USE OF *Lucilia cuprina* WIEDEMANN (1830) LARVAE FOR DETECTION OF HEROIN METABOLITES AND IDENTIFICATION OF HOST DNA

by

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cyt	Cytochrome
DNA	Deoxyrobonucleic Acid
HV	Hypervariable
IUPAC	International Union of Pure and Applied Chemistry

PENGGUNAAN LARVA *LUCILIA CUPRINA* WIEDEMANN (1830) UNTUK MENGESAN METABOLIT HEROIN DAN PENGENALPASTIAN DNA

ABSTRAK

Kajian ini dijalankan untuk mengkaji keberkesanan larva peringkat 3 *Lucilia cuprina* Wiedemann (1830) dalam analisis toksikologi untuk mengesan metabolit heroin dengan menggunakan aplikasi GCMS dan dalam analisis molekul dengan menggunakan *Cytochrome b* gen (mtDNA) untuk mengenalpasti jujukan DNA daging kerbau cincang (yang telah digunakan sebagai bahan eksperimen dalam kajian ini) tanpa heroin. Kajian yang dilakukan juga menyiasat kesan heroin kepada tempoh kitaran hidup *Lucilia cuprina*. Selepas itu kaedah yang dibangunkan ini digunakan dalam kes-kes forensik untuk mengkaji keberkesanan kaedah tersebut dalam mengendalikan kes-kes forensik manusia dan haiwan. Analisis toksikologi yang dilakukan ke atas larva peringkat 3 telah memberi keputusan morfin, iaitu metabolit heroin. Heroin yang telah dicampurkan bersama dengan daging kerbau cincang (*Bubalus bubalis*, Linnaeus, 1758) di dalam kepekatan yang berbeza telah mempercepatkan pertumbuhan larva *L. cuprina* berbanding dengan larva pada daging kerbau cincang tanpa heroin yang mempunyai pertumbuhan yang lebih panjang. Analisis molekul yang dijalankan pada sistem usus larva peringkat 3, berjaya mengamplifikasi jujukan DNA daging kerbau cincang iaitu *Bubalus bubalis*. Penambahan heroin pada daging kerbau cincang didapati memberi perubahan pada nukleotida pada jujukan DNA daging kerbau cincang yang diperolehi. Terdapat sebelas (11) polimorfisme DNA yang berlaku pada jujukan DNA kerbau yang diperoleh daripada sistem usus larva peringkat 3. Perubahan yang berlaku pada jujukan DNA kerbau juga boleh menyebabkan perubahan pada jujukan asid amino

dan juga jujukan protein. Analisis molekular yang dijalankan pada larva instar peringkat 3 yang dijumpai di dalam kes forensik berjaya mengamplifikasi jujukan DNA *Gallus gallus*, Linnaeus, (1758); *Sus scropa*, Linnaeus, (1758); Civet (*Paradoxurus hermaphrodite*, Pallas, 1777); dan *Bubalus bubalis*, Linnaeus, (1758). Selain daripada dapat mengesan jujukan DNA bagi kes-kes forensik haiwan, sistem usus larva peringkat 3 yang ditemui dalam kes-kes forensik manusia juga berjaya mengesan jujukan DNA manusia dan bahan bukti ini telah digunakan untuk prosiding mahkamah. Larva peringkat 3 digunakan dalam kedua-dua kes forensik ini disebabkan oleh larva peringkat 3 mempunyai kepanjangan yang maksimum dan semua hasil makanan akan disimpan di dalam sistem usus larva peringkat 3 sebelum memupa. Panjang larva peingkat 3 merupakan satu kriteria penting kerana pada peringkat ini, tanaman dengan mudah boleh dilihat di bawah mikroskop dan dibedah keluar. Kajian yang dilakukan dalam kes-kes forensik menunjukkan bahawa bukti entomologi terutamanya pada peringkat larva boleh digunakan dalam mengenal pasti DNA tuan rumah dalam keadaan ketiadaan bukti-bukti biologi.

USE OF *LUCILIA CUPRINA* WIEDEMANN (1830) LARVAE FOR DETECTION OF HEROIN METABOLITES AND IDENTIFICATION OF HOST DNA

ABSTRACT

This study investigates the capability of 3rd instar larvae of *Lucilia cuprina* Wiedemann (1830) in toxicology analysis to detect the heroin metabolites by using GCMS application and in molecular analysis by utilizing the Cytochrome b gene (mtDNA) for precise identification of minced buffalo meat (which had been used as experimental materials in this study) in the absence of heroin. The study conducted also investigates the effect of heroin on the *Lucilia cuprina* duration of life cycle. Then the developed method was applied in exact forensic cases to examine the effectiveness of the developed method in handling both human and animal forensic cases. The toxicology analysis conducted on the 3rd instar larval crop was successfully detecting a metabolite of heroin, such as morphine. The heroin that was mixed with the minced buffalo (*Bubalus bubalis*, Linnaeus, 1758) meat in different concentrations, accelerated the duration of life cycle of *L. cuprina* larvae compared to the larvae that were collected from the minced buffalo meat without heroin, which had a longer life cycle. The molecular analysis conducted on 3rd instar larval crop, succesfully amplify desired results of minced buffalo meat (with heroin) DNA sequence, which is *Bubalus bubalis*. The addition of heroin was found to give an impact, which reflected the nucleotide changes to the buffalo DNA sequences obtained. Eleven (11) DNA polymorphisms were detected to occur in the buffalo DNA sequences, which derived from the 3rd instar larval crop. The nucleotide changes in the buffalo DNA sequence also led to some changes in protein sequences.

