observed on their carrion. On the other hand, according to a study by Jales et al. (2020), the decomposition of rat carcasses treated with terbufos (organophosphate) by gavage was sped up by 24 hours when compared to control carcasses. According to previous studies (Cycon & Piotrowska-Seget, 2007; Jales et al., 2020), the composition of terbufos increased the metabolism of decomposing microorganisms, hence increased cellular respiration and ammonia production, ultimately speeding up the decomposition of rat carcasses.

On the other hand, previous research using warfarin intoxicated carcasses as their animal model had shown that the decomposition rate of intoxicated carcasses was faster than the control group (Abd El-Gawad et al., 2019). This finding was in contrast with the current

study, as the coumarin-intoxicated rat carcasses was slower and took longer time to decompose which about five days delayed compared to the control group. According to Abd El-Gawad (2019), rapid carcass decomposition that was treated with warfarin relative to control group was because the toxin did not mask the odor of the carcasses. Besides, high ambient temperature in Cairo has also contributed to the rapid decomposition of the carcasses (range: 28 to 43 °C). Warfarin is a derivative of coumarin which also an active ingredient in rat poison (Verhoef et al., 2014).

Insect activity plays a vital role during decomposition process (Abd. El-Bar & Sawaby, 2011). A study on the effect of insect exclusion on pig decomposition had shown that the delayed insect access to carrion has retards the carrion decomposition (Pechal et al., 2014). In previous literature, it was stated that the presence of toxins may have masked the decay odour, in which making the carcass less attractive to the insects (Voss et al., 2008). It was also indicated that gravid flies are normally attracted to the chemicals (ammonia and sulphur-containing compounds) emanated by the decayed carcasses (Abd. El-Bar & Sawaby, 2011). Based on the outcome of current experiment, chlorpyrifos might have masked the decay odour, making the decay smell subtler on T2 carcasses. Therefore, the number of colonising flies visiting the carcasses has reduced and no oviposition occur on the carcasses.

Another similar study using malathion (organophosphate insecticide) has caused a delay in oviposition and prolongs the fly development of both *Ch. rufifacies* and *Ch. megacephala* (Mahat et al., 2009). There is no fly eggs presence on carcasses intoxicated with chlorpyrifos. Previously, a study has reported that there was a delay in fly oviposition on carcasses that were sprayed with the insecticide. The presence of eggs was only observed on a second day, although eggs' eclosion into larvae was not observed (Denis et al., 2018).

This is slightly different with our current study, as the fly eggs are not found at all on chlorpyrifos-intoxicated carcasses. The environment might be too toxic for the fly to oviposit and develop (Gunatilake & Goff, 1989). Review from other literature has revealed that there was a change in fly behavior in terms of delay in egg-laying, decreased in egg production and failure of the egg to hatch for house fly species, *Musca domestica* when exposed to chlorpyrifos (Dad & Yousuf, 2011). Furthermore, the ability of fly to produce offspring was reduced in muscid fly (*Drosophila melanogaster*) when exposed to insecticides (Gupta et al., 2007).

In current study, the decomposition of T3 carcasses which were intoxicated with coumarin was delayed for about five days. This may be caused by insecticidal activity of coumarin (Sharma et al., 2006). Naturally, coumarin can be found in several plant families include; Apiaceae, Miliaceae and Asteraceae (Chiasson et al., 2001; Gonzales-Coloma et al., 2005; Gupta & Misra, 2006; Prasad et al., 2006; Sharma et al., 2006) which are known to have potential insecticidal constituents. According to Sharma et al. (2006), coumarins caused high percentage mortality of eggs and larvae of insects. Although there were no dead insects found on the T3 carcasses, the decomposition progression from decay to dry stage was longer.

This may be due to the slow feeding of the larvae, where more activity was concentrated at the head region rather than the abdomen. The abdomen infestation came later after about four days since the carcass's placement at the site. Similar observation was reported where the larvae activity was concentrated at the chest area of the test animal (chicken) that was soaked with household insecticide. The larvae colonisation continue later

for the whole carcasses as the insecticide was washed away by rainstorms. Due to the slow feeding of the larvae, the decomposition was delayed (Tellez, 2015).

However, the presence of glyphosate has no effect on the arrival of insects for the T1 group. Similar colonising insects was observed on the carcasses relative to the control group. The flies were able to colonise and successfully infesting the carcasses' tissue until the end of decomposition process. The decomposition process was slightly longer during advanced decay stage in relative to control carcasses (~24 hours). The findings were consistent with a prior study from Kota Samarahan where the insect colonisation and duration of decomposition process of rabbit carcasses intoxicated with paraquat dichloride (herbicide) was similar with the control carcasses, which took about a week. (Lawai et al., 2015). Both pesticides are herbicides that are not toxic to insects and do not mask the odour of decaying carcasses. Therefore, has no impact on the insect's arrival and the decomposition time of the carcasses.

5.5 Conclusion

The decomposition was delayed in pesticides-intoxicated rat carcasses. The most prolonged decomposition time was from the chlorpyrifos-intoxicated carcasses, followed by coumarin and glyphosate. The duration of decomposition of rat carcasses in glyphosate intoxicated carcasses were delayed for ~14 hours in glyphosate-intoxicated carcasses, and about 5-10 days for chlorpyrifos and coumarin-intoxicated carcasses respectively in relation to control carcasses which took about 7 days. The delay in the rate of decomposition of carcasses intoxicated with chlorpyrifos and coumarin was due to their insecticidal activity which caused by a lack or slow larvae activity on both groups of carcasses. Meanwhile, the insect succession pattern of rat carcasses was almost similar for all groups of carcasses,

although the number of insects visiting were varied. The highest number of insects collected were in group T3, followed by control group, T1 and T2. There is a reduced number of insects in T2 group due to the absence of immatures on the carcasses. To conclude, the presence of pesticides inside the carrion body does affect the decomposition and insect activity. The changes in these two factors are vital as it can influence the post-mortem interval estimation in forensic investigation where pesticides occurrence is suspected. The entomological data in control group can also be used as an added knowledge to the forensic entomological baseline data in Sarawak specifically for carrion insects' diversity in secondary forest.

CHAPTER 6

SECONDARY BIOACCUMULATION OF PESTICIDES IN BLOWFLY LARVAL TISSUES INFESTING ON PESTICIDE-INTOXICATED RAT CARCASSES

6.1 Introduction

The study that involves in determination of chemical substance (poisons or drugs) in insects recovered from decomposing remains is relatively a new branch of forensic entomology field which known as entomotoxicology (Gagliano-Candela & Aventaggiato, 2001). The insects are used as the alternative species for toxicological analysis in cases where the human samples are not available (Verma & Paul, 2013). The unavailability of body samples for toxicological analysis might be due to the badly decomposed body caused by delayed in the discovery of the body.

The cases of delayed body discovery are possible when an individual died due to drug abuse in isolated places, suicide or in cases where the body is concealed (Archer et al., 2006). In these scenarios, the usual body samples used in toxicological analysis; tissues, body fluids and internal organs may already have degraded and not suitable for analysis. Therefore, the insects are the only reliable specimen for analyses in the investigation (Gosselin et al., 2011; Badenhorst & Villet, 2018).

Insects are vital in forensic investigations as they utilise the dead body as food source for their larvae (Goff, 2009). While feeding, the poisons or drugs in the body systems were transferred into the larvae metabolic system. Furthermore, the substance that were now inside the larvae can also be transferred through the food chain when other arthropods predate on the larvae (Introna et al., 2001).

The entomotoxicological studies that were carried out worldwide mostly concentrated on prescribed drugs. Several publications have reported the analyses of toxic and controlled substances in arthropods (Goff et al., 1989; Bourel et al., 2001b). Besides drugs, death due to abuse of insecticides, pesticides and other toxins are common worldwide. Bachmann (2018), in his report on suicidal epidemiology, reported that about 450,000 people died around the world due to drug abuse in 2015. It was also estimated that about 800,000 people die every year due to suicide, in which 20% of the death are caused by pesticide ingestion (Bachmann, 2018).

