INFLUENCE OF RAIN, MALATHION, SUNLIT AND SHADED HABITATS ON THE DURATION OF DEVELOPMENT AND MOLECULAR IDENTIFICATION OF *Chrysomya megacephala* (FABRICIUS) (DIPTERA: CALLIPHORIDAE) IN KELANTAN, MALAYSIA

by

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UNIVERSITI SAINS MALAYSIA

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LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

ADD	Accumulated Degree Days
ADH	Accumulated Degree Hours
ANOVA	Analysis of Variance
A	Adenine
Apr	April
ATP	Adenosine triphosphate
ATSDR	Agency of Toxic Substances and Disease Registry
a.m	*Ante meridiem*
Aug	August
bp	Base pair
C	Cytosine
CO1	Cytochrome oxidase 1
cm	Centimeter
CM	*Chrysomya megacephala*
CR	*Chrysomya rufifacies*
CV	Coefficient variation
CNKK	*Chrysomya nigripes* collected in Kubang Kerian
DNA	Deoxyribonucleic acid
Da	Dalton
Dec	December
dNTPs	Deoxyribonucleotide triphosphate
E	East
Exp	Experiment
Feb	February
G	Guanine
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HLKK	*Hemipyrellia ligurriens* collected in Kubang Kerian
JKM	Jabatan Kimia Malaysia
Jan	January
Jul	July
Jun	June
KL	Kuala Lumpur
KT	Kuala Terengganu
KK	Kubang Kerian
LCKK	*Lucilia cuprina* collected in Kubang Kerian
LOD	Limit of detection
LOQ	Limit of quantitation
Mar	March
MMD	Malaysian Meteorological Department
MS	Mass Spectrometry
mtDNA	Mitochondrial DNA
N	North
n	Sample size/replicate
NIST	National Institute of Standards and Technology
NC	Negative control
Nov	November
Oct	October
PMI	Postmortem interval
PCR	Polymerase Chain Reaction

PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
PC	Positive control
PG	Penang
p	Probability
pH	Potential of hydrogen
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RAPD	Random amplified polymorphic DNA
rpm	Revolution per minute
SD	Standard deviation
SDKK	*Sarcophaga dux* collected in Kubang Kerian
Sept	September
T	Thymine
T1	Malathion-treated 1
T2	Malathion-treated 2
T3	Malathion-treated 3
UV	Ultraviolet
USM	Universiti Sains Malaysia
USA	United States of America
UK	United Kingdom

SYMBOLS

A_{260}	Absorbent at 260 nm wavelenght
A_{280}	Absorbent at 280 nm wavelenght
g	Gram
Kg	Kilogram
Km	Kilometre
$MgCl_2$	Magnesium chloride
m	Metre
mL	Mililitre
mm	Milimetre
m/z	Mass-to-charge ratio
ng	Nanogram
µL	Microlitre
µg	Microgram
°C	Degree Celcius
r^2	Regression
r	Correlation coefficient
Psi	Pound per square inch pressure unit
V	Volt

LIST OF PUBLICATIONS AND AWARD RECEIVED

PUBLICATIONS

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Mahat, N.A., Jayaprakash, P.T. & Zafarina, Z., 2008. Necrophagous infestation in rabbit carcasses decomposing in Kubang Kerian, Kelantan, *Mal. J. Med. Sci.* 15 (supplement 1), 124.—Abstract for the 13th National Conference on Medical Sciences, 22-23 May 2008, Kota Bharu, Kelantan.

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PENGARUH HUJAN, MALATHION, HABITAT TERBUKA DAN TERLINDUNG TERHADAP TEMPOH PERKEMBANGAN DAN PENGENALPASTIAN *Chrysomya megacephala* (FABRICIUS) (DIPTERA: CALLIPHORIDAE) SECARA MOLEKULAR DI KELANTAN, MALAYSIA

ABSTRAK

Chrysomya megacephala (Fabricius) merupakan spesies nekrofagus terawal dan paling lazim menginfestasi mayat di Malaysia, justeru memberikan anggaran selang masa kematian (PMI) yang paling tepat. Di Malaysia, hujan lebat/hujan berterusan merupakan suatu fenomena yang biasa manakala malathion ialah racun yang paling lazim digunakan untuk membunuh diri. Dalam kajian ini, pengaruh hujan dan kehadiran malathion secara berasingan dan bersama pada bangkai arnab yang mereput di habitat terbuka dan terlindung terhadap pengovipositan dan perkembangan *Ch. megacephala* dikaji selama setahun di Kelantan, Malaysia bagi menyediakan data asas untuk menganggar PMI. Jujukan bes gen CO1 digunakan bagi tujuan pengenalpastian spesies dan pengaruh malathion terhadap jujukan bes gen CO1 *Ch. megacephala* juga dikenalpasti. Kebolehgunaan larva dan pupa *Ch. megacephala* dalam mengesan kehadiran malathion bagi mendiagnos sebab kematian terutamanya apabila organ-organ visera telah mengalami pereputan juga dikaji. Secara amnya, bergantung kepada intensiti, hujan boleh menunda pengovipositan awalan selama 1-2 hari dan memanjangkan tempoh pempupaan selama 1-3 hari. Kehadiran malathion dalam bangkai melambatkan pengovipositan awalan selama 1-3 hari dan memanjangkan tempoh pempupaan selama 2-3 hari. Kehadiran hujan dan malathion secara kumulatif memanjangkan tempoh pempupaan sehingga 8 hari. Malathion didapati tidak memberi kesan terhadap pengenalpastian spesies menggunakan jujukan bes fragmen gen CO1 yang diamplifikasi menggunakan primer C1-J-2495/C1-N-2800. Pengesanan malathion dalam larva dan pupa berkemungkinan boleh digunakan bagi mendiagnos sebab kematian, memandangkan malathion merupakan racun yang boleh menyebabkan kematian berbanding dadah-dadah yang disalahguna. Hasil kajian ini perlu dipertimbangkan semasa menganggar

PMI bagi mayat yang dijumpai di Malaysia semasa musim hujan dan musim kurang hujan serta dalam keadaan di mana sebab kematian dijangkakan oleh keracunan malathion.