The molecular analysis carried out on the 3rd instar larval crop found in actual forensic cases successfully amplify DNA sequences of *Gallus gallus*, Linnaeus, (1758); *Sus scropa*, Linnaeus, (1758); Civet (*Paradoxurus hermaphrodite*, Pallas, 1777); and *Bubalus bubalis*, Linnaeus, (1758). Besides being able to detect the animal DNA sequences in animal forensic cases, the 3rd instar larval crop found in human forensic cases also managed to determine human DNA sequence and this evidence has, in fact, been used for court proceedings. The 3rd instar larval crop was used in both cases because the 3rd instar larvae have a maximum length and all the food are stored in the 3rd instar larval crop before larvae start to pupate. The length of 3rd instar larvae, indeed, is an important criterion because at this stage, the crop can easily be seen under a microscope and dissected to remove the crop. The study, which had been conducted in forensic case, displayed that entomological evidence, especially the larval stages, could be used in identifying the host DNA in conditions where biological evidence was absent.

CHAPTER 1

INTRODUCTION

1.1 General Introduction.

In the decomposition process of human remains, every stage of the decaying process is associated with specific insects, starting from flies to beetles, and finally, moths (Adair, 1999; De Jong & Chadwick, 1999; Wickham, 1902). However, the most important insects in this process are flies from family Calliphoridae, especially several genera from subfamily *Chrysomyinae*, as well as *Lucilia* spp. and *Hemilucilia* spp. (Reed, 1958). This is because; they are the first insects to colonize a human body post-mortem (Wells *et al*. 2001a). Flies play an important role in the decomposition of carcasses or human remains because fly larvae consume dead soft tissue. Thus, this makes the fly larvae important in forensic investigation because the tissue they consume can be utilized in toxicological and DNA analyses, especially in cases where other tissue samples are not available or viable. However, larvae specimens for toxicological and DNA analysis in forensic cases can only be viable if live larval specimens are collected and properly preserved to ensure that the integrity of the specimens is not tempered with during the decomposition process.

Apart from analyzing biological evidence such as blood and tissue, more recently, forensic investigations have begun applying entomological evidence based on the larval crop (the foregut region of the larvae digestive system that stores food) to determine the identity of the corpse by performed the sequencing analysis on the larval crop. Besides identify the human identity, larval crop also can assess in drug abuse cases by determine the level and type of drug that had consume by the victim

through the toxicology analysis conducted on the larval crop. By using the larval crop found at the crime scenee in both analyses is the right decision in the absent of biological evidence because the entomological evidence can be utilized in various way to meet the forensic investigator purposes.

Furthermore, the approach of using larval crop in DNA and toxicological analyses complements other approaches, such as blood analysis, which is expectly not available at crime scenes that involve decomposed corpses. Moreover, the use of entomological evidence namely larval crop (foregut area) in DNA and toxicology analyses is practical because of its function as a food store, which in terms of forensic investigation provides a valuable source of information. The data obtained from the analysis of crop, such as the sequences of the victim's DNA (which can be tested with possible relatives), could help investigators solve forensic cases by pinpointing the exact crime scene through the larvae feeding period based on the crop. In addition, the information gained from larval crop can also be used to determine the human identity.

In the DNA and toxicology analysis, the 3rd instar larvae are selected among the 1st and the 2nd instar larvae. This is because; the crop (food storage) of the 3rd instar larvae would have reached a maximum size of body length and the crop is visible and easy to remove for purposes of analysis. Moreover, at this stage, the 3rd instar larvae would have stopped eating and would be preparing themselves to pupate, as these attributes are absent in the 1st and the 2nd instar larvae. When the process occurs indirectly, all the food would be retained in the crop. This implies that all the information desired by the authorities also remained in the larval crop. Nonetheless, this feature is not characterised by the 1st and the 2nd instar larvae. The

factors mentioned above are among the reasons on why the 3rd instar larvae are suitable to be applied in most DNA analyses.

The changes in developmental rates, the extended periods of insect feeding and the variation in size of the insect during the different stages of development actually enables an investigator to analyse the larval crop in order to identify the presence of toxic substances (Introna *et al*., 2001). The utilization of the 3rd instar larval crop in toxicology analysis is now being considered as a useful substitute tool in detecting drugs consumed by the human that caused the death. A lot of literature have reported that different drugs can have different effects on insect developmental rates, which would result in inaccurate PMI estimations that will, in turn, jeopardize the outcome of a forensic investigation (Goff *et al*., 1989; Goff *et al*., 1991; Goff *et al*., 1992, Goff *et al*., 1993; Goff *et al*., 1994).

In this study, heroin was used to validate the effect of the drug on *L. cuprina* life cycle by using minced buffalo meat as an experimental subject. Heroin was chosen because this drug can be easily obtained in the black market and besides, it is being used as a recreational medicine to relief pain widely in Malaysia. Additionally, there are various methods of taking this drug, for instance, snorting or injection, and sometimes, overdose of heroin could lead to death. When death occurs and no biological evidence is present, entomological evidence can be applied in identifying the drug and in aiding the forensic investigation. Hence, this study was conducted to validate the potential of applying larval crop in toxicology analysis for drug determination.