Therefore, in several cases, dead bodies are only recovered after several weeks to months. In these particular cases, entomotoxicology can be an important tool in solving cases when the bodies are badly decomposed and recovered after a long period of time (Chophi et al., 2019). The detection of drugs and poisons in insects and other arthropods have been documented using several techniques such as immunoassays (Kintz et al., 1990), high performance liquid chromatography (HPLC) (Sadler et al., 1995; Gunn et al., 2006), liquid chromatography-mass spectrometry (LCMS) (Tracqui et al., 2004) and gas chromatography-mass spectrometry (GCMS) (Campobasso et al., 2004; Definis-Gojanovic et al., 2007) have been routinely used to detect xenobiotics both from cadaver and insects' samples. Besides chromatography methods, the pesticide detection has also been carried out using spectrophotometry method (Venugopal et al., 2012; Catrinck et al., 2014), although most of the method development studies are focusing on detection of pesticides in fruit and vegetable samples (Chowdhury et al., 2013; Sahu et al., 2022). This current study aimed to identify and quantify the secondary bioaccumulated glyphosate and coumarin inside the blowfly larval tissue using UV spectrophotometer and GCMS.

6.2 Materials and methods

6.2.1 Pesticide and animal model preparation

The blowfly larvae were collected from the field experiment using rat carcasses as presented in detailed in Chapter 5 (section 5.2.2 and 5.2.3, pg 106-107).

6.2.2 Blowfly larvae collection

The predominant larvae (species: *Chrysomya rufifacies*) infesting the carcass body were collected by using hand and spatula. About 20 to 30 live larvae were cleaned and killed in 80 °C water. The individual larvae were then put into a 50 ml centrifuge tube in dry condition and kept in a -20 °C freezer for subsequent toxicological analysis (Gosselin et al., 2011). The analysis to detect glyphosate was carried out using UV-spectrophotometer, meanwhile detection of coumarin was analysed using GCMS. The analysis of chlorpyrifos from larvae sample was not conducted due to the absence of larvae on carcasses during field experiment as mentioned in Chapter 5 (section 5.3.3.3, pg 119).

6.2.3 Glyphosate detection analysis

6.2.3.1 Stock solution and buffer preparation

The 500 mg/L glyphosate stock solution was prepared by diluting 250 mg of glyphosate analytical standard (Sigma Aldrich, Germany) in 500 ml of distilled water. Meanwhile, 500 mg/L of derivatising agent, 9-fluorenylmethyl chloroformate (Fmoc-Cl) (Sigma Aldrich, Germany) solution was prepared by diluting 250 mg of Fmoc-Cl in 500 ml of acetonitrile. The buffer system used in this analysis was sodium borate buffer (pH 8.5). Sodium borate buffer was prepared by diluting 0.915 g of sodium tetraborate (HmbG®, Malaysia) and 0.469 g of boric acid (Sigma Aldrich, Germany) in 100 ml of distilled water.

6.2.3.2 The standard curve of glyphosate

An initial 500 mg/L of glyphosate analytical standard (Sigma Aldrich, Germany) was prepared by diluting 250 mg of glyphosate in 500 ml of distilled water. Then, a series of 100, 200, 300, 400 and 500 mg/L of glyphosate concentrations were prepared from the initial stock solution. Then, several concentrations of glyphosate standard solution underwent a derivatisation procedure as detailed in Chapter 6 (section 6.2.3.4, pg 152). The mixture was analysed by a UV-Vis spectrophotometer and the absorbance were read at 240 to 310 nm region. The optimal absorbance found in the UV-Vis spectrum were used to plot a glyphosate standard curve.

6.2.3.3 Larvae sample preparation

The larval sample (1 g) was homogenised manually using pestle and mortar. Then, the homogenised sample was put into 50 ml centrifuge tube and added with distilled water (ratio 1:2). The centrifuge tube was vortexed for one minute. Then, 6.5g of QuEChERS Extract Pouch (Agilent Technologies, USA) was added and mixed vortex for another one minute. Next, the mixture was centrifuged at 2,500 *g* for five minutes at room temperature. Then, the supernatant was subjected to clean-up process using SPE kit (Agilent Technologies, USA). The supernatant (1 ml) was transferred into 1.5 ml microcentrifuge tube containing a mixture of anhydrous MgSO₄: PSA (6:1). The tube was mixed vortex for one minute, and then centrifuged again at 9,500 *g* in room temperature for five minutes. The supernatant was collected and filtered through 0.46 µm syringe filter (Biopore, Malaysia). Sample preparation following method by Vetter et al. (2017) with modification. The process was summarised in Figure 6.1.

6.2.3.4 Derivatisation of glyphosate in larvae samples

The procedure for glyphosate derivatisation was following the methods described by Catrinck et al. (2014). The derivatisation agent used was 9-fluorenylmethyl chloroformate (Fmoc-Cl) (Sigma Aldrich, Germany). The reaction medium was prepared by mixing 500 mg/L of larvae sample with 100 mM sodium borate buffer (HmbG®, Malaysia) and 500 mg/L of Fmoc-Cl solution in 2:1:2 ratios. In a 25 ml centrifuge tube, the mixture was homogenised by vortexing for five minutes. Then, two successive wash was performed to remove the excess derivatising agent (Fmoc-Cl) with 7.5 ml of ethyl acetate (Fisher, Denmark). Next, the sample was diluted in sodium borate buffer and analysed using UV visible spectrophotometer (Denovix DS-11 Spectrophotometer) and the absorbance was measured in a wavelength of 265 nm. The procedure for sample preparation and derivatisation was summarised in Figure 6.1.

The details of reagent concentration and volumes are shown in Table 6.1. The glyphosate analytical standard was used as positive control by mixing the 0.5 g/L of glyphosate stock solution and negative control larvae samples in 1:1 ratio. The negative control used was larvae sample collected on rat carcasses which administered with distilled water. The absorbance value reported were interpolated in glyphosate standard curve to determine the trace amount of glyphosate in the larvae samples.

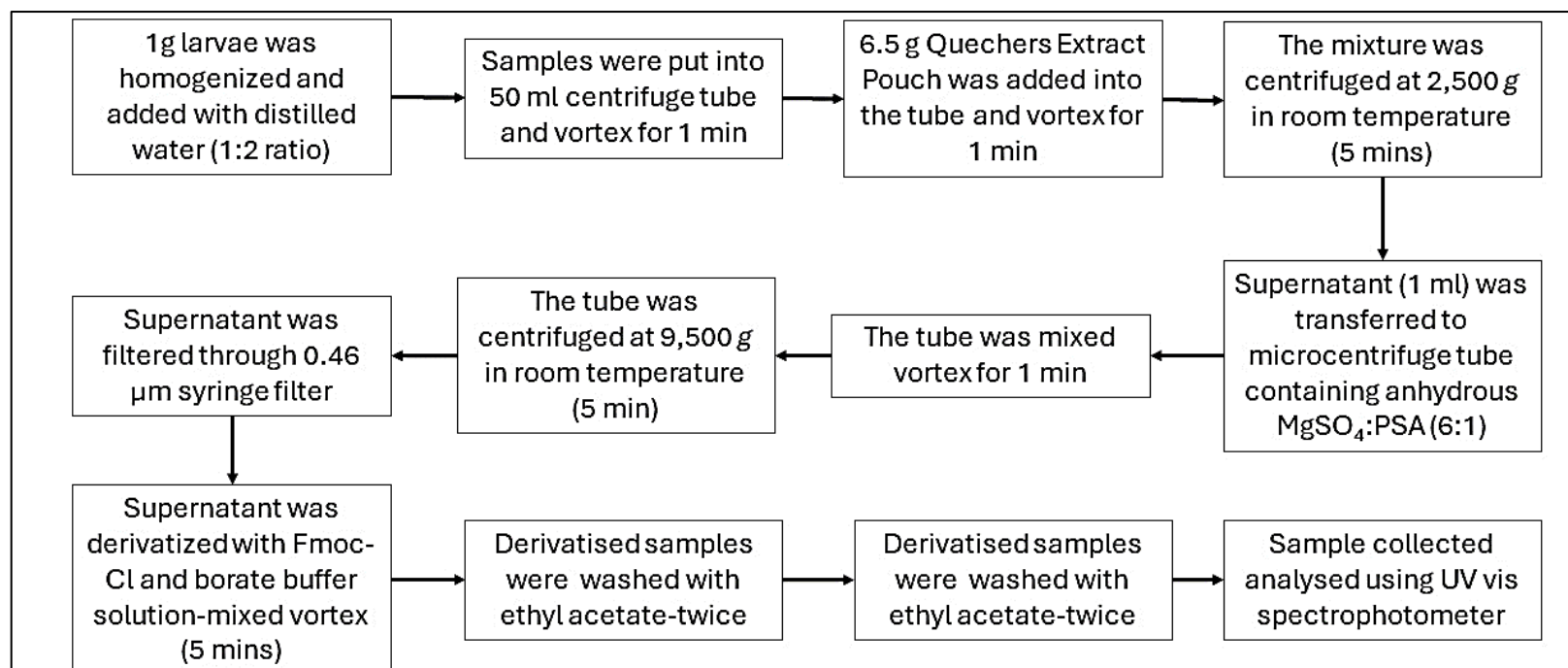


Figure 6.1: Sample preparation and derivatisation for glyphosate detection in larvae tissues.