INFLUENCE OF RAIN, MALATHION, SUNLIT AND SHADED HABITATS ON THE DURATION OF DEVELOPMENT AND MOLECULAR IDENTIFICATION OF *Chrysomya megacephala* (FABRICIUS) (DIPTERA: CALLIPHORIDAE) IN KELANTAN, MALAYSIA

ABSTRACT

Chrysomya megacephala (Fabricius) is the earliest and the most prevalent necrophagous species infesting dead bodies in Malaysia and thus, may provide the best estimate of minimum postmortem interval (PMI). In Malaysia, heavy and/or incessant rain is commonplace occurrence and malathion is the most prevalent poison used for committing suicide. In this research, the independent and combined influences of rain and presence of malathion in whole rabbit carcasses decomposing in sunlit and shaded habitats on oviposition and development of *Ch. megacephala* were studied over a period of 1-year in Kelantan, Malaysia for providing baseline data for estimating PMI. Base sequence of CO1 gene was used for species identification and the influence of malathion on the sequence of CO1 gene of *Ch. megacephala* was determined. The possible use of larvae and pupae of *Ch. megacephala* for detecting malathion for diagnosing the cause of death especially when visceral organs have liquified was also investigated. In general, rain, depending on its intensity, delayed initial oviposition by 1-2 days and prolonged the pupation period by 1-3 days. The presence of malathion in the carcasses delayed initial oviposition by 1-3 days and prolonged the pupation period by 2-3 days. Presence of rain and malathion cumulatively prolonged pupation period that extended to a maximum of 8 days. It was found that malathion did not impede the accuracy of species identification while using the base sequence of CO1 gene fragment amplified by C1-J-2495/C1-N-2800 primers. The detection of malathion in larvae and pupae *per se* may prove useful in diagnosing the cause of death, since malathion is a poison as against the other drugs that are abused. These findings deserve consideration while estimating PMI relating to decomposed bodies recovered in

Malaysia during both the less rainy and rainy months of a year as well as when malathion poisoning is suspected.

CHAPTER 1

INTRODUCTION

1.1 Proposed problems and their relevance for forensic practice in Malaysia

Chrysomya megacephala (Fabricius) is the earliest and the most prevalent necrophagous species found infesting dead bodies in Malaysia followed by *Chrysomya rufifacies* (Macquart). Similar to the other dipterans, the developmental stages of *Ch. megacephala* is prone to alter in response to environmental conditions. However, the possible influences of the environmental conditions prevailing in Malaysia on the development of this species have not been reported. In addition, malathion being the most prevalent poison used for suicidal purposes in Malaysia, its influence on the larvae of *Ch. megacephala* and the possible impact on death investigations deserve to be studied. Consequently, the effect of malathion in the larvae has to be studied when the larvae are used for molecular-based technique for identification of species since malathion is a known genotoxic substance and the technique of molecular-based species identification is gaining global popularity. Considering the potential scope for utilizing entomological data in practical crime investigations in Malaysia, this research was designed covering the three broad aspects detailed below.

1.1.1 Problem 1: Influences of rain, habitats and malathion on infestation and development of *Ch. megacephala*

The significant contribution of forensic entomology in crime investigation is in providing reliable estimation of the minimum postmortem interval (PMI), especially in cases where dead bodies are found during the later stages of

decomposition (Greenberg & Kunich, 2002; Gennard, 2007). The reliability of the estimated PMI depends on infestation and development patterns of necrophagous insects, which are influenced by biogeoclimatic factors such as rain (Mann et al., 1990; Grassberger & Frank, 2004), sunlit and the shaded habitats (Sharanowski et al., 2008) and presence of toxic substances (Gunatilake & Goff, 1989; Gennard, 2007). In Malaysia, rain is a commonplace occurrence (Malaysian Meteorological Department, MDM, 2007) and malathion (an organophosphorus insecticide) is the most prevalent poison used for committing suicides (Maniam, 1988; Jabatan Kimia Malaysia, JKM, 2009, personal communication). However, research on the effects of the independent and combined influences of rain and presence of malathion on the infestation and development patterns of necrophagous species has not been reported. The first part of this research was a year long study on the effects of the independent and combined influences of rain, sunlit and the shaded habitats and, presence of malathion on the infestation and development of *Ch. megacephala* for providing the baseline data that can be useful for estimating PMI.

1.1.2 Problem 2: Influence of malathion on molecular-based technique for species identification of *Ch. megacephala* in Malaysia

Estimation of PMI requires accurate taxonomic identification mostly involving the immature forms of necrophagous insects (Benecke & Wells, 2001; Greenberg & Kunich, 2002; Gennard, 2007). It has also been recognized that the taxonomic identification of larvae of necrophagous species using morphological characteristics is difficult, especially when they are closely related (Gennard, 2007; Wells & Stevens, 2008). In addition, the available rearing procedure is time consuming and may even cause considerable problems if rearing fails (Amendt et al.,

2004). To overcome these problems, molecular-based identification techniques such as the use of cytochrome oxidase 1 (CO1) gene has been suggested and is gaining global popularity (Gennard 2007; Wells & Stevens, 2008). In Malaysia, the application of such techniques for forensic use is in its nascent stage. Considering the potential for utilizing molecular-based technique for identification of species in Malaysia, it becomes pertinent to investigate the effect of the prevalently used malathion on the identification process since it is a known genotoxic substance (e.g. Flessel et al., 1993; Bonilla et al., 2008). Furthermore, the stability of the base sequence in the fragment of the CO1 gene that is chosen for amplification in the different geographical locations in Malaysia also deserves to be studied for assuring the accuracy of the molecular-based technique for species identification at the national level.