Traditionally, fly larvae are used in estimating PMI because forensic flies usually complete their entire life cycle from larvae to pupa on a corpse (Ross *et al*.,

1982). During the larval stages, the corpse tissues ingested by the larvae would fill its crop before being digested. Therefore, the larval stage is the most suitable stage for use in molecular analysis to identify host DNA (Linville *et al*., 2004). This can be done by analysing the larval crop, especially to identify the identity of a corpse, because what the larvae have fed on is stored in the crop (Coulson *et al.*, 1990; Gokool *et al.*, 1993; Lord *et al*., 1998; Wells *et al*., 2001).

Besides, based on previous studies conducted by several scientists, it can be concluded that the 3rd instar larvae offer a suitable stage to serve as an alternative specimen in DNA analysis for determining species identification. For this critical criterion, the 3rd instar larvae are indeed suitable to be applied, and moreover, with the lack of biological evidence, larval crop is definitely the right choice and it is able to answer all questions in forensic decomposed corpses. Therefore, a study was conducted in DNA analysis to utilise the information gained from the larval crop for minced buffalo meat identification.

Even though the use of larval crop may help in identifying the identity of the victim, a few factors could hinder the accuracy of the DNA sequence analysis of the victim. These include weather, temperature, storage conditions, preservation solution, humidity, and the presence of drugs, which may alter the sequences of DNA. Although a number of studies have been done on the effect of these factors, the effect of the presence of drugs on the DNA sequence of flies is lacking. Thus, in order to investigate if DNA sequence is altered in the presence of drugs, an a study project was designed to investigate this parameter.

Furthermore, most of the previous studies were carried out abroad, hence, there in a basis to increase awareness on the importance of larval crop, especially in

Malaysia. The data obtained are valuable to the Royal Malaysian Police because they can help to solve forensic cases, especially those cases involving decomposing corpses. Information gained from the DNA analysis on larval crop can also be used to build the human identification database and reconnect with the possible relatives.

In addition, it is important for investigators to determine if there are drugs or toxic substances in corpses before estimating PMI because the presence of drug or toxic substances will alter the life cycle of the insect, thus jeopardising the PMI estimation. Therefore, the present study was conducted to determine the effects of different heroin concentrations on the developmental growth rate, the life cycle duration of *Lucilia cuprina* larval on minced buffalo meat, and if the method developed could be applied in forensic investigation as a new tool in Malaysia.

Generally, the objectives outlined in this study applied the larval of *L. cu*prina for detection of heroin metabolites and identification of host DNA. In addition, this study also observed the effects of heroin onto the length of *L. cuprina* larvae and in the DNA sequence of *B. bubalis*. Then, the application developed in this study was demonstrated in the real forensic case to examine the efficiency of the developed method.

1.2 General Objectives.

i. To study the utility of *L. cuprina* larval crop in toxicological analysis of forensic cases.

ii. To study the effect of heroin on the *L. cuprina* developmental time

iii. To study the utility of *L. cuprina* larval crop in molecular analysis of forensic cases.

iv. To study the effect of heroin on DNA sequence of *Bubalus bubalis*.

CHAPTER 2

LITERATURE REVIEW

2.1 Forensic toxicology and entomotoxicology.

Toxicology can be narrowly defined as the study of the adverse effects of chemicals, including drugs and poisons, on living organisms (Hodgson, 2004). However, it is actually a broad field of study that encompasses various other fields such as biology, biochemistry, histology, pharmacology, pathology and forensics (Goff & Lord, 1994; Williams *et al*., 2000; Hodgson, 2004). For example, cases of poisoning or drug overdoses might require the expertise of forensic pathologists, biochemists, biologists and those from various other fields of study (Williams *et al*., 2000). The applications of toxicology are numerous and one of the most important applications of toxicology is in the field of forensic science which is generally referred to as forensic toxicology. Forensic toxicology involves the application of toxicology to cases and issues that have administrative or medicolegal consequences and where the results are likely to be used in court (Levine, 2006).

According to Goff and Lord (1994), drug related deaths have been on the rise in several countries. However, a cadaver is often found in various stages of decomposition under different conditions and this makes the forensic investigation difficult because of the absence of biological samples such as blood. In order to overcome this problem, there are many toxicological analyses, techniques and methodologies available to forensic scientists and investigators (Kintz *et al*., 1990a; Pounder, 1991). Despite the availability of such methods, investigators still face problems in obtaining an appropriate sample for the toxicology analysis because of

the aforementioned complications involving the condition of the cadaver. Therefore, in this context, insects especially those that are in the immature stages are often used as alternatives in the absence of body fluids or other biological samples from the cadaver for toxicological analysis (Beyer *et al*., 1980; Kintz *et al*., 1990b). The mere presence of insects at a crime scene provides valuable information such as post mortem interval (PMI) estimation which can be used to facilitate a criminal investigation (Beyer *et al*., 1980).

Insects have long been known for their significant role in determining post-mortem interval in forensic investigations. However, the role of insects in forensics has been expanded, whereby they are now used as tools in toxicological analysis for detection of drugs to proof cause of death by drugs, especially in cases where there is a lack of other biological evidence. This role expansion has come to be known as entomotoxicology.

Entomotoxicology refers to the use of insects in toxicological analysis to detect the presence of drugs or poisons (Introna *et al*., 2001). This field also includes the study of the side effects caused by drugs and toxins on the development of insect life cycles which is vital in estimating post mortem interval (PMI) (Goff & Lord, 1994). In addition, carrion insects present in cadavers can also be used in toxicological analyses if there is no other biological evidence such as blood or urine. This is because drugs, toxins and other chemical substances that are ingested, inhaled or injected by the deceased prior to death will be present in the body post-mortem and will be passed on to the carrion insects colonizing the corpse, starting from the blow fly which is the first insect that colonises a corpse, to coleopterans which are abundant during the advanced decay stage (Tracqui *et al*., 2004)

In certain circumstances, insects are proven to be a more suitable or optimal sample for toxicological analysis compared to human tissue samples. Studies by Kintz *et al*. (1990b) and Nolte *et al*. (1992) both demonstrated such a circumstance clearly. In these studies, tissue degradation or products from the decomposition process interfered with quantification of foreign substances in the human tissue samples. The insect samples proved to be a more suitable sample for toxicological analysis due to the lack of any decomposition interference.