Table 6.1: Concentration and volumes of reagents used to glyphosate derivatisation.

Reagents	Concentration	Volume
Larvae samples	0.5 g/L	3.750 ml
Sodium borate buffer	100 mM	1.875 ml
Fmoc-Cl	0.5 g/L	3.750 ml
Solvent: ethyl acetate	100%	7.5 ml

6.2.4 Coumarin detection analysis

6.2.4.1 Larvae sample preparation

The extraction procedure was following methods by Vetter et al. (2017) with modifications. Frozen larvae samples were thawed and homogenised manually using mortar and pestle. Ten millilitre of homogenised samples and ceramic homogenisers (Agilent Technologies, USA) were placed in a 50 ml centrifuge tube and 10 ml acetonitrile (Fisher, Korea) was added. The ceramic homogenisers are added to facilitate breaking up matrix and salt agglomerates for higher extraction recoveries of target analytes. The mixture was then vortexed for one minute. Then, 6.5 g of QuEChERS Extract Pouch (Agilent Technologies, USA) was added and vortexed for another one minute and centrifuged for five minutes at 2,500 *g* in room temperature.

The supernatant was subjected to clean-up process using SPE kit (Agilent Technologies, USA). The supernatant (1 ml) was transferred into 1.5 ml vortexed for one minute, and then centrifuged again at 9,500 *g* in room temperature. The supernatant was then transferred into GCMS vial and analysed by GCMS. The positive control was prepared by diluting analytical grade coumarin in acetonitrile in concentration of 0.5 mg/ml. The sample preparation procedure was summarised in Figure 6.2.

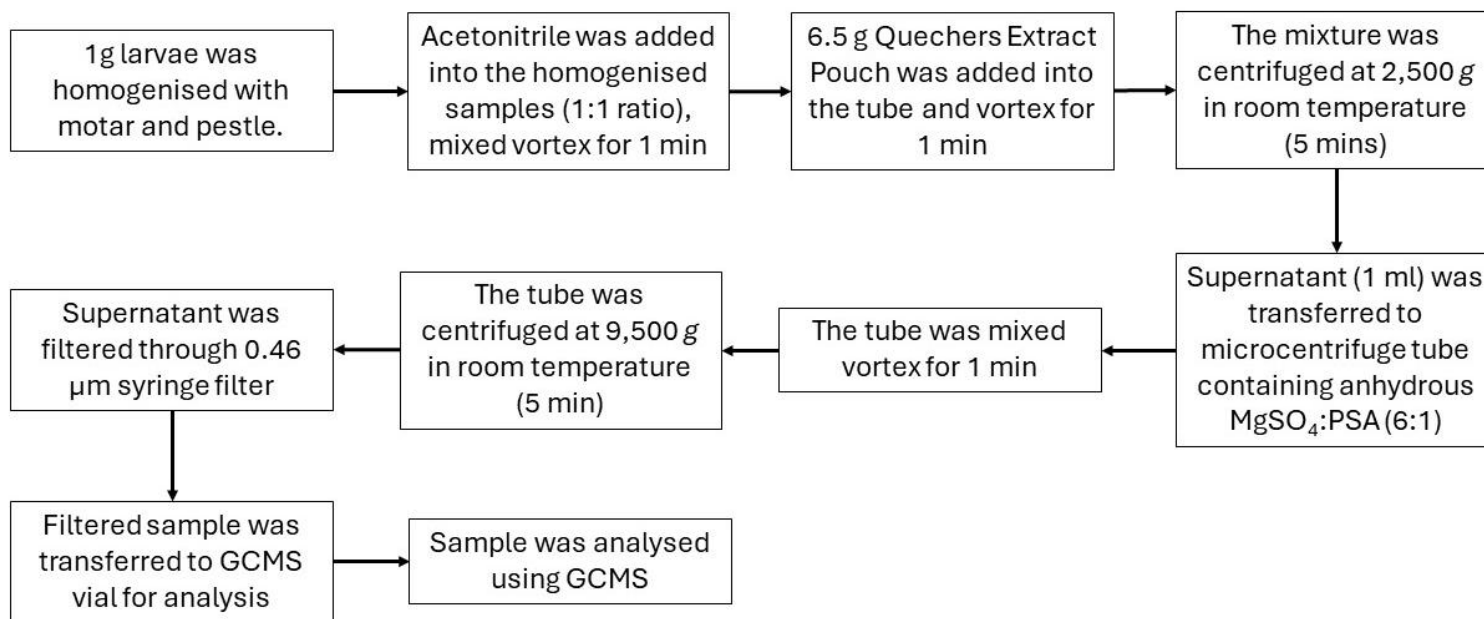


Figure 6.2: Sample preparation procedure for coumarin detection from larvae tissues.

6.2.4.2 Coumarin detection by gas chromatography-mass spectrometry (GC-MS)

The samples of each trial were analysed using Gas Chromatography-Mass Spectrometry (GC-MS) (Shimadzu QP GC-2010 Plus). One microlitre of the sample was injected into GC-MS at a temperature of 270 °C and flow rate of 1.4 ml/min of helium as carrier gas. The separation was carried out using a non-polar column, cross-linked BPX-5 (30 m x 0.25 mm x 0.25 µm film thickness composed of non-polar 5% phenyl methyl siloxane) with the following oven programme: The initial temperature was 50 °C for 2 minutes, then ramping up to 150 °C (25 °C/min), ramping up to 240 °C (10 °C/min) and finally up to 300 °C (50 °C/min) where the temperature was held for one minute. Data was collected in selected ion monitoring (SIM) technique at selected ions (m/z 50-250). The GC-MS method for coumarin detection followed procedure by Vetter et al. (2017). The identification of the analyte was performed by matching the peak obtained from the database in National Institute of Standard and Technology (NIST) library based on the values of the retention time, molecular formula, molecular weight and concentration.

6.2.4.3 Coumarin standard calibration curves

Coumarin standard solution was prepared by diluting 1g of coumarin into 100 ml of acetonitrile. Three concentrations of coumarin standard solution were prepared by diluting several aliquots from the stock solution to make up; 2, 6 and 10 mg/ml standard solutions. The standard solutions were analysed in GC-MS systems by using SIM mode as detailed in previous section 6.2.4.2. The resulting peak area in the chromatograms were used to plot calibration curve to calculate the trace amount of the analyte in the samples.

6.3 Results

6.3.1 Glyphosate detection by ultra-violet (UV) visible spectrophotometer

6.3.1.1 Calibration curve of glyphosate

Five concentrations of analytical standard glyphosate were derivatised in Fmoc-Cl and the mixture was read in spectrophotometer at a wavelength ranged from 240 to 310 nm. The optimal absorbance was read at wavelength of 265 nm (Appendix J1). Therefore, the absorbance at 265 nm was used to plot calibration curve (Appendix J2) and sample analysis in subsequent experiment.

6.3.1.2 Detection of glyphosate compound in larvae samples.

The larvae samples from three trials of experiment that were analysed using ultra-violet (UV) visible spectrophotometer have shown a trace amount of glyphosate compound. The absorbance at 265 nm of the larvae samples were interpolated into the calibration curve. Table 6.2 shows the concentration of the trace amount of glyphosate detected in larvae samples, collected from three trial of experiments. The highest trace amount detected was in T3 larvae samples (9.87 ± 0.18 mg/ml), followed by T1 larvae samples (9.26 ± 0.11 mg/ml) and 6.63 ± 0.55 mg/ml in T2 larvae samples.

Table 6.2: Trace amount of glyphosate detected in larvae samples by ultra-violet (UV) visible spectrophotometer.

Samples	Trace amount (mg/ml)
T1	9.26 ± 0.11
T2	6.63 ± 0.55
T3	9.87 ± 0.18

6.3.2 Coumarin detection by GC-MS

Initial analysis was performed on the standard coumarin at 0.5 mg/ml. shows the ion was eluted at 15.1 minutes (Appendix K1). The analysis of larval samples that were collected on negative control rat carcasses shows separation of several compounds, but there is no coumarin compound detected (Appendix K2). From the GC-MS analysis, it can be observed that all three samples showed the presence of coumarin compound in the larvae tissues (Appendix K3, K4 and K5).