1.1.3 Problem 3: Malathion extraction from the larvae of *Ch. megacephala* for determining death due to malathion

The use of entomological specimens for diagnosing the presence of drugs in decomposing bodies has been demonstrated by many researchers (Introna et al., 1990; Kintz et al., 1990; Miller et al., 1994; Sadler et al., 1997a; Goff et al., 1997; Hedouin et al., 2001). Those studies focused on drugs of abuse and/or therapeutic drugs that seldom caused death and hence the results were useful in indicating the circumstances surrounding death such as addiction, rather than the actual cause of death. A significant contextual difference introduced in this research is that the detection of malathion in larvae, in contrast to the detection of drugs of abuse, would enable diagnosing the cause of death since malathion is a known poison and not a drug of abuse. The circumstances necessitating the use of larvae for malathion

detection would include similar situations reported for other entomotoxicological analysis such as advanced stages of decomposition where organs are not identifiable for sampling (Goff & Lord, 2001; Greenberg & Kunich, 2002; Gennard, 2007) or absence of body in instances of secondary disposal.

1.2 Objectives

The objectives of this research were:

1. To determine the independent and combined influences of rain, sunlit and shaded habitats as well as the presence of malathion on initial oviposition and development of *Ch. megacephala* and to provide baseline data that can be useful for calculating PMI.
2. To study the utility of a CO1 fragment for identifying *Ch. megacephala* and other dipteran species of forensic importance infesting rabbit carcasses in Kelantan as well as *Ch. megacephala* and *Ch. rufifacies* sampled from several geographical locations in Malaysia, in view of recent popularization of molecular-based techniques for taxonomic identification.
3. To evaluate the utility of the CO1 fragment for taxonomic identification of larvae of *Ch. megacephala* grown in the presence of malathion.
4. To study the potential use of larvae and pupae for detecting malathion for the purpose of diagnosing the cause of death in the context of suicide.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to the literature review

Utilization of insects' developmental data, particularly Calliphoridae in providing accurate estimation of PMI has been extensively reported in literature (Wells & LaMotte, 2001; Greenberg & Kunich, 2002; Gennard, 2007) and in Malaysia, *Ch. megacephala* is the first and the most prevalent Calliphorid species recovered from decomposing corpses (Hamid et al., 2003; Lee et al., 2004; Kumara et al., 2010). It has been shown that the development of Calliphorids is influenced by variations in climatological conditions viz. temperature and rain (Mann et al., 1990; Grassberger & Frank, 2004), differences in sunlit and the shaded habitats (Joy et al., 2006; Sharanowski et al., 2008) as well as the presence of toxic substances (Introna et al., 2001; Amendt et al., 2004). For estimating PMI, the accuracy in taxonomic identification is critical and in this context, molecular-based techniques for species identification of insects are gaining global popularity (Greenberg & Kunich, 2002; Gennard, 2007; Wells & Stevens, 2008). Malathion a known genotoxic substance is found to be a poison prevalently used for suicides in many countries (Thompson et al., 1998; Pannell et al., 2001) and especially in Malaysia (JKM, 2009, personal communication). The following review of literature relates to three broad aspects, all pertaining to the development of *Ch. megacephala* in Malaysia namely; (1) influence of the climatological factors, (2) influence of the presence of drugs especially malathion and (3) stability of the characteristic gene sequence useful for molecular-based identification when larvae were grown in the presence of the genotoxic substance, malathion.

2.2 Utility of forensic entomology in estimating PMI

2.2.1 Role of insects in death investigation

It has been reported that insects are attracted to a body within minutes of death (Anderson & VanLaerhoven, 1996; Dillon, 1997; Greenberg & Kunich, 2002). This has been attributable to various factors such as the presence of ammonia-rich compounds, hydrogen sulphide and amount of moisture in the decomposing body (Fisher et al., 1998; Anderson, 2001). Smith (1986) divides insects that are associated with a decomposing body into four ecological categories viz. necrophagous species, predators and parasites of necrophagous species, omnivorous species and adventive species. Necrophagous species that include Calliphoridae, Dermestidae and Silphidae are insects that feed on the corpse itself, responsible for majority of the biomass loss and within this category Calliphoridae are considered as the most important insects for estimating PMI (Amendt et al., 2004). Predators and parasites of necrophagous species include Silphidae, Staphylinidae and some necrophagous species that becomes predacious during the later instar stage (Smith, 1986; Omar et al., 1994a; Amendt et al., 2004). Omnivorous insects that include wasps, ants and some Coleoptera feed on the corpse and on its inhabitants, while the adventive insects (springtails and spiders) use the corpse as an extension of their environment and may become incidental predators (Campobasso et al., 2001).

In Malaysia, *Ch. megacephala* is the first and the dominant necrophagous species recovered from dead bodies as well as in animal carcasses followed by *Ch. rufifacies* (Lee, 1989; Omar et al., 1994b; Hamid et al., 2003; Lee et al., 2004; Heo et al., 2007). Since the infestation of other necrophagous species such as *Chrysomya nigripes* (Aubertin) and *Chrysomya villeneuvi* (Patton) occurs following that of *Ch.*

megacephala (Omar et al., 1994a; Lee, 1996; Lee et al., 2004), the applied values of utilizing these species for estimating PMI in Malaysia are limited. *Chrysomya megacephala* are widely distributed throughout the Oriental and Australasian regions and has recently been introduced in the Afrotropical and Neotropical regions (Kurahashi et al., 1997; Byrd & Castner, 2001). In Malaysia, *Ch. megacephala* is found throughout the year at various altitudes, up to 2000 meter above sea level (Kurahashi et al., 1997; Omar et al., 2003a). Byrd and Castner (2001) described the general appearance of *Ch. megacephala* adult as similar to that in *Ch. rufifacies*, but with a noticeably larger head and prominently red eyes. Detailed description on the morphology of *Ch. megacephala* adults provided by Kurahashi et al. (1997) included: fuscous (grey-black) prothoracic spiracles, orange-yellow gena and postgenal areas with pale-yellow hairs except in the area immediately around vibrissae, at least the posterior lower squama being distinctly infuscated and eye facets being conspicuously enlarged. Later, Omar (2002) provided a detailed description on the morphology of the third instar larvae of *Ch. megacephala* that included: mildly sclerotized incomplete peritremes of posterior spiracles, absence of conical tubercles on body segments, presence of dorsal arch and 'dot or club-shaped' structure in cephalopharyngeal skeleton and the anterior spiracles consisting of 11-13 papillae.

