DAILY RHYTHMS IN INSECTICIDE SUSCEPTIBILITY, METABOLIC ENZYME ACTIVITY AND GENE EXPRESSION IN THE COMMON BED BUG, *Cimex lectularius* L. (HEMIPTERA: CIMICIDAE)

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

$2^{-\Delta\Delta C_T}$	Comparative CT value
7-EC	7-ethoxycoumarin
AI	Active ingredients
CDNB	1-chloro-2, 4-dinitrobenzene
CE	Carboxylesterases
C_T	Cycle threshold
CT	Circadian Time
CYP	Cytochrome P450 Monooxygenases
DD cycle	Dark-dark cycle
DDT	dichloro-diphenyl-trichloroethane
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
GST	Glutathione S-Tranferase
LD cycle	Light-dark cycle
PCR	Polymerase chain reaction
RH	Relative humidity
RNA	Ribonucleic acid
RNAi	RNA interference
ZT	Zeitgeber Time
cDNA	complementary DNA
g	Gram
mM	Millimolar
mg	Milligram

ml	Milliliter
nm	Nanometer
qRT-PCR	Quantitative Real Time PCR
rpm	Revolutions per minute
µl	Microliter
α-NA	Alpha-naphtyl acetate
β-ME	Beta-mercaptoethanol
β-NA	Beta-naphtyl acetate

RITMA HARIAN DALAM KERENTANAN RACUN SERANGGA, AKTIVITI METABOLIK ENZIM DAN EKSPRESI GEN DALAM PEPIJAT UMUM, *Cimex lectularius* L. (HEMIPTERA: CIMICIDAE)

ABSTRAK

Penggunaan berterusan racun serangga secara tidak langsung mendorong kepada perkembangan kerintangan serangga di kalangan serangga perosak termasuk pepijat. Beberapa gen enzim pengekodan yang diketahui memetabolismekan racun serangga seperti Cytochrome P450, Glutathione S-tranferase (GST), dan esterase didapati mempunyai ritma harian pada beberapa serangga seperti nyamuk, lalat dan lipas. Sehingga kini, maklumat ini masih tidak jelas dalam pepijat. Pemahaman yang lebih baik terhadap ritma harian akan memberikan gambaran mengenai strategi kawalan asli untuk spesis perosak yang penting dalam perubatan. Lanjutan daripada itu, kami meneroka ritma harian dalam kerentanan racun seranggan bagi pepijat umum, *Cimex lectularius*. Ini dilakukan dengan mendedahkan pepijat kepada deltamethrin (pyrethroid) dan imidacloprid (neonikotinoid) pada masa tertentu sepanjang hari dan aktiviti 'knockdown' (KT_{50}) ditentukan. Ujian biokimia enzim untuk CYP450, GST dan esterase diuji untuk menentukan ritma harian dalam aktiviti detoksifikasi enzim, manakala kajian ekspresi gen dijalankan untuk menentukan ritma harian dalam ekspresi gen mRNA detoksifikasi racun serangga. Keputusan menunjukkan bahawa *C. lectularius* adalah paling toleran dengan deltamethrin dan imidacloprid semasa hujung fasa siang, masing-masing pada ZT9 dan CT9 dalam kitaran cahaya gelap (LD) dan kegelapan yang berterusan (DD). Walau bagaimanapun, analisis lengkung kemandirian menunjukkan bahawa pepijat mempunyai masa hidup

berselang-seli terhadap imidacloprid pada keadaan LD dan DD. Ekspresi ritma *GSTs1* dan *CYP397A1* menunjukkan corak ekspresi yang sama dengan aktiviti enzim GST dan CYP450 masing-masing dalam keadaan LD dan DD. Aktiviti enzim dan ekspresi gen ini secara konsisten menunjukkan aktiviti tertinggi pada ZT 9. Ekspresi mRNA *CE3959* menunjukkan corak ekspresi yang sama dengan gen *GSTs1* dalam keadaan LD dan DD. Pengetahuan ini boleh digunakan dalam merangka strategi yang berkesan untuk mengawal populasi pepijat dengan melakukan rawatan pada waktu tertentu yang diselaraskan dengan kerentanan racun serangga.