6.3.2.1 Quantification of trace amount of coumarin in the larvae samples

The total peak area obtained from GC-MS analysis was interpolated into the calibration curve of standard coumarin (Appendix L). The highest trace amount of coumarin compound detected was T3 sample (0.355 ± 0.01 mg/ml) and the least coumarin compound detected was in T2 sample (0.222 ± 0.01 mg/ml). Table 6.3 shows the concentration of coumarin compound found in larvae samples in three trials of experiment.

Table 6.3: Quantification of coumarin detected in larvae samples by GC-MS.

Samples	Trace amount (mg/ml) (Mean, $\pm$ S.D.)
T1	0.305 ± 0.00
T2	0.222 ± 0.01
T3	0.355 ± 0.01

6.4 Discussion

The secondary bioaccumulation studies on glyphosate and coumarin from insect samples has not been conducted previously. Therefore, the sample preparation of blowfly larvae was conducted based on the method by Lawai et al. (2015) which investigated secondary bioaccumulation of paraquat with modifications. The blowfly larvae collected

from decomposing rat carcasses were kept in a -20 °C. Specimen storage and preservation is important as it can influence the stability of the analyte for subsequent toxicological analysis. Insect specimens should be kept in -4 °C (Nolte et al., 1992) and the toxicological analysis can be carried out in the same method as human tissues or fluids (Gagliano-Candela & Aventaggiato, 2001). However, it was recommended to keep the specimens in -20 °C (in dry condition) for the stability of the compound of interest (Gosselin et al., 2011). Other method of storage was by preserving it in 70 to 95% ethanol (Amendt et al., 2007), which are not recommended as the analyte of interest can be extracted out from the specimens' matrix into the alcohol.

In past decades, the toxicological samples were analysed using various methods including, radioimmunoassay (RIA), gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), thin-layer chromatography (TLC) (Goff & Lord, 2001), high performance liquid chromatography-mass spectrometry (HPLC/MS) (Heinrikson & Meredith, 1984; Goff & Lord, 2001), infrared spectroscopy (Barbosa et al., 2018), fluorescence analysis (Azab et al., 2015; Feng et al., 2018) and ultra violet (UV) visible spectroscopy (Bazrafshan et al., 2017; Ahmed et al., 2018). In this study, due to the high polarity characteristic of glyphosate, it was analysed using UV visible spectrophotometer, meanwhile for coumarin, it was analysed using GC-MS.

The methods on pesticides determination in fruits and vegetables have been widely published, but very limited literature published on the methods in the analysis of arthropods, such as insects, snails and spiders (Stöckelhuber et al., 2017). The analyses from the previous study were carried out using GCMS/MS have detected several pesticides which include, cyflufenamide, difenoconazole, dimethomorph, fluopicolide, fluopyram, metrafenone,

myclobutanil, quinoxifen, tebuconazole (Stöckelhuber et al., 2017). In Malaysia, several studies have been conducted to detect traces of pesticides in insect tissues. For example, malathion traces were detected in the larvae of *Chrysomya megacephala* which infesting on rabbit carcasses poisoned with malathion (Rashid et al., 2008; Mahat et al., 2012). Another study has also detected the presence of secondary bioaccumulated paraquat in blowfly larvae tissues (Lawai et al., 2015).

Detection and quantification of glyphosate is challenging and cannot be detected using methods that simultaneously detect other chemicals and their metabolites in the same assay (Valle et al., 2019). This is due to its high polarity and solubility in water, poor evaporation, poor solubility in common organic solvents and low volatility (Ibanez et al., 2006; Skeff et al., 2016). Therefore, in conventional detection systems, such as using an ultraviolet (UV) detector, derivatisation reaction is necessary.

Derivatisation is a chemical process that led to a new product with improved chromatographic properties (Shummer et al., 2009). For example, the glyphosate samples were reacted with derivatising agent such as, 9-fluorenylmethyl chloroformate (FMOC-Cl) (Khrolenko & Wieczorek, 2005; Qian et al., 2009; Garba et al., 2018), 2,2,2-trifluoroethanol, trifluoroacetic anhydride (Connolly et al., 2020) or N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) (Hori et al., 2003). Based on the derivatisation reaction methods used, the samples can be analysed by HPLC (Garba et al., 2018), liquid chromatography/mass spectrometry (LCMS) (Malysiak & Kiljanek, 2022), GC-MS (Hori et al., 2003) or UV spectrophotometer (Catrinck et al., 2014; Felton et al., 2018).

Analysing using UV spectrophotometer was fast, easy and efficient compared to analysing using chromatography spectrometry which require longer time for

homogenization and complicated procedure (De Llasera et al., 2005; Peruzzo et al., 2008). In this study, the larvae samples were reacted with FMOC-Cl and analysed using UV visible spectrophotometer. Although this method has been used to detect glyphosate in water, soil and foods (Catrinck et al., 2014; Felton et al., 2018), the trace amount of glyphosate was successfully quantified from insect samples in this study. Besides FMOC-Cl, other compound such as, bis 5-phenyldipyrinate of nickel (II) (Ni (PhDP)₂) (Romero-Natale et al., 2019) and ninhydrin (Bhaskara & Nagaraja, 2006) have been reacted with glyphosate and analysed by UV spectrophotometer.

Coumarin and its derivatives is a naturally occurring compound in plants such as, *Apiaceae* and *Rutaceae*. Coumarin is known to exhibit several biological activities, with a wide range of applications (Matos et al., 2015; Yang et al., 2013) which include as rodenticides (Suzuki, 2005) and various pharmaceutical characteristics (Venugopala et al., 2013), such as, antibacterial (Raja et al., 2012), antifungal (Wang et al., 2009), antiviral (Patil et al., 1993), anticancer (Kostova et al., 2007; Luo et al., 2012) to name a few.

Although coumarin has been identified in a number of plants and food items (Vetter et al., 2017), its detection in fly larvae tissue has not yet been explored. In this study, the detection of secondary bioaccumulated coumarin in larvae tissues was detected using GC-MS by single/selected ion monitoring method (SIM). SIM refers to the use of compound of interest in an expected retention time window. The usage of SIM is useful for quantitative analysis and have high sensitivity and specificity (Sparkman et al., 2011). The molecule peak for coumarin was selected at m/z 146 and the analyte was eluted at 15 minutes.

6.5 Conclusion

This study has confirmed that the glyphosate and coumarin are bioaccumulated inside the blowfly larvae tissues and the detection is possible with the utilization of UV spectrophotometer and GCMS respectively. Quantitatively, glyphosate compound detected was ranged from 6.63 ± 0.55 to 9.87 ± 0.18 mg/ml. Meanwhile for coumarin, the compound was detected in the range of 0.222 ± 0.01 to 0.355 ± 0.01 mg/ml. Other methods should also be explored with consideration on the polarity and non polarity of glyphosate and coumarin.

CHAPTER 7

GENERAL DISCUSSION

The abuse of chemicals and toxins often result in death and are common all over the world. It was reported that in 2015, over 450, 000 people died as a result of drug abuse, and about 160, 000 people die of suicide as a result of pesticide ingestion (Bachmann, 2018). After death, variety of insects are attracted to the body due to odour released from the decomposed body (Rivers & Dahlem, 2022). Blowflies lay their eggs on the body, and the immatures feed on the decomposed tissues and developed until they reach adulthood. Due to their continuous feeding on the carrion, the larvae, pupae and puparial cases are the organic materials that were used as specimen in toxicological analysis (Gagliano-Candela & Aventagglato, 2001).

In entomotoxicology, the field of studies were not only limited to the detection of organic compound in the insect tissues, it also includes the effect of the organic compound or chemicals on the post-mortem interval estimation (Chophi et al., 2019). In forensic entomology, insect, especially flies are used to estimate the PMI time. Several criteria such as the fly development, insect succession pattern and decomposition process were used as tools to calculate the minimum PMI (Zanetti et al., 2015). These tools are heavily influenced by external factors, for example environment conditions, body conditions and the presence of toxins or chemicals (Goff & Lord, 2009). Therefore, the presence of chemicals or organic compounds may alter these tools, hence change the PMI estimation.