In the context of infestation by *Ch. megacephala* and *Ch. rufifacies* in the same corpse/carcass, it has been found that *Ch. rufifacies* infestation invariably succeed that by *Ch. megacephala* (Lee, 1989; Omar et al., 1994b; Hamid et al., 2003; Lee et al., 2004; Heo et al., 2007). Furthermore, researchers have indicated that maggots of *Ch. rufifacies* develop predatory and cannibalistic behaviours during its

third instar stage (Omar et al., 1994a, 1994b; Bharti & Singh, 2003; Ahmad & Ahmad, 2009). In Malaysia, predatory behaviour of third instar larvae of other necrophagous species such as *Chrysomya villeneuvi* and *Ophyra spinigera* has been observed in the field (Omar et al., 1994a; 1994b).

2.2.2 Factors influencing the development of Calliphorids

Two factors that influence the development of calliphorids are biogeoclimatic factors (Mann et al., 1990; Campobasso et al., 2001; Greenberg & Kunich, 2002; Grassberger & Frank, 2004; Gennard, 2007) and presence of toxic substances (Gunatilake & Goff, 1989; Bourel et al., 1999; Musvaska et al., 2001; O'Brien & Turner, 2004; Arnaldos et al., 2005). In geographical regions with defined seasons such as summer, spring, autumn and winter in a year, the growth rate of insects has been found to be influenced by sunlit and the shaded habitats as well as the difference in seasons, largely due to the differences in ambient temperature (Joy et al., 2006; Sharanowski et al., 2008). Moreover, rain in general, inhibits adult activity (Nuorteva, 1977; Mann et al., 1990; Campobasso et al., 2001; Greenberg & Kunich, 2002; Grassberger & Frank, 2004; Gennard, 2007) and prolongs the development of Calliphorids (Nuorteva, 1977; Mann et al., 1990; Greenberg & Kunich, 2002; Grassberger & Frank, 2004). In the context of Malaysia being different in its yearly seasons as well as annual rainfall, review of the literature does not reveal research on the development of calliphorids as influenced by less rainy and rainy seasons or heavy and/or incessant rain that are commonplace in this country.

In addition, studies have shown that the development of Calliphorids can be influenced by drugs and poisons (Utsumi, 1958; Gunatilake & Goff, 1989; Goff et

al., 1989, 1991; Bourel et al., 1999; Musvaska et al., 2001; O'Brien & Turner, 2004; Arnaldos et al., 2005). It has been reported that opiates (Goff et al., 1989; Lord, 1990; Goff et al., 1991; Bourel et al., 1999) and amphetamines (Goff et al., 1992, 1997) accelerate the development of both *Lucilia sericata* (Calliphoridae) and *Parasarcophaga ruficornis* (Sarcophagidae). On the other hand, no difference was observed in the development of *Parasarcophaga ruficornis* larvae reared on tissues that contained therapeutic drugs (amitriptyline and phencyclidine) when compared with that in controls (Goff et al., 1993, 1994). In a case of suicide using malathion, Gunatilake and Goff (1989), stressed on the importance of considering the possible effect of the ingested malathion on the development of *Ch. megacephala* and *Ch. rufifacies*. In a laboratory-controlled study, the larval stages of *Ch. megacephala* reared on dissected liver tissues obtained from the malathion-exposed rats, were observed to be longer when compared with that in control (Abd-Rashid et al., 2008). In this context, it is pertinent to quote that any factors mitigating insect development have the potential of affecting subsequent insect-based estimates of postmortem interval (Goff & Lord, 2001). However, review of the literature does not indicate data relating to the influence of the different concentrations of ingested malathion in a whole decomposing carcass on initial oviposition and development of calliphorids under field conditions. On the other hand, it has been found that there has been an apparent increase in the incidence of drug related death and in many of such cases the bodies are not recovered for a substantial period of time (Goff & Lord, 2001). In the context of poison related death in Malaysia, it is seen that malathion is more prevalent (JKM, 2009, personal communication). Thus, it was found pertinent to study the influence of ingested quantities of malathion in decomposing carcasses on calliphorids development.

2.2.3 Estimating PMI: historical perception

The first case which utilized entomological evidence in estimating PMI was reported by Bergeret in 1855, who analyzed the insect assemblage in an infant corpse and concluded, perhaps incorrectly, that the infant died two years ago leading to the prosecution of the murderer (Smith, 1986; Catts & Goff, 1992; Hall, 2001; Greenberg & Kunich, 2002; Amendt et al., 2004; Gennard, 2007). Later, Megnin (1894) characterized eight stages of human decomposition and the insect taxa that were associated with them and this knowledge about insect succession became the basis for estimating PMI (Lee, 1989; Hall, 2001; Gennard, 2007). During the first 72 hours after death, estimation of PMI based on pathological changes is reasonably accurate (Gennard, 2007). However, as the time since death increases, the pathological changes become less useful for estimating PMI, while necrophagous insects recovered from the corpse may provide more accurate PMI estimate (Wells & LaMotte, 2001; Amendt et al., 2004; Gennard, 2007). Estimation of PMI depends on the accuracy in estimating the age of the larvae and for that purpose, various approaches have been suggested (Wells & LaMotte, 2001; Amendt et al., 2004). Pertinently, all these attempts are based on the fact that insects are poikilothermics and therefore, their developments depend on the prevailing ambient temperature (Wells & LaMotte, 2001; Amendt et al., 2004). The relevant approaches for estimating PMI are discussed below.