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ABSTRACT

The continuous usage of insecticides inadvertently leads to the development of insecticide resistance among insect pests including bed bugs. Several genes encoding enzymes known to metabolize insecticide such as Cytochrome P450, Glutathione S-tranferase (GST) and esterase are found to have daily rhythm in some insects such as mosquitoes, flies and cockroaches. To date, this information remains unclear in bed bugs. An improved understanding of bed bug chronotoxicity will yield insights into developing novel control strategies for this medically important pest species. To this extend, we explored daily rhythm in insecticide susceptibility of the common bed bug, *Cimex lectularius*. This was done by exposing the bed bug to deltamethrin (pyrethroid) and imidacloprid (neonicotinoid) at specific time across the day and knockdown activity (KT_{50}) was determined. Biochemical assays for CYP450, GST and esterase were tested to determine daily rhythm in enzymatic activities of detoxification, while gene expression study was conducted to determine the daily rhythm of mRNA expression level of insecticide detoxification genes. Results showed that the *C. lectularius* was most tolerant towards deltamethrin and imidacloprid during the late photophase at ZT9 and CT9 in light-dark cycle (LD) and constant darkness (DD), respectively. However, survival curve analysis revealed that the bed bugs have alternating survival time towards imidacloprid in both LD and DD condition. Rhythmic expressions of *GSTs1* and *CYP397A1* displayed similar expression patterns

with total GST and CYP450 enzyme activities in LD and DD conditions, respectively. This enzyme activities and gene expressions consistently displayed the highest peak activity at ZT9. *CE3959* mRNA expression show similar expression pattern with *GSTs1* gene in both LD and DD condition. This knowledge can be utilized in devising an effective strategy of controlling bed bug population by performing treatment during specific time of day that synchronizes with insecticides susceptibility.

CHAPTER 1

INTRODUCTION

The earth's rotations around its axis and the resulting daily environmental changes have caused most organisms to develop circadian (24hr) rhythms. These rhythms alter the activity levels of an organism including physiological processes, molecular expression and behavior throughout the day to adapt to diurnal changes in the environment (Hardin, 2005). In relation to this, a significant number of physiological and behavioral functions that are observed for 24-hour oscillations is mainly driven by endogenous circadian rhythms. Pittendrigh (1981) stated the circadian rhythms controls daily biological rhythms that play an important role in adjusting the physiological function into a necessary level when required in order to increase their fitness for survival. In order to synchronize with environmental changes, organisms process external environmental changes, called zeitgebers literally from German: time-givers. Environmental light is the main time that regulates the timing of circadian rhythms in most organisms (Daan *et al.*, 1976). Several published studies reported that some insects and mites tend to display circadian rhythm of susceptibility to toxic chemical (Beck, 1963; Polcik *et al.*, 1964).

A considerable amount of studies pointed out that many insects tended to show daily rhythm in detoxifying insecticides (Eesa and Cutkomp, 1995), such as those observed in *Aedes aegypti* (Linnaeus), *Anopheles gambiae* (Giles), *Drosophila melanogaster* (Meigen), *Blattella germanica* (Linnaeus), and *Bombyx mori* (Linnaeus) (Yang *et al.*, 2010; Balmert *et al.*, 2014; Hamby *et al.*, 2013; Lin *et al.*, 2014; Egi and Sakamoto, 2016). These studies however, are still limited to only a few taxa

considering the long history of this discovery, its importance, and the number of pest species around us. Moreover, the time of day expression of the detoxification genes and the respective detoxification enzyme activities at their minimum level may correspond to the time that the insects are most susceptible to insecticides considering that the up-regulation of genes involved in the detoxification pathway may contribute to the metabolic resistance of insecticides (Halberg *et al.*, 1974).

Several number of genes that are known to play a role in insecticide detoxifications such as the Glutathione S-transferase (GST), Cytochrome P450 (CYP450), and esterase are reported to be found in several insects such as mosquitoes (Li *et al.*, 2016), moths (Karthi and Shivakumar, 2016), and bees (Claudianos *et al.*, 2006). CYP450, GST, and esterase gene are suspected to be involved with xenobiotic resistance in insects by decreasing target site protein sensitivity that contributes to insecticide resistance for several classes of insecticides such as pyrethroid (Li *et al.*, 2007), DDT (Dombrowski *et al.*, 1998) and organophosphate (Feyereisen, 2005). In response to this issue, the group of detoxification genes was further investigated in other insect pest in the study of chronotoxicity. In addition, study conducted on malaria mosquitoes (*An. gambiae*), proved that Glutathione S-transferase gene (*GSTE2*), and several Cytochrome P450 genes (*CYP6P3*, *CYP6M2*, *CYP6Z1*) were up-regulated and managed to show insecticide resistance, especially in DDT and pyrethroids (Hemingway *et al.*, 2004; Stevenson *et al.*, 2011). Genome studies on common bed bugs reveal several genes coding for metabolizing enzyme which play a vital role in insecticide resistance. According to the study conducted by Adelman *et al.* (2011), only one GST gene (*GSTs1*) and two esterase gene (*CE3959* and *CE21331*) are over expressed in the resistant strain. In addition, Zhu *et al.* (2013) manage to identify four CYP450 genes (*CYP397A1*, *CYP398A1*, *CYP6DN1*, *CYP4CM1*) that show significant

up-regulation in the resistant strain which successfully knocked down the target gene found in RNAi studies.