In present study, the influence of pesticides, which consists of herbicide, insecticide and rodenticide against the entomological parameters associated with decomposing intoxicated rat carcasses was investigated. The secondary bioaccumulation of the pesticides inside the larvae tissues was subjected into toxicological analysis. From the outcome of the experiment, it was showed that direct exposure of pesticides during larval stage, has delayed the rate of development and affects the size of the larvae compared to the control larvae. Depends on the type of the chemicals tested, the rate of blowfly development can either be decreased or increased. For example, Liu et al. (2009) has reported, the presence of malathion delayed the development of blowfly larvae by 36 hours. In contrast, metamphetamine has caused the increase in the development rate of larvae by 18 hours (Goff et al., 1992).

In this study, the initial experiment was conducted in a laboratory setting to study the effects of the pesticides on fly development. Investigation in laboratory setting is important as it gives the initial outcome on the impact of pesticides or any chemical substance on fly development (Abd Al Galil et al., 2021). The present study was then continued on the field to investigate the effects of these pesticides on the animal carcasses decomposition and the associated insects. The entomological parameters that were observed include, the insect composition and succession pattern of the insects arriving and infesting on the carcasses.

During this experiment, it was found that the decomposition process was delayed for pesticide treated carcasses. The delayed in the decomposition process might be related to the composition of the pesticides and also the lack of insects on the carcasses (de Souza Sandoval & de Ascensão Medeiros, 2013). Among the three pesticides tested in this study, rat carcasses treated with chlorpyrifos-treated rat carcasses showed the longest

decomposition period. In addition, the lack of fly larvae infesting on the carcasses has contributed to the delayed in the decomposition process by ~10 days compared to control carcasses. This observation was in agreement with the previous study where the organophosphate (pirimiphosmethyl)-treated rabbit carcasses did not decompose completely even after 40 days of post-killing (Abd El-bar & Sawaby, 2011). Other study conducted in Kelantan, Malaysia had shown the presence of malathion on the rabbit carcasses has delayed the insect infestation by two days, which eventually caused delay carrion decomposition (Mahat et al., 2008). However, other study where rat carcasses treated with higher dose of terbufos had accelerated the decomposition process in 24 hours compared to the control carcasses (Jales et al., 2011). This shows that different types of chemical compounds affect decomposition process duration in carrion.

Different types of chemicals can act in various ways and produce changes in the life cycle pattern of insects such as shortened or prolonged insects' development time thus, altering the insect succession pattern of the carcasses (Oliviera-costa & Mello-Patiu, 2004). In this study, the insect succession pattern was similar in all groups, which follows the same pattern found in both tropical and temperate areas (Galal et al., 2009). The insect's invasion started with dipteran flies; family Caliphoridae and Sarcophagidae, follows by Muscidae and few adult coleopterans. Blowflies have been observed as the primary coloniser during carcass decomposition in Malaysia (Chin et al., 2007; Azwandi et al., 2013; Maramat & Rahim, 2015; Silahuddin et al., 2015; Adrus & Rahim, 2018), to name a few. Meanwhile for the coleopterans, they colonised later than the flies (Varatharajan & Sen, 2000; Kulshrestha & Satpathy, 2001; Schroeder et al., 2002).

The abundance of colonising insects was influenced by the chemical substance presence on the carcasses, for example calliphorid flies were reduced in chlorpyrifos treated carcasses' group, compared to other groups. The presence of chlorpyrifos may repelled the arrival of flies toward the carcasses, and the flies that arrived were found dead when they tried to burrowed inside the carcasses especially at natural orifices area. This may be due to the strong smell of the insecticide which has masked the decay odour of decomposing carcasses. The use of common household products with strong odours such as; kerosene, insecticide and perfume has altered the arrival of insects toward the carcass and eventually altered the decomposition stages (Ghandour & Attia, 2013). Other compounds such as paint or gas on clothing can delay the carrion insect colonisation Marchenko (2001). Previous study that administered organophosphate by injection has not observe any insect repellent towards the carcasses, as the pesticides did not mask the decay odour of the carcasses. However, the decomposition process was delayed as the flies that arrived were found dead, caused by the pesticides (Abd El-bar & Sawaby, 2011) which was similar to the present study.

The secondary bioaccumulation analysis of blowfly larvae was carried out to detect glyphosate and coumarin in third instar larvae tissues. The toxicological analysis has showed the presence of trace amount of both analyte and was quantified. In entomotoxicology, insect specimens in both larvae and pupae can be subjected to toxicological analysis. However, according to Sadler et al. (1995), the peak concentration of the analyte can be detected during larval stage as a result of rapid feeding of larvae during the second and third instar stage. The concentration of the analyte decreases during prepupal stage and was not detected at all during puparial stage. This may be because both prepupal and puparial stage were non feeding stage (Liu et al., 2009).

The insect's response towards the pesticides is different when compared with the previous studies. This shows that although the pesticides were from the same class, the effects on the insects' activity and development were influenced by the active ingredient of the pesticides. Therefore, further investigations on various pesticides on insects' development needed to be commenced. The interpretation of insects' development and succession must be considered in the case of pesticides or other toxicants intervention during legal investigation.

CHAPTER 8

CONCLUSION AND RECCOMENDATIONS

8.1 Conclusion

The implication of three types of pesticides; glyphosate (herbicide), chlorpyrifos (insecticide), coumarin (anticoagulant rodenticide) on forensic investigation and pesticides secondary bioaccumulation in blowfly tissues were explored. The study was divided into three sections; (1) the effects of pesticides on *Chrysomya megacephala* development; (2) the decomposition and insect succession pattern of pesticide-intoxicated rat carcasses and (3) the detection of secondary bioaccumulated pesticides in blowfly tissues. From the first section of the study, the development of *Ch. megacephala* was delayed when exposed to three different concentrations of pesticides. The rate of blowfly development was similar to the control group when exposed to a lower concentration of pesticides. However, at higher concentration, the rate of larval development was longer, and eventually the larvae died before it reaches the third stage larvae. In a situation where the larvae survive until the puparial stage, the adults failed to emerge. The experiment was conducted in the laboratory, at room temperature.

The second section of the study involved the observation of the decomposition and insect succession pattern of rat carcasses intoxicated with pesticides. Throughout the study period, the daily surface temperature ranged from 23.2 ± 0.2 to 32.5 ± 0.6 °C, with 83.2 ± 1.3 % of humidity. Fluctuated rainfall was observed over the study period, where the highest accumulated rain was recorded during the first trial in January. From the field experiment, the carcasses underwent five stages of decomposition; fresh, bloated, active decay, advanced decay and dry remains. The decomposition was delayed in pesticides-treated carcasses, with

the longest duration for the carcasses to decompose was carcasses treated with chlorpyrifos followed by coumarin-treated carcasses and glyphosate-treated carcasses. The insect succession pattern was similar in all groups, even though smaller population of insects were observed and collected on chlorpyrifos-treated carcasses. The earliest insects to arrive was Diptera (Calliphoridae, Sarcophagidae, Muscidae), and coleopterans beetles were seen on the carcasses during the late stage of decomposition. The predominant species colonising the rat carcasses was *Chrysomya rufifacies*, followed by *Ch. megacephala* and *Hemipyrellia ligurriens*.

The third section of this study was the detection of secondary bioaccumulated pesticides in blowfly larvae tissue. The third stage larvae specimens were collected from glyphosate-treated carcasses and coumarin-treated carcasses. The specimens for chlorpyrifos group were absent due to the lack of larvae present in chlorpyrifos-treated carcasses. The detection of glyphosate and coumarin analyte was carried out using UV visible spectrophotometer and GCMS respectively. The finding showed the presence of trace amount of the analytes, which indicated that the pesticides bioaccumulated inside the larvae tissues which caused decreased development rate of blowfly, hence delayed the carcasses' decomposition process.

The study on the effect of pesticides on the forensic investigation is important as it can alter the post-mortem interval estimation. This is due to the changes in the rate of insects' development and the decomposition duration. Pesticides formulation can also repel the arrival of insects, thus changing the insect succession pattern. Therefore, the presence of pesticides in the insects need to be considered when estimating the age of the insects presents at the site.

8.2 Recommendations

This study aims were to investigate the influence of pesticides on the carcasses' decomposition and insect activities and development. However, the findings can be used as entomological baseline data on the diversity of insects associated with carrion in Sarawak. Forensic entomology study is very limited in Sarawak as well as in Sabah. Generally, in Malaysian Borneo, the entomological data on forensic entomology is still lacking. Most of the studies (including current research) were centralized in Kuching and Kota Samarahan division and have been carried out either in secondary forest or mangrove forest. Therefore, it is recommended in future studies, other habitats such as coastal and interiors area, especially in northern Sarawak such as in Kapit, Belaga, or Limbang where the insects associated with carrion were to be investigated.