2.2.3(a) Thermal summation approach

Thermal summation approach for estimating PMI, introduced by Wigglesworth (1972), is based on the assumption that the relationship between the developmental rate and the ambient temperature is linear within the mid-range of the

sigmoidal developmental curve (Greenberg & Kunich, 2002). It relates to the fact that insects are poikilothermics, using environment as the source for heat for their growths and the thermal units are known as the accumulated degree days (ADD) or the accumulated degree hours (ADH) (Gennard, 2007). In this aspect, the ambient temperature data covering the period when the deceased was last seen alive until the time the corpse was recovered must be obtained from the nearest meteorological station (Amendt et al., 2004; Gennard, 2007). The obtained ambient temperature data must be corrected using a 'correction factor' obtained from the regression equation between the ambient temperature data from the meteorological station versus the half-hourly ambient temperature data recorded at the crime scene for three to five consecutive days after the corpse was discovered (Amendt et al., 2004; Gennard, 2007). Upon obtaining all the relevant climatological data and the base temperature for the observed insect species, the ADH or ADD can be calculated and ultimately, PMI can be estimated.

2.2.3(b) Degree of development approach

This approach for estimating PMI is based on the assumption that larvae of the same age hatch and molt in a relative synchrony (Davies & Ratcliffe, 1994; Wells & Kurahashi, 1994; Wells & LaMotte, 1995). Hence, the age of a larva can be estimated from its size and dry weight using the appropriate growth curve (Wells & LaMotte, 2001) and this approach may be useful in countries where the observed fluctuation in ambient temperature is less. Since most of the development data were obtained under constant temperatures, while temperature at crime scene is fluctuating, the time period under consideration must be divided into shorter intervals (e.g. 12 hours) (Williams, 1984) and the closest developmental model to the mean

temperature recorded during each period can be applied for estimating PMI (Wells & LaMotte, 2001).

Interestingly, Schoenly (1992) prescribed estimation of PMI using the computer model that they developed. In this aspect, it is pertinent to indicate that the data obtained in laboratory need to be compared with the data gathered under field conditions for utilization in human death investigation (Byrd & Allen, 2001).

2.2.4 Forensic entomology in Malaysia

Researches relating to forensic entomology in Malaysia include retrospective compilation of the larval species recovered from corpses referred to major hospitals (Hamid et al., 2003; Kumara et al., 2010) and to the Institute for Medical Research, Kuala Lumpur (Lee et al., 2004) apart from the following experimental studies. Ecologically, Omar et al. (2003b) studied the synanthropic index of flies in Malaysia and identified seven species as hemisynanthropes and the highest level being *Ch. megacephala*. Six monkeys were studied in two seasons for recovering the species composition of adult dipterans and their arrivals (Ahmad & Ahmad, 2009). Heo et al. (2007) used a single pig for studying the species composition of adult dipterans. Later, Heo et al. (2008) decomposed one piglet in a pond to study the species composition of adult dipterans. Lee and Marzuki (1993) used two monkey carcasses and reported the stages of development of dipterans although the season was not specified. Later, Omar et al. (1994a) reported the developmental stages of diptera in a monkey carcass in a single occasion during October-December 1991. In another study, Omar et al. (1994b) used three monkey and three cat carcasses to study the behavioural patterns of dipteran larvae. Lee (1989) analyzed specimens received

from various hospitals in Malaysia and concluded that maggots of *Ch. megacephala* and *Ch. rufifacies* were predominantly found and indicated his unpublished findings that at temperature of 30±2°C and relative humidity of 85±5%, one generation time for *Ch. megacephala* and *Ch. rufifacies* were 7.15 days and 9, respectively. Thus, it is seen that the previous researches in forensic entomology in Malaysia pertained to studies of adult species composition attracted to carcasses (Heo et al., 2007, 2008; Ahmad & Ahmad, 2009), studies of developmental stages in a single instance using naturally dead monkey carcasses (Lee & Marzuki, 1993; Omar et al., 1994a), a study of larval behaviour (Omar et al. 1994b) and a study using malathion-exposed dissected liver as rearing medium (Abd-Rashid et al., 2008). As such, research findings on the development of dipterans throughout the period of one year in carcasses decomposing in defined and varying climatological conditions and in the presence of malathion has not been reported so far.

2.3 Molecular-based technique for species identification

2.3.1 Application of molecular-based technique in forensic entomology

Accurate identification of species is critical in estimating PMI (Greenberg & Kunich, 2002; Harvey et al., 2003a; Amendt et al., 2004; Wells & Stevens, 2008). It has been shown that larvae of species that are closely related may differ substantially in their developmental rates (Higley & Haskell, 2001; Stevens et al., 2002; Wells & Stevens, 2008) and may be difficult to identify, morphologically (Tantawi & Greenberg, 1993; Wells & Stevens, 2008). Furthermore, the identification keys for the immature forms are sparse when compared with that for adult insects (Wallman et al., 2005; Zehner et al., 2004). In this context, Sperling et al. (1994) were the first to suggest the application of molecular-based techniques to identify insect of forensic

importance. The important advantages of using DNA for taxonomic identification include its enormous diagnostic information and its resistant to environmental degradation (Benecke & Wells, 2001). Recently, a wide array of molecular-based techniques for species identification has been reported and these techniques are tabulated in Table 2.1.

Variations in base sequences in two forensically important species i.e. *Chrysomya bezziana* and *Cochliomyia hominivorax* due to differences in geographical regions have been reported (Vargas & Espin, 1995; Hall et al., 2001). It has been suggested that the geographic variation in phenotype for a widespread species presumably associated with genetic variation (Wells & Stevens, 2008). Forensic entomologist would be interested in this aspect due to (1) the importance of replicating the entomological studies in various geographical regions since data obtained in one region may not be useful for other regions and (2) the possible inference on the postmortem relocation of a corpse (Wells & Stevens, 2008). Tan et al. (2009) reported for the first time in Malaysia the complete DNA sequence of CO1 and CO11 gene in *Ch. megacephala* (from six localities) and *Ch. rufifacies* (from three localities), indicating 0.26% and 0.17% intraspecific variations for both species, respectively. These authors concluded that the DNA sequence can facilitate and complement the morphology-based species identification. Thus, it is seen that the application of molecular-based techniques for taxonomic identification of insects of forensic importance may be useful in speeding up the identification process and ultimately the estimation of PMI using entomological data for forensic practice.