The use of insects in detecting poisons has been documented as early as 1958, in a study by Utsumi, in which he observed different groups of flies were attracted to carcasses of rats that were previously killed by different poisons. In the late 70s, Sohal & Lamb (1977; 1979) discovered accumulation of metals such as copper, iron and zinc in adult housefly, *Musca domestica*. Nuorteva & Nuorteva (1982) demonstrated a similar condition of metal accumulation in larvae, pupae and adults of Calliphoridae and Staphylinidae. Several studies have shown that drugs present in animals or humans prior to death could be detected in the various life stages of necrophagous insect collected post-mortem (Goff *et al*., 1993; Miller *et al*., 1994). These findings prove that foreign substances that enter the digestive system of insects can be passed on through to the subsequent life stages in their life cycle and thus provide information useful to medicolegal or forensic investigations.

2.2 Entomotoxicology as a tool in medicolegal or forensic investigations.

Entomotoxicological analysis can be applied to detect poisons and drugs to facilitate and support medicolegal or forensic investigations. Nuorteva (1977) reported that entomotoxicology findings were applied to a forensic case in Finland involving a corpse found in a state of advanced decay. Based on the results of the entomotoxicology analysis of flies collected from the corpse, investigators were able to trace the geographical origin of the victim. Beyer *et al*. (1980) reported that entomotoxicological analysis of carrion flies colonising the corpse of a woman who had committed suicide showed the presence of barbiturates. In another case of suicide by poisoning, toxicological analysis using gas chromatography showed the presence of organophosphates (Malathion) in larvae of Calliphorids, *Chrysomya megacephala* and *Chrysomya rufifacies* (Gunatilake & Goff, 1989).

Entomotoxicology analysis can even be applied in cases involving mummified cadavers found long after death. Miller *et al*., (1994), found two types of tricyclic antidepressants, amitriptyline and nortriptyline, in Phoridae puparial cases as well as beetle (Dermestidae) skin casts and faecal material collected from the mummified remains of a woman whose death occurred two years prior to finding the remains. Leclercq & Brahy (1985) showed that entomotoxicology can also be applied in investigation of accidental death due to overdose drug consume.

An insect is a treasure trove of information, not only for entomotoxicologists but for forensic investigators as well. In the late 20th century, a myriad of studies detected a variety of drug usage through toxicology analysis of larval tissue extracts, larval crop contents, pupae and puparial casings (Kintz *et al*., 1990a, 1990b; Manhoff *et al*., 1991; Nolte *et al.*, 1992; Goff *et al*., 1992; Wilson *et al*., 1993; Kintz *et al.*,

1994; Miller *et al*., 1994; Sadler *et al*., 1995; Hedouin *et al*., 1999) and even in insect faeces (Goff, 2000). Studies involving toxicological analysis of insects have been continuous, whereby in the 21st century, Bourel *et al.* (2001) and other more recent studies have focused on furthering knowledge in the field of entomotoxicology (Gosselin *et al*., 2011; Souza *et al*., 2013; Verma & Paul, 2013).

These and many other studies show that insects can provide valuable and vital information for forensic investigations. Through entomotoxicological studies, various investigators have reported drug usage including heroin or morphine (Bourel *et al*., 1996; Bourel *et al*., 1999); morphine and cocaine (Goff *et al*., 1989; Goff *et al*., 1991; Kintz *et al*., 1994; Hedouin *et al*., 1999; Bourel *et al*., 2001), benzoylecognine (Goff *et al*., 1989; Nolte *et al*., 1992), tricyclic antidepressants (Wilson *et al*., 1993; Goff *et al*., 1993; Miller *et al*., 1994; Sadler *et al*., 1995), amphetamine (Goff *et al*., 1992; 1997), benzodiazepines and phenotiazines (Kintz *et al*., 1990a, 1990b; Sadler *et al*., 1997;. Carvalho *et al*., 2001), barbiturates and other salicylates (Beyer *et al*., 1980; Sadler *et al*., 1997), bromazepam and levomepromazine (Kintz *et al*., 1990a), malathion (Gunatilake & Goff, 1989), phenobarbital, brazotam, oxazepam (Kintz *et al*., 1990b) and diazepam (Calvalho *et al*., 2001).

2.3 Heroin.

2.3.1 Origin and history.

Diacetylmorphine, systematic IUPAC name (5α, 6α)-7,8-didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol diacetate, was developed in 1874 by the English chemist, C.R. Wright and is derived from morphine. The compound was later synthesized for commercial production by chemist Felix Hoffman who was working for the German company Aktiengesellschaft Farbenfabriken, which today is the pharmaceutical company Bayer (United Nations, 1953; The Sunday Times, 1998). Diacetylmorphine did not become commonly known as "Heroin" until Bayer commercially produced and marketed it under the trademark name "Heroin" from 1898 to 1910 as a cough suppressant and cure for morphine addiction. Unfortunately, it was only later discovered that the drug rapidly metabolises into a form of morphine that was twice more addictive. Although Bayer's role in introducing heroin to the world has become a lesser known fact today, this historical blunder was a source of great embarrassment for the company (The Sunday Times, 1998). Heroin was eventually classified as an illegal drug in the United States as well as many other countries (United Nations, 1953).