In this study, the pesticides tested were agricultural pesticides (glyphosate and chlorpyrifos), and also the active ingredient for anticoagulant rodenticide (coumarin). The pesticides were chosen based on the epidemiological study on pesticides poisoning by Kamaruzaman et al. (2020), where high percentage of pesticides involved were glyphosate, chlorpyrifos and rodenticide. Previous entomotoxicological studies have used several household pesticides (Ortho brand) (Tellez, 2015) and household products such as bleach, perfume, caustic soda and gasoline (Aubernon et al., 2015). However, these studies were conducted outside of Malaysia. It would be a significant finding to explore these local household products and pesticides in Malaysia as an added knowledge in documenting the effects of various chemical substance and its implication on decomposition and insects' activities in Sarawak.

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APPENDICES

Appendix A: Statistical analysis to compare larvae development duration of *Ch. megacephala* between control and glyphosate-treated groups.

ANOVA

PMI

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	86419.667	3	28806.556	155.152	.000
Within Groups	1485.333	8	185.667		
Total	87905.000	11			

Appendix A1: One-way ANOVA analysis showing there is a significant difference of mean development duration of *Ch. megacephala* between control and glyphosate-treated groups.

Multiple Comparisons

Dependent Variable: PMI

Dunnett t (2-sided)^a

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T1	control	-.66667	11.12555	1.000	-32.7045	31.3711
T2	control	44.66667*	11.12555	.010	12.6289	76.7045
T3	control	-176.66667*	11.12555	.000	-208.7045	-144.6289

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Appendix A2: Multiple comparison analysis was performed using Dunnett test. The mean development duration of T2 and T3 glyphosate-treated larvae are significantly different from control group.

Appendix B: Statistical analysis to compare the mean of larvae length of *Ch. megacephala* between control and glyphosate-treated groups.

ANOVA					
Larvae length					
	Sum of	df	Mean	F	Sig.
	Squares		Square		
Between Groups	11.792	2	5.896	.304	.740
Within Groups	620.598	32	19.394		
Total	632.390	34			

Appendix B: One-way ANOVA analysis showing there is no significant difference of mean larvae length of *Ch. megacephala* between control and glyphosate-treated groups.

Appendix C: Statistical analysis to compare larvae development duration of *Ch. megacephala* between control and chlorpyrifos-treated groups.

ANOVA

Development_duration

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	23168.000	3	7722.667	144.800	.000
Within Groups	426.667	8	53.333		
Total	23594.667	11			

Appendix C1: One-way ANOVA analysis showing there is a significant difference of development duration of *Ch. megacephala* between control and chlorpyrifos-treated groups.

Multiple Comparisons

Dependent Variable: Development_duration

Dunnett t (2-sided)^a

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T1	Control	13.3333	5.9628	.130	-3.838	30.504
T2	Control	-10.6667	5.9628	.246	-27.838	6.504
T3	Control	-98.6667*	5.9628	.000	-115.838	-81.496

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Appendix C2: Multiple comparison analysis was performed using Dunnett test. The mean development duration in T3 larvae is significantly different from control group.

Appendix D: One-way ANOVA analysis showing there is no significant difference of mean larvae length between control and chlorpyrifos-treated groups.

ANOVA

Larvae_length

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.035	3	4.345	.233	.873
Within Groups	877.410	47	18.668		
Total	890.445	50			

Appendix E: One-way ANOVA analysis showing there is a significant difference of development duration of *Ch. megacephala* between control and coumarin-treated groups.

ANOVA					
Development_duration					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	70826.667	3	23608.889	134.141	.000
Within Groups	1408.000	8	176.000		
Total	72234.667	11			

Appendix E1: One-way ANOVA analysis showing there is a significant difference of development duration of *Ch. megacephala* between control and coumarin-treated groups.

Multiple Comparisons						
Dependent Variable: Development_duration						
Dunnett t (2-sided) ^a						
(I) Group	(J) Group	Mean	Std. Error	Sig.	95% Confidence Interval	
		Difference (I-J)			Lower Bound	Upper Bound
T1	Control	21.3333	10.8321	.191	-9.859	52.526
T2	Control	-144.0000*	10.8321	.000	-175.193	-112.807
T3	Control	58.6667*	10.8321	.002	27.474	89.859

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Appendix E2: Multiple comparison analysis was performed using Dunnett test. The mean development duration in T2 and T3 larvae is significantly different from control group.

Appendix F: One-way ANOVA analysis showing there is no significant difference of mean larvae length between control and coumarin-treated groups.

ANOVA

Larval length mm

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	251.510	3	83.837	4.717	.005
Within Groups	977.497	55	17.773		
Total	1229.006	58			

Appendix F1: One-way ANOVA analysis showing there is a significant difference of mean larvae length of *Ch. megacephala* between control and glyphosate-treated groups.

Multiple Comparisons

Dependent Variable: Larval_length

Dunnett t (2-sided)^a

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
t1	control	-1.43818	1.74772	.756	-5.7317	2.8553
t2	control	-.49343	1.67916	.983	-4.6185	3.6316
t3	control	-7.52561*	2.08021	.003	-12.6359	-2.4153

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Appendix F2: Multiple comparison analysis was performed using Dunnett test. The mean larvae length of T3 glyphosate-treated larvae are significantly different compared to control group.

Appendix G: Statistical analysis of daily surface temperatures in three trials of experiment.

ANOVA

Daily surface temperature

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	28.542	2	14.271	11.211	.000
Within Groups	61.099	48	1.273		
Total	89.640	50			

Appendix G1: One-way ANOVA analysis showing there is a significant difference of daily surface temperatures in three trials of experiments.

Multiple Comparisons

Dependent Variable: Ambient_temperature

Tukey HSD

(I) Trial	(J) Trial	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T1	T2	-.8235	.3870	.095	-1.759	.112
	T3	-1.8294*	.3870	.000	-2.765	-.894
T2	T1	.8235	.3870	.095	-.112	1.759
	T3	-1.0059*	.3870	.033	-1.942	-.070
T3	T1	1.8294*	.3870	.000	.894	2.765
	T2	1.0059*	.3870	.033	.070	1.942

*. The mean difference is significant at the 0.05 level.

Appendix G2: Multiple comparison analysis was performed using Tukey test. The mean daily surface temperatures in the third trial (T3) is significantly different from every other trials.

Appendix H: Statistical analysis of relative humidity (RH) in three trials of experiment.

ANOVA

RH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	438.621	2	219.311	10.291	.000
Within Groups	1022.965	48	21.312		
Total	1461.586	50			

Appendix H1: Appendix G1: One-way ANOVA analysis showing there is a significant difference of RH in three trials of experiments.

Multiple Comparisons

Dependent Variable: RH

Tukey HSD

(I) Trial	(J) Trial	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T1	T2	1.6588	1.5834	.551	-2.171	5.488
	T3	6.8824*	1.5834	.000	3.053	10.712
T2	T1	-1.6588	1.5834	.551	-5.488	2.171
	T3	5.2235*	1.5834	.005	1.394	9.053
T3	T1	-6.8824*	1.5834	.000	-10.712	-3.053
	T2	-5.2235*	1.5834	.005	-9.053	-1.394

*. The mean difference is significant at the 0.05 level.

Appendix H2: Multiple comparison analysis was performed using Tukey test. The mean RH in the third trial (T3) is significantly different from every other trials.

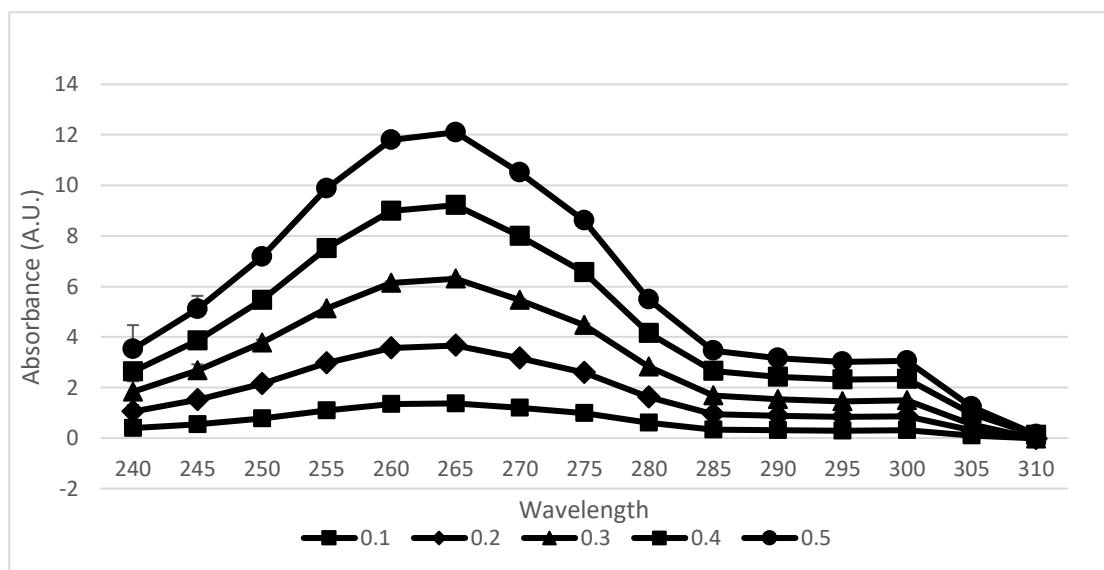
Appendix I: Statistical analysis on comparison of insect composition between groups of carcasses.