Table 2.1 Summary of the researches conducted using molecular-based techniques for taxonomic identification of forensically important insects

Molecular-based techniques used	Researchers
Sequencing of mtDNA control region	Stevens and Wall (1997a); Lessinger et al. (2000); Lessinger and Azeredo-Espin (2000); Lessinger et al. (2004); Junqueira et al. (2004)
Sequencing of cytochrome oxidase subunits 1 and 2	Sperling et al. (1994); Gleeson and Sarre (1997); Malgorn and Coquoz (1999); Wells and Sperling (1999); Vincent et al. (2000); Lessinger et al. (2000); Wallman and Donnellan (2001); Wells and Sperling (2001); Stevens et al. (2002); Harvey et al. (2003a, 2003b); Schroeder et al. (2003); Stevens (2003); Junqueira et al. (2004); Wells et al. (2004); Saigusa et al. (2005); Pai et al. (2007); Ames et al. (2006); Caine et al. (2009); Desmyter and Gosselin (2009); Alessandrini et al. (2008); Tan et al. (2009)
Sequencing of cytochrome b gene	Hall et al. (2001); Ramos de Pablo et al. (2006)
Sequencing of mitochondrial ribosomal RNA (rRNA) genes 12S	Stevens and Wall (1996); Stevens and Wall (1997a, 1997b); Lessinger et al. (2000); Junqueira et al. (2004)
Sequencing of nuclear ribosomal RNA (rRNA) genes 28S	Stevens and Wall (2001); Stevens et al. (2002); Stevens (2003)
PCR-RFLP of nuclear DNA (internal transcribed spacers)	Ratcliffe et al. (2003); Song et al. (2008); Nelson et al., 2008
PCR-RFLP of mtDNA	Sperling et al. (1994); Narang & DeGrugillier (1995); Taylor et al. (1996); Gleeson and Sarre (1997); Malgorn and Coquoz (1999); Litjens et al. (2001); Schroeder et al. (2003)
Random Amplified Polymorphic DNA (RAPD)	Stevens and Wall (1996); Stevens and Wall (1997a); Benecke (1998)

In addition, the molecular-based techniques have also been shown useful in identifying the identity of a corpse using human DNA recovered from maggots' crop (Wells et al., 2001; Luise et al., 2008).

2.3.2 Mitochondrial DNA (mtDNA): the relevance of using cytochrome oxidase 1 (CO1) gene for species identification in the presence of malathion

Mitochondrion is an organelle in which adenosine triphosphate (ATP) is produced through oxidative phosphorylation, a process catalyzed by the cytochrome c oxidase (Anderson et al., 1981; Gennard, 2007). Studies (Lewis et al., 1995; Lessinger et al., 2000) describe insect mtDNA as a small circular genome that contains 37 genes i.e. transfer RNA (22 genes), ribosomal RNA (2 genes) and enzymes (13 genes). mtDNA is considered useful for taxonomic identification since it has greater DNA copies than the nuclear DNA (Parsons & Coble, 2001) and lower occurrence of insertion (Moritz et al., 1987). Consequently, the CO1 gene that is located at nucleotide position 7-1539 in the mitochondrion is among the largest protein-coding genes in insects and well conserved throughout metazoan organisms, allowing the use of universal primers for PCR amplification (Lunt et al., 1996; Gennard, 2007). Therefore, in the context of molecular-based technique for species identification, CO1 gene has been acquiring considerable interest in many forensically oriented studies (e.g. Wells & Sperling, 1999, 2001; Harvey et al., 2003a; Wallman et al., 2005; Wells et al., 2007; Nelson et al., 2007; Caine et al., 2009).

Pertinently, mitochondria are the primary subcellular targets for organophosphorus compounds such as malathion (Carlson & Ehrich, 1999). Studies have demonstrated that in acute exposure malathion disrupts mitochondrial function especially in the expression of cytochrome oxidase subunits (Bonilla et al., 2008). Malathion has also been shown to cause DNA damages, chromosomal aberrations and sister chromatid exchange in both acute and chronic exposures (Flessel et al., 1993; Blasiak et al., 1999; Giri et al., 2002; Reus et al., 2008). Although malathion as the parent compound does not appear to induce point mutations, its active metabolite, malaoxon is capable at inducing point mutations in mammalian cell tests (Flessel et al., 1993). Additionally, studies have reported that malathion being more toxic for insects than mammals, attributable to lower level of malathion carboxylesterase activity in insects compared with that in mammals (Broadley, 1996; Wheelock et al., 2005). However, the ability to use CO1 gene for taxonomic identification of necrophagous larvae in the presence of malathion has not been reported in literature.

2.4 Utility of entomological specimens for diagnosing the cause of death

Entomotoxicology, a relatively new branch of forensic entomology deals with the qualitative and/or quantitative determination of drugs and/or poisons in insects recovered in decomposing remains (Gagliano-Candela & Aventaggiato, 2001; Introna et al., 2001). Summary of the drugs and/or poisons analyzed in the previous entomotoxicological studies is presented in Table 2.2. It has been indicated that while feeding on a decomposing corpse, larvae may accumulate drug and/or poison consumed by the person before death (Amendt et al., 2004; Gennard, 2007). It has been indicated that in instances where the body is badly decomposing and when the