Like other opioids, heroin is used as a recreational drug which inevitably leads to chronic addiction. Legally, diacetylmorphine (heroin) is prescribed as a controlled drug and can only be supplied in tablet or injectable form with the same indications as morphine (Besacier & Chaudron-Thozet, 1999). Normally, in medicolegal investigations relating to drugs, blood or urine will be sampled and quantitated to search for metabolites associated with drugs such as diacetylmorphine,

6-MAM, morphine, morphine-3-glucuronide and morphine-6-glucuronide (Baselt, 2008). There are a few chemical steps in the conversion of heroin to morphine as shown in Figure 2.1- one group of OH (carboxyl) in the morphine chemical structure is eliminated and replaced with one group of O (oxygen) producing hydromorphone metabolite. Hydromorphone is one part of the minor metabolic pathways of heroin.

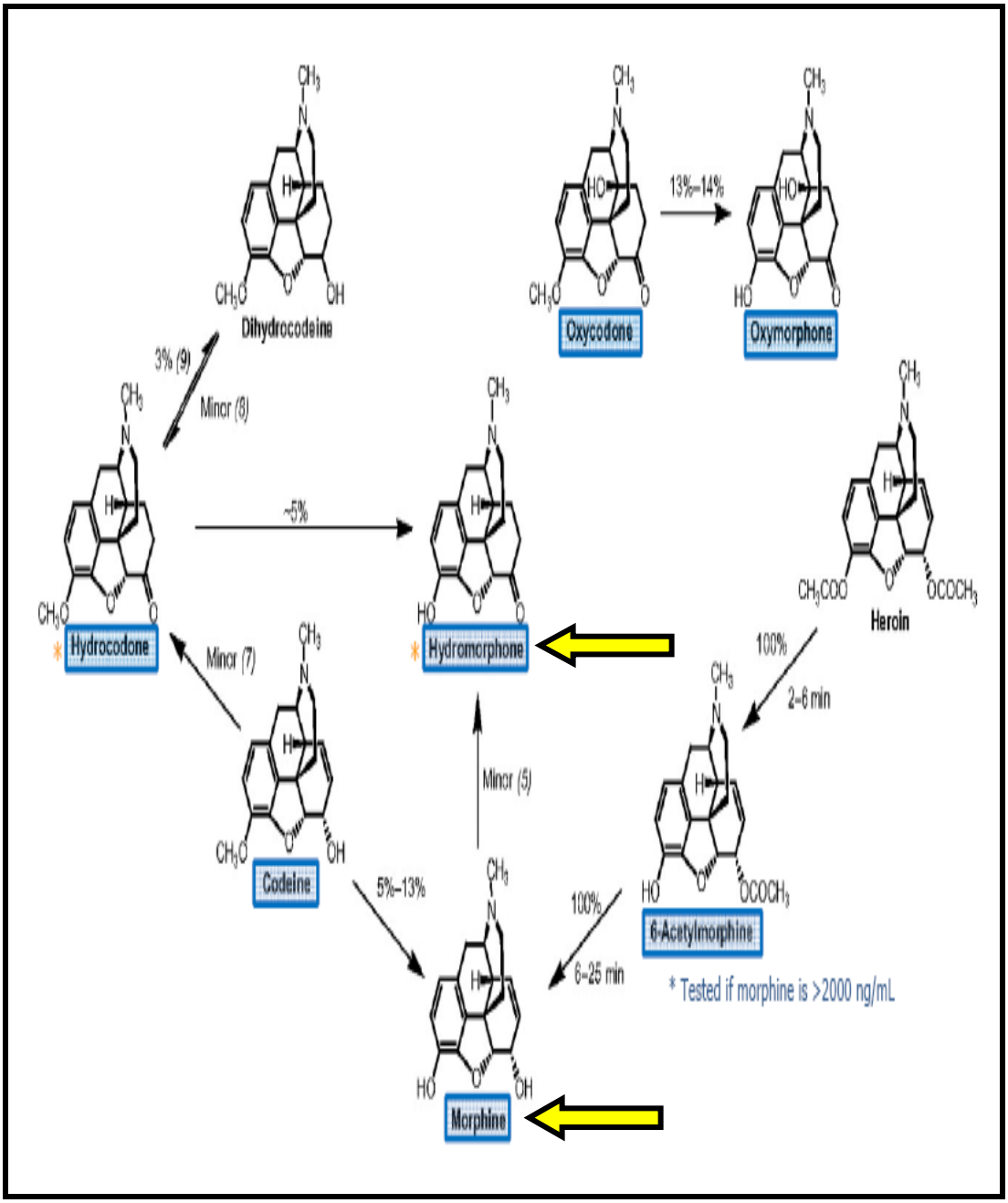

Figure 2.1: The common heroin metabolic pathway (Reisfield *et al*., 2009). The arrow in yellow colour indicates the elimination of the chemical group of heroin resulting in hydromorphone and morphine.