Generally, bed bugs are known to be blood-sucking ectoparasites that feed on human and other warm-blooded animals. In most cases, bed bugs are found to be very intolerable among individuals, in which their distress is often manifested by general anxiety. The main factors that lead to the resurgence of bed bugs are the repeated exposure and excessive application of insecticides which then cause them to become resistant. More importantly, the medically important insect pest is known to be resistant to various type of insecticides such as DDT, organophosphate, and pyrethroids (Koganemaru and Miller, 2013). It is crucial to note that insects are capable to overcome the insecticides effects through several mechanisms. Basically, bed bugs evolve and have the adaptive strategy to be resistant towards insecticide through different mechanisms which include the modification of target site insensitivity (Dang *et al.*, 2015a), decreased cuticular penetration (Koganemaru *et al.*, 2013), behavioural response by avoiding insecticides (Romero *et al.*, 2009), and metabolic mechanism to detoxify insecticides (Lilly *et al.*, 2016).

To our knowledge, no study has been performed on the chronotoxicity of bed bugs; hence, further investigation is deemed necessary to obtain more information on this issue. In this study, it is believed that bed bugs will show specific changes in the time of day relating to insecticide susceptibility as shown in many other insects such as mosquitoes, cockroaches, and flies. In relation to this, this study finds that it is important to explore the time of day changes in insecticide susceptibility of the common bed bug, *Cimex lectularius* (Linnaeus).

Hence, the aims of this study are:

1. to investigate the daily rhythms of insecticide susceptibility of the common bed bugs, *C. lectularius* towards deltamethrin and imidacloprid,
2. to study the daily rhythms of biochemical enzyme activities of the common bed bugs, *C. lectularius*, and
3. to determine the daily rhythms of metabolic detoxification gene expression of the common bed bugs, *C. lectularius*,

It is hoped that an improved understanding of bed bug chronotoxicity can be achieved to yield insights into the development of novel control strategies for this medically important pest species.

CHAPTER 2

LITERATURE REVIEW

2.1 Bed Bugs

Generally, bed bugs are polyphagous nocturnal ectoparasites that feed on a broad range of hosts from birds to humans (Potter, 2011; Usinger, 1966). According to Koganemaru and Miller (2013), bed bugs can be categorized into two cryptic species in this world: (1) the common bed bug, *C. lectularius* that are mainly found in temperate region, and (2) the tropical bed bug, *C. hemipterus* (Fabricius) which can be found in tropical and subtropical regions.

2.1.1 Biology

Bed bugs belong to the family Cimicidae with over 75 hematophagus species within the order of Hemiptera (Doggett *et al.*, 2012). Nevertheless, there are two cimicid species that are known to be associated with human, namely the common bed bug (*C. lectularius*) and the tropical bed bug (*C. hemipterus*). Adult bed bugs are small in size which is approximately 3-5 mm long. They are oval-shaped insects that are flattened dorso-ventrally which will turn pale brown when unfed. However, they become engorged and elongated after feeding which then turn reddish brown with a 3-segmented beak, 4-segmented antennae, and vestigial wings. According to Bernardeschi *et al.* (2013), a mature *C. lectularius* is approximately the size of an apple seed based on microscopic evidence. Generally, unfed adult can achieve about 130% to 200% of their body weight with every blood meal taken (Johnson, 1941).

Adult bed bugs are very interested to mate with high rate of copulation directly after blood feeding. The mating of bed bug is performed through traumatic

insemination which involves puncturing the cuticles (Stutt and Siva-Jothy, 2001). Specifically, adult male will deliver the sperm into female haemolymph by piercing and then introducing an intromittent organ to the cuticle of female abdomen (Reinhardt *et al.*, 2003). A study conducted by Stutt and Siva-Jothy (2001) suggest that the females lifetime reproductive success will increase when the females copulate