ANOVA

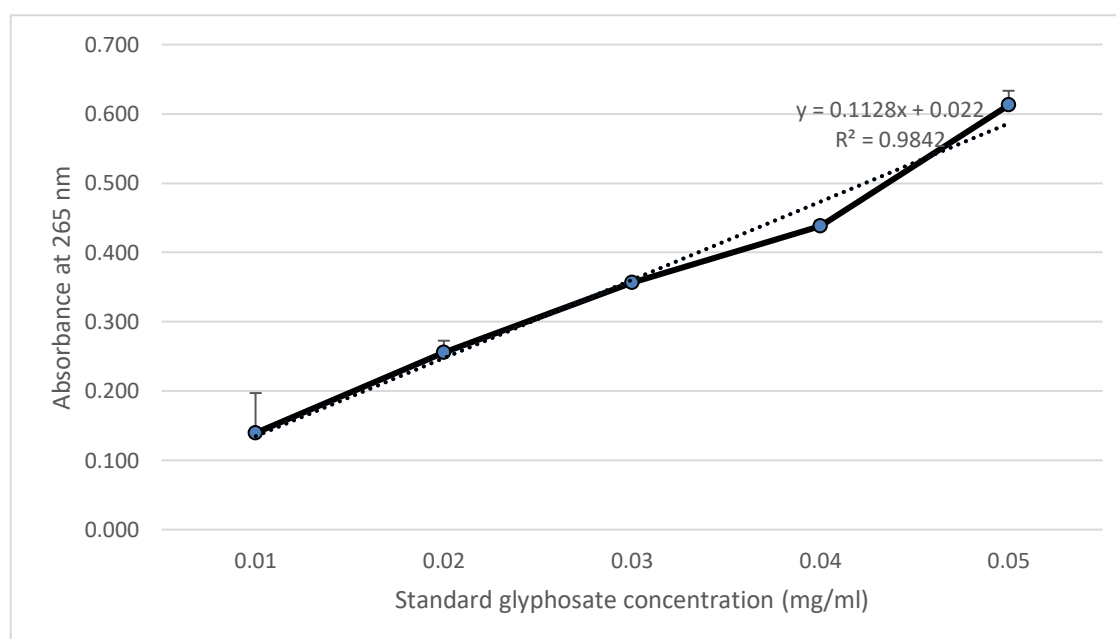
Insects

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5112.054	3	1704.018	.248	.862
Within Groups	356578.786	52	6857.284		
Total	361690.839	55			

Appendix J: Calibration curve of standard glyphosate.

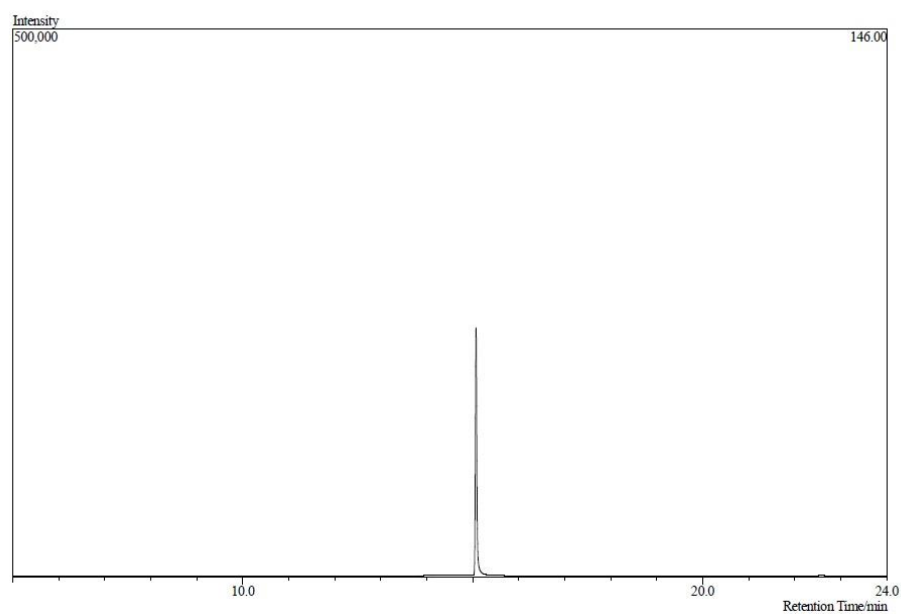


Appendix J1: The spectrophotometer absorbance of five concentrations of analytical standard glyphosate derivatised with Fmoc-Cl was read in wavelength range from 240 to 310 nm, showing the maximum absorbance was read at 265 nm.

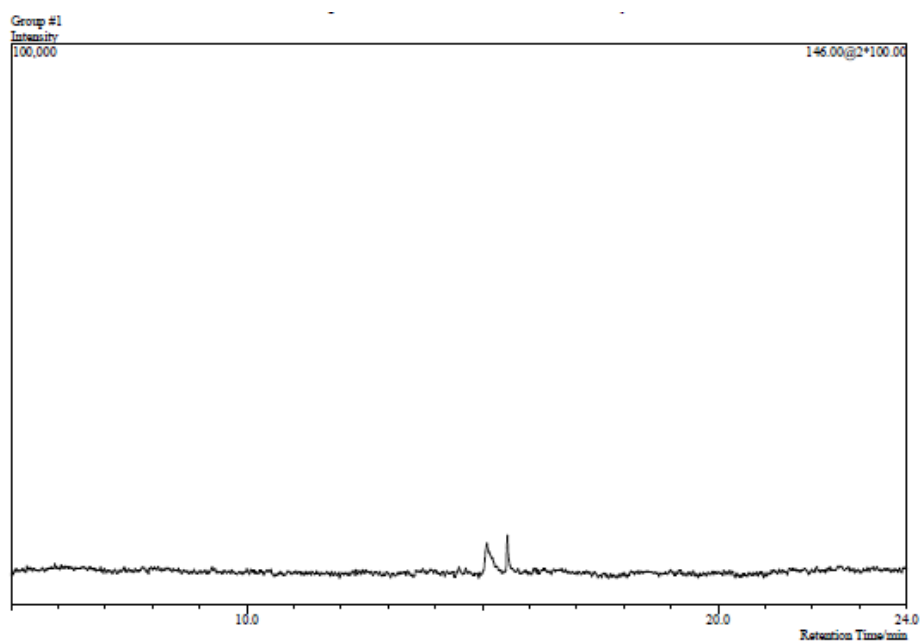


Appendix J2: Calibration curve of standard glyphosate at different concentration.