2.3.2 Heroin addiction and medicolegal cases associated with heroin in Malaysia.

Globally drugs have been widely used for many centuries among Arab traders and the phenomenon started the 8th century. Around this period, Arab traders introduced drug usage to many Asian cultures during their explorations, trading or war activities. This problem escalated with the colonisation of the Asian region by many European countries, especially during the British rule. Malaysia was also very much affected. Being a British colony, many immigrants were brought in to what was then Malaya as cheap workers. Heroin was commonly used by these labourers to maintain their strength doing hard labour. Unfortunately, it also came with devastating after effects. The active compound of heroin is altered during usage and cause addiction among the workers leading to further widespread abuse (Maisto *et al*., 1995; Anonymous 1997; Kalant, 1997).

As this problem grew, since 1929 to 2015 almost 52313 heroin addicts were registered in the Federal Malay States and this is estimated to reach 250000 by year 2015. In part the rapid progress and urbanization brought brought about by the social, economical and political changes within the country (Khairuddin, 1994; Rusdi *et al*., 2008). The increasing trend in drug addiction results in economic loss due to ineffectiveness of many employees or potential ones leading to social ills and therefore poses a threat to the future of nation. The increase in number of drug addicts in Malaysia also causes an increase in drug related deaths. Most of the time when a corpse is discovered after a period of time upon death, it is already in a highly decomposed or skeletonized stage (Chawarski & Schottenfeld, 2006). In such particular situation, there is in insufficient tissue for toxicology analysis and it is

impossible to detect drug or toxic substances in the absence of biological evidence. However, the problem can be solved by analyzing the insects such as larvae or pupal skin that are present around the corpse.

2.4 Blowflies.

2.4.1 The importance of blow flies in forensics.

Order Diptera is a group of insects with only a single pair of wings (fore-wings) while the hind-wings are reduced to small club-like structures called halteres. The order is divided into three suborders; Brachycera, Nematocera and Cyclorrhapha. However, the suborder that is most important in the field of forensic science is Cyclorrhapha, while the others have their own importance as vectors of diseases. In the suborder Cyclorrhapha there are three different families of flies, which are of forensic importance; Calliphoridae (blowflies), Sarcophagidae (flesh flies) and Muscidae (houseflies). The family Calliphoridae, also known as carrion breeding flies, can be found during the early stages of decomposition, while Sarcophagidae can be found during the subsequent stages of decomposition, followed by Muscidae (Carvalho & Linhares, 2000).

According to Introna *et al*. (1998), the most common fly species occurring on human corpses are from the family Calliphoridae. Each stage of human decomposition is often associated with a certain insect, and it begins with flies through to beetles, but Calliphorids have the most important role in forensic investigations, especially in estimating post-mortem interval (Greenberg, 1991). Among the most forensically important flies of the family Calliphoridae are the

genera *Chrysomya, Lucilia* and *Hemilucilia* due to these species being the first colonizers of a human body upon death (Reed, 1958). Once flies colonise a corpse, females will lay eggs which will eventually hatch and develop into the larvae, pupal and adult stages. Thus, forensic investigators can estimate the post-mortem interval based on the current life stage of the flies found on a corpse.

2.4.2 *Lucilia cuprina* (Diptera: Calliphoridae).

There are many fly species of importance in forensic science. However, one of the main species for this purpose is the Australian sheep blowfly, *Lucilia cuprina* (formerly *Phaenicia cuprina*) due to it being one of the first species that colonizes a human body post-mortem. Generally, species of the genus *Lucilia* have a size range of between 4.5 - 10 mm and a shiny green or reflective bronze-copper coloured abdomen and therefore are also collectively known as the bronze bottle flies (Dress & Jackman, 1998). The adults are easy to differentiate due to bristles present on the meron of the thorax, and plumose or feathery aristae and the prominent hair on the terminal segment of each antenna. *Lucilia cuprina* are most easily identified by their strong dorsal setae and black thoracic spiracle (Durden, 1999.

2.4.3 Blowfly life cycle in relation to develop on corpses.

Like all dipterans, blowflies undergo holometabolous development with an egg, three larval instars, and a pupal and adult stage (Ross *et al*. 1982). The eggs, which are about 2 mm in length and white in colour, are usually oviposited in

clusters, after which they undergo a short embryonic developmental stage. On fresh corpses, these clusters are usually found in the mouth, ears, nasal openings, and any area where mucous membranes come into contact with the outside air. Wounds and bruises are also common sites for the eggs to be deposited (Byrd & Castner, 2001). After hatching, the larvae undergo three larval instar stages, commonly referred to as larvae (Parr, 1962; Skidmore, 1985). Generally, fly larvae lack functional legs, are cream in colour and soft bodied (Ross *et al*. 1982). The first instar is approximately 5 mm long after 1.8 days; the second instar is approximately 10 mm long after 2.5 days while the third instar reaches approximately 17 mm long after 4-5 days (Parr, 1962; Skidmore, 1985).

Identifying the correct instar stage is relatively easy based on the size of the larvae, the size of their mouthparts and morphology of the posterior spiracles. A larva has a pair of "hooks" in its mouth to help attach itself to its food source while it feeds. Differences in the fine structure of the mouth hooks and spiracles are used to differentiate among species as well as to determine the number of times it has shed its skin (Salimi *et al*., 2010).

The third instar larva will shed its skin and form a dark brown puparia measuring about 12-14 mm in length. This takes place 18-24 days after oviposition. The skin contracts to a capsule-like shape and becomes stiff and hardened (newly-molted). Inside the capsule the living insect cannot move or feed, is pale white and has rudimentary legs and wings and antennae. The hardened skin surrounding it is called puparium (Apperson *et al*., 2011).