Table 2.2 Summary of the previous entomotoxicological studies

Drugs of abuse and therapeutic drugs	Researchers
Amphetamines	Goff et al. (1997)
Barbiturates	Beyer et al. (1980); Kintz et al. (1990); Sadler et al. (1997a)
Opiates	Goff et al. (1989); Introna et al. (1990); Goff et al.(1991); Manhoff et al. (1991); Nolte et al. (1992); Kintz et al. (1994); Hedouin et al. (1999)
Benzodiazepines	Kintz et al. (1990)
Tricyclic antidepressants	Kintz et al. (1990); Goff et al. (1993); Miller et al. (1994)
Acetylsalicylic acid	Sadler et al. (1997a)
Poisons and toxicants	**Researchers**
Heavy metal (mercury)	Nuorteva (1977); Nuorteva and Nuorteva (1982); Schott and Nuorteva (1983)
Metals (copper, iron and zinc)	Sohal and Lamb (1977, 1979)
Arsenic	Leclercq and Brahy (1985)
Malathion	Gunatilake and Goff (1989); Abd-Rashid et al. (2008)

traditional toxicological specimens such as blood and tissues are unavailable or not suitable for toxicological sampling, it may be useful to analyse necrophagous insects as the alternative specimens for detecting the presence of drugs (Goff & Lord, 2001) using standard toxicological techniques (Gagliano-Candela & Aventaggiato, 2001). The influence of toxic substances on insect development has been reported with reference to different drugs as well as in different species of insects (Utsumi, 1958; Gunatilake & Goff, 1989; Goff et al., 1989, 1991, 1992, 1993, 1994, 1997; Lord, 1990; Bourel et al., 1999; Musvaska et al., 2001; O'Brien & Turner, 2004; Arnaldos et al., 2005; Abd-Rashid et al., 2008).

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While dealing with drugs of abuse and therapeutic drugs in human corpses as well as in arthropod larvae, Tracqui et al. (2004) indicated that the drugs identified in larvae can also be found in cadaver and commented that larvae analysis is of almost no interest for practical forensic casework. However, it was observed that the detection of poisons such as malathion (Gunatilake & Goff, 1989) and mercury (Nuorteva & Nuorteva, 1982) using maggots may prove useful in diagnosing the cause of death since these substances can readily cause death. It has been indicated that the incidence of suicides in remote areas is increasing (Page et al., 2007) and that suicidal victims tend to choose private places for committing suicide (Hayati et al., 2008). In this context, the application of entomological specimens for detecting the presence of poisons such as malathion can be useful for forensic practice when dealing with highly decomposed dead bodies in remote or private areas wherein the traditional toxicological specimens are unsuitable or unavailable for sampling.

2.5 Malathion

Malathion ($C_{10}H_{19}O_6PS_2$) is a widely used organophosphorus insecticide in agriculture, household applications and for the treatment of head lice (Roberts, 2002). In the market, malathion is available in the forms of emulsifiable concentrate, dust, wettable powder, ready-to-use liquid as well as pressurized liquid (Suleiman, 1995). In its pure form, malathion is colourless, while in lower purity solutions the colours range from deep brown to yellow (Cheremisinoff & King, 1994; Budavari et al. 1996). Malathion also known as Cythion, Cekumal, Fyfanon, Malixol and Maltox (Agency for Toxic Substances and Disease Registry, ATSDR, 2003) has a molecular weight of 330.36 Da (Howard & Neal, 1992) with melting and boiling points of 2.85°C and 156-157°C, respectively (Budavari et al., 1996), water solubility (at 20°C) of 145 mg/L (Tomlin, 1997) and it is miscible in alcohols, esters, ketones, aromatics as well as vegetable oil (Budavari et al., 1996).

2.5.1 Malathion fatal poisoning

Cases of human fatalities due to malathion, both suicidal and poisonings have been reported in many countries (Farago, 1967; Namba et al., 1970; Jusic & Milic, 1978; Morgade & Barquet, 1982; Jadhav et al., 1992; Zivot et al., 1993; Thompson et al., 1998; Pannell et al., 2001). In Malaysia, malathion is the most prevalent poison used for committing suicide, accountable for 43 and 26 cases in year 2007 and 2008, respectively (JKM, 2009, personal communication). Carlton et al. (1998) indicated the estimated fatal oral dose of malathion for human being as 60 g/70 kg.

2.5.2 Malathion toxicity

Malathion toxicity in acute doses relates to cholinergic activity, which involves the inhibition of acetylcholinesterase enzyme by malaoxon since its molecular structure is similar to acetylcholine (Lin et al., 1984). Malaoxon the immediate metabolic product of malathion binds to active serine residue of acetylcholinesterase producing a relatively stable phosphorylated acetylcholinesterase (Ecobichon, 1994) and consequently causing the overstimulation of postsynaptic receptors due to excessive amount of acetylcholine (Liu & Pope, 1998; Bauchard et al., 2003; Pope et al., 2005). The symptoms of acute poisoning include increase salivation, lacrimation and perspiration, constriction of pupils, nausea, vomiting, abdominal cramps, diarrhea, loss of muscle regulation such as muscle fasciculation, muscle weakness, flaccid and rigidity and, paralysis (ATSDR, 2003; Pope et al., 2005).

Malathion poisoning has been shown to affect several human and animal body systems such as causing respiratory difficulties (Ojha et al., 1992; Zivot et al., 1993; Choi et al., 1998), hemorrhage and hyperemia of lungs (Piramanayagam et al., 1996) and dyspnea (Prabhakaran et al.,1993). It is pertinent to mention here that the paralysis of the respiratory muscles would result in respiratory failure and consequently, death (Pannell et al., 2001). Furthermore, malathion is known to cause tachycardia (Ojha et al., 1992), cardiac congestion and hemorrhage (Piramanayagam et al.,1996).

Apart from causing deviation in the parameters of the differential blood counts, (Aaron & Howland, 1998) malathion has also been reported to induce

oxidative stress on erythrocytes (Durak et al., 2009). Additionally, malathion is known to decrease the glutathione content and increase lipid peroxidation in liver in rats (Prabhakaran et al., 1993) as well as to elevate the serum alanin aminotransferase and aspartate aminotransferase (Husain et al., 1987). It has been reported that malathion induces teratozoospermia, and significant damages to the Sertoli cells in mice (Contreras & Bustos-Obregon, 1999). The genotoxic effects of malathion have also been reported (Flessel et al., 1993; Kumar et al., 1995; Abraham et al., 1997; Blasiak et al., 1999; Amer et al., 2002; Giri et al., 2002; Reus et al., 2008; Bonilla et al., 2008).