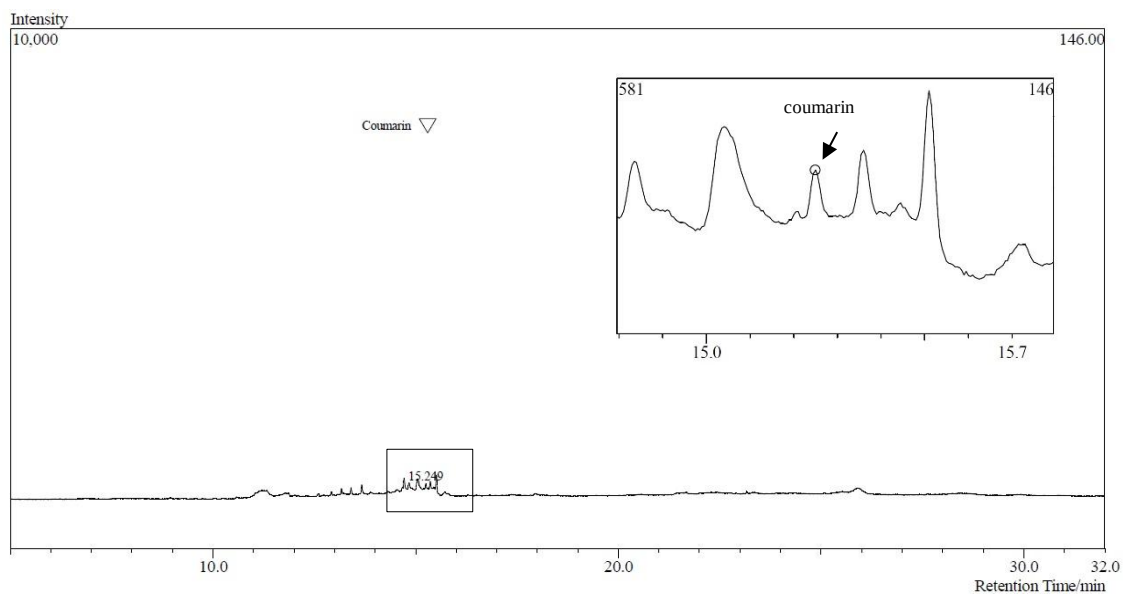
Appendix K: Ion chromatogram of coumarin.



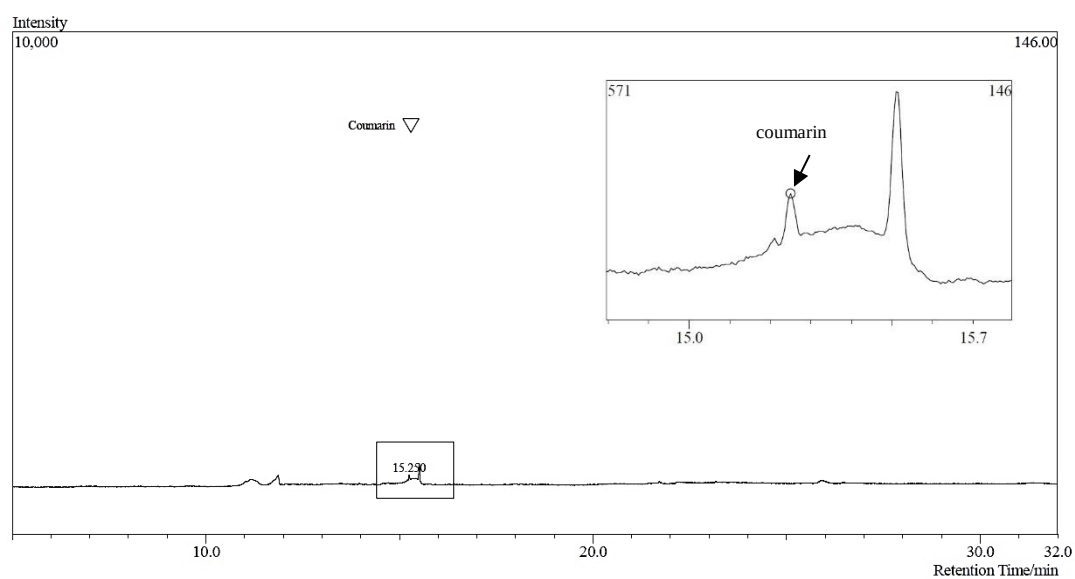
Appendix K1: Ion chromatogram of standard coumarin, eluted at 15.01 minutes.



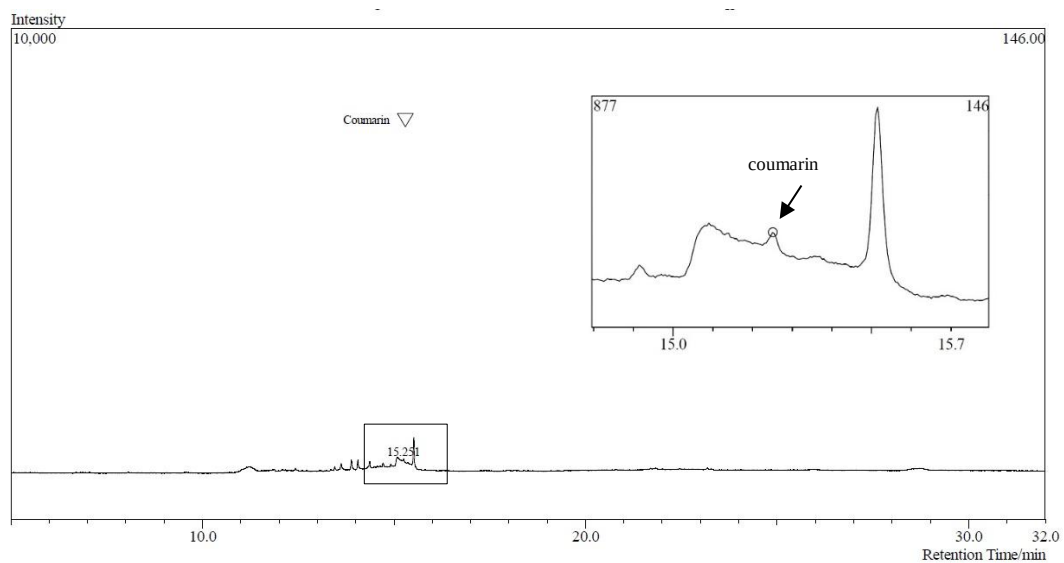
Appendix K2: Ion chromatogram of negative control larvae samples, showing there is no coumarin compound detected in the samples.



Appendix K3: GC-MS ion chromatogram shows the peak of coumarin compound detected at 15.240 minutes in T1 larvae sample.

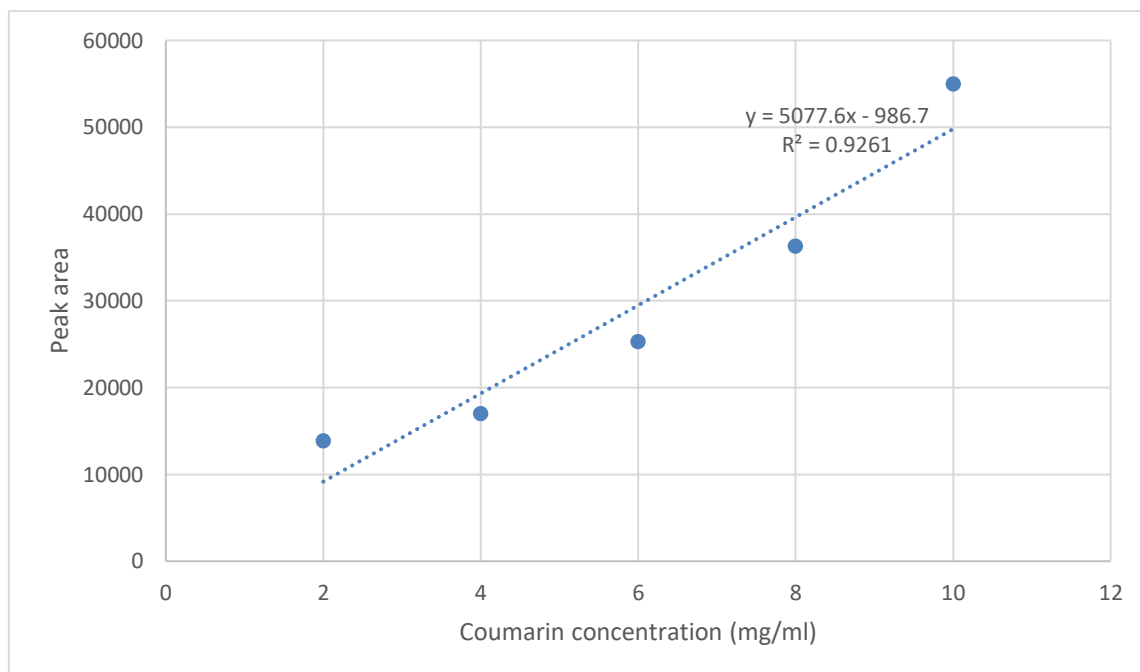


Appendix K4: GC-MS ion chromatogram showing the coumarin compound detected at 15.290 minutes in T2 larvae samples.



Appendix K5: Ion chromatogram shows the peak of coumarin compound detected at 15.251 in T3 larvae sample.

Appendix L: Calibration curve of coumarin based on the standard coumarin detected by GCMS.



Appendix M: The flies (Order: Diptera) collected at the study site



Appendix N: Other insects collected at the study site.