In forensic investigation the presence of pupae on a corpse, usually in the folds of clothing, is critical evidence because the pupal stage being the oldest stage

would indicate to the forensic entomologist the precise stage of decomposition which can then be accurately linked with the time of death. However, their presence is sometimes overlooked or the investigator fails to detect the presence of pupae (Rueda *et al*., 2010).

Newly emerged adult flies are pale in colour and soft to the touch, and have crumpled, unexpanded wings upon emerging from the puparium. They later expand their wings while the body becomes a green, bronze or blue colour. After they had fully hardened (tanning process), the adult flies will begin to mate and produce the next generation (Apperson *et al*., 2011). Once they have mated they are among the most mobile creatures on earth (due to their size), and can travel for great distances to find suitable sites to lay their eggs. Adult flies are always found around a corpse but due to their mobility fully mature adults are worthless as forensic indicators as their source is doubtful (Salimi *et al*, 2010).

2.5 The importance of 3rd instar larval crop.

The usage of larval insect crop has been potentially demonstrated in DNA analysis for forensic purposes and this includes those of the lice (Mukabana *et al*., 2002), mosquito, (Mumcuoglu *et al*., 2004) and carrion feeding flies (Zehner *et al.*, 2004). Among the larval stages, the 3rd instar larva which is the last stage before changing into a quiescent pupa is the suitable stage to be applied in molecular analysis and it has proven successful, especially in human identification process and toxicological analyses. This is because food materials contained in larval crop provide are directly associated with its last food intake before it transforms into a

pupa (Wells *et al*., 2001a; Wells *et al*., 2001b; Wells & Steven, 2008; Zehner *et al*., 2004).

Due to its function a as food storage, larval crop can also be utilized in DNA and toxicological analyses to detect the identity of the corpse, chemicals and drugs that are present in the body. Since the larvae are the first arthropods that come to the bodies after death, it can be concluded that the larvae are the first to consume the biological tissues of the corpse. Thus, important information can be extracted from the food materials are that stored in the larval crop. Therefore, because of this factor, the larval crop is suited to be applied in both analyses mentioned above. From these two analyses, the identity of the corpse and the presence of drug or chemical substances can be traced.

Referring to the anatomy of the larval crop (Figure 2.2), the crop is located between the mouth and stomach. All the materials consumed by the larvae will pass through the crop and be stored until pupation. Thus, all materials contained in the crop are maintained. So the whole tissue can be removed which can facilitate the process of DNA extraction and deriving DNA (Campobasso *et al*., 2005). Therefore, it is highly useful in forensic investigation if larvae are found at a crime scene in the absence of body and biological tissues. The Royal Malaysia Police can employ this application to resolve a chain of custody disputes, if when there are question whether the larvae used in the post-mortem interval estimation has been moved from another crime scene.