The biotransformation of malathion primarily involves oxidative reaction catalyzed by the mixed function oxidase producing malaoxon, the most important toxicological product responsible for the toxic effects of malathion (Hazarika et al., 2003). The subsequent steps in the biotransformation of malathion include the hydrolysis of one of the two carboxylic ester linkages that abolishes the potential acute toxicity by malathion (Buratti & Testai, 2005) and elimination of a methyl group catalyzed by gluthation-S-transferase (Malik & Summer, 1982; Hazarika et al., 2003).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Analytical apparatus

The apparatus used for the entomological studies included dissecting microscope, oven, freezer, balance, digital camera and image analyzer. The apparatus that were used for the molecular part of this research were distillation set, E-pure set, electrophoresis set, fumehood, set of hotplate and magnetic stirrer, ice machine, incubator, microwave oven, thermocycler (Gradient Cycler), UV hood and UV transilluminator. The apparatus used for the toxicological part of this research were Gas Chromatography-Mass Spectrometry, pH meter and heating and evaporating unit. The items of apparatus that were used for both the molecular and toxicological parts of this research included autoclave, balance, deep freezer (–20^{o}C), microcentrifuge, refrigerator, vortex mixer and waterbath. The items of apparatus that were used along with detail of the manufacturers are presented in Table 3.1.

3.1.2 Laboratory wares, chemicals, reagents and analytical kits

The laboratory wares and chemicals, reagents as well as the analytical kits along with detail of the manufacturers are presented in Tables 3.2 and 3.3. Photograph of the malathion commercial used in this study is presented in Plate 3.1.

Table 3.1 List of analytical apparatus

Items	Manufacturer/Supplier
Autoclave steam sterilizer model MC-30LDP	ALP Co. Japan.
Balance	Sartorius, Germany
Canon digital camera A 400	Canon, Malaysia
Dissecting microscope SFC-11	Motic, Hong Kong
Distillation set	Nalgene / Syborn Corp.
Deep freezer (–20^{o}C) FIOCCHETTI model Super Freezer 340WID Fisher 7 payker N308Freeze	 FIOCCHETTI, Italy Fisher & Payker, Germany.
E-pure (Multistage in-exchange system)	Barnsted, USA.
Electrophoresis apparatus: Power pack ELITE 300 PLUS Easy Cast ^{TM} horizontal minigel system (B1 A and B1 model)	 WEALTEC, USA. OWl separation systems, USA.
Fumehood model RICO	ERLA, Malaysia.
Gas Chromatography-Mass Spectrometry GC-MS (HP6890 GC coupled with HP5973 mass spectrometer detector)	Agilent Technologies, Australia
Heating and evaporating unit (Reacti-Therm Model 18790 and Pierce Reacti-Vap Model 18780)	Rockford, USA
Hotplate and magnetic stirrer EMS-HP-7000	ERLA, Germany.
Ice machine Scotsman AF10	Scotsman Frimton, Italy.
Image Analysis System BX41/CVXS	Olympus, USA
Image Master Digital Imaging system	Hoefer, UK.
Incubator, shaker Innova 4400	New Brunswick Scientific, USA.
Microcentrifuge, Spectrafuge 16M	National Labnet Co, USA.
Microwave oven	Sanyo, Japan.
Mercury thermometer	Wuxi Medical instrument Factory, China
Oven, Memert	Memert, Germany.
Oven, incubation, model 115	Binder, UK
pH Meter cyberscan1000 model RS232	Eutech Instruments, Singapore.
Refrigerator, Hitachi model R170	Hitachi, Japan.
Thermocycler (Gradient Cycler) MJ Research PTC-100 and PTC-200 models	Eppendorf, Germany.
UV Hood model	ERLA, Malaysia.
UV Transilluminator, Macrovue UV-25	Hoefer, UK.
Vortex Mixer EVM-6000	ERLA, Germany.
Water - bath, Memert	Memert, Germany.

Table 3.2 List of laboratory wares

Items	Manufacturer/Supplier
Animal surgical set	TW Medical Veterinary Supply, USA
Beakers (250 mL)	Schott Duran, Germany
Beakers (50 mL)	Schott Duran, Germany
Bijou bottles	Schott Duran, Germany
Calibrated volumetric flasks (100 mL)	Schott Duran, Germany
Calibrated volumetric flasks (10 mL)	Carolina, USA
Capped Jars (100 mL)	Merck Ltd, United Kingdom
Centrifuge tubes (1.5 mL)	Odonixx Labware, USA.
Eppendorf tubes (1.5 mL)	Eppendorf Netheler-Hinz-Gmbt, Germany
Filter flask	Schott Duran, Germany
Hypodermic syringe (5 mL)	Becton Dickinson Medical Pte Ltd, Singapore
Latex examination gloves	Terong Wusa Sdn. Bhd, Malaysia.
Magnifying glass	Chongqing Yangguang Photoelectric Co. Ltd, China
Micropipettes	Gilison, USA.
Microscope Plain Glass Slides	Chongqing Yangguang Photoelectric Co. Ltd, China
Pasteur pipette	BD Bioscience Discovery Labware, USA
PCR tubes (0.5 mL and 0.2 mL)	Odonixx Labware, USA.
Petri dishes	Primula Sdn Bhd, Malaysia
Pestle Tissue Grinder (Thomas®): Size BB (chamber internal diameter 15.9 mm) Size O (chamber internal diameter 4.8 mm)	Thomas Scientific, USA
Pipette tips (10 µL, 200 µL, 1000 µL)	Odonixx Labware, USA.
Plain blood tubes (5 mL)	Primula Sdn Bhd, Malaysia
Scalpels	Ghia Surgiblades PVT, Ltd, India.
Screw capped glass test tubes	The Lab Depot, Inc, USA
Specimen containers	Permula Sdn Bhd, Malaysia
Sterile needles (21 and 23-gauge)	Becton Dickinson Medical Pte Ltd, Singapore
Synthetic 3-0 absorbable suture	Shandong Sinorgmed Co., Ltd., China
Universal containers (60 mL container with white screw cap)	ZheJiang GongDong Medical Plastic Factory, China