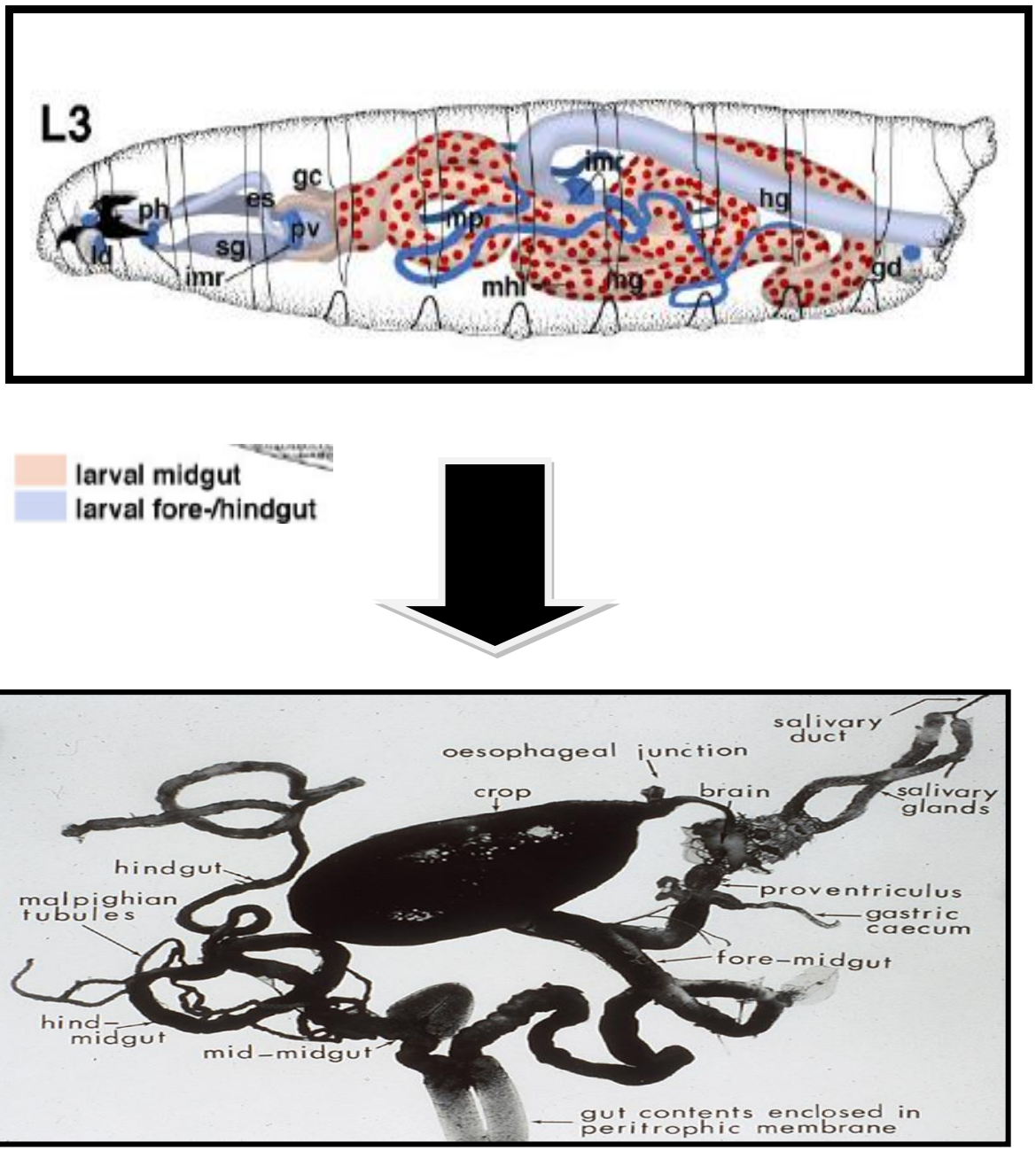

Figure 2.2: The anatomy of fly digestive system (Taken from Imms, 1934).

2.6 Effects of drugs on fly development.

There is no denying the fact that insects, particularly flies, are important in forensics. However, various studies have also shown that drugs and toxic foreign substances present in a corpse can affect the development of insects that colonise it. This will expectedly affect the information obtained from insects, especially when estimating postmortem interval which depends on the insect life cycle. A study conducted by Gunatilake & Goff (1989), showed that the presence of Malathion interrupted the post-mortem interval (PMI) estimation of a female corpse. They observed that based on the development of the blowflies, *Chrysomya megacephala* and *C. rufifacies* found on the corpse, post-mortem interval was estimated to be five days. However, based on the stage of decomposition of the corpse, it was determined to be eight days. The difference of PMI estimation was due to the presence of a toxic substance which is Malathion.

Besides Malathion, Goff *et al*. (1989) have also studied the effects of cocaine on the development of *Boettcherisca peregrina* (Sarcophagidae). They discovered that *B. peregrina* developed more rapidly on rabbit tissue that contained cocaine and concluded that if cocaine was present in human tissue, the PMI estimates that are based on larval development could be in error by up to 24 hours. Mustava *et al*., (2001) studied the effects of different concentrations of hydrocortisone and sodium methohexital on the development of *Sarcophagi tibialis* (Sarcophagidae) on chicken liver. In the study, larvae of *S. tibialis* reared on chicken liver injected with different concentrations of the two drugs had significantly different effects on the pupal developmental time. *Sarcophagi tibialis* exposed to hydrocortisone experienced a longer pupal stage while those exposed to sodium methohexital displayed a shorter

pupal stage. This shows that drugs and foreign substances can have different effects on the developmental rate of the various life stages of flies.

In a further study, Goff *et al*. (1991) studied the effects of drugs, specifically heroin on the development of *B. peregrina* (Sarcophagidae) and concluded that the differences observed in the rates of development were sufficient to alter PMI estimates based on larval development by up to 29 h and estimates based on the pupal development by 18 to 38 h. Other studies by Goff *et al*., (1989, 1991, 1992) were focused on various other types of drugs such as methamphetamine and cocaine and their effects on the development rate and growth of *Parasarcophaga ruficornis* (Sarcophagidae).

Other studies have provided further proof which supports the findings of Goff and Gunatilake (1989) and Goff *et al*. (1989), that drugs can accelerate fly development. For example, Lord (1990) have estimated the post-mortem interval of decomposed body of a female aged 20 was 7 days based on the life cycle of *Lucilia sericata* (Meigen) (Calliphoridae) and *Cynomyopsis cadaverina* (Robineau-Desvoidy) (Calliphoridae) that were collected from the female decomposed body. However, the estimated PMI was questionable because basically these two flies' species take approximately 3 weeks to complete their life cycle. After a few investigations, they found out that the decomposed female had taken an overdose of heroin and thus accelerated the life cycle of these two flies. Bourel *et al*. 1999 studied the effects of morphine on the growth rate of *L. sericata* (Calliphoridae) on rabbit tissue and similar to Goff *et al*. (1989), concluded that an underestimation of PMI of up to 24 h can occur if the presence of morphine is not considered.

Many of the aforementioned research have been conducted on flies that are predominant to a geographical region or climate other than that of Malaysia. For example, *Lucilia sericata* is not found in the tropics including Malaysia. Although they are closely related, they are inherently different species with probable differing habitual and geographical adaptations and therefore the results of studies on the effects of drugs on *L. sericata*, which is more prevalent in the literature, cannot be simply applied to *L. cuprina* which is more predominant in Malaysia. Thus, in order to develop a locally applicable baseline for estimation of vital information such as PMI, studies on the effects of drugs on insect development needs to be not only specific to the common drugs found in Malaysia but also to the species of flies, especially those in relation to medicolegal and forensic cases.

2.7 The importance of proper sample collection and selection of toxicological analysis.

When conducting sample collection for forensic toxicological analysis it is vital that a systematic and quality-assured procedure be strictly followed (Catts & Haskell, 1990). This is to guarantee the chain of custody of evidence and prevent contamination of the crime scene and destruction of evidence especially when dealing with live insect samples. While at the crime scene, forensic entomologists should wear overalls, gloves and shoe covers or boots (Amendt *et al*. 2007). There are several effective ways to avoid sample contamination and wastage of which the investigator needs to be aware, especially during sample collections. Blood, tissue or dead organic samples should be immediately stored in appropriate containers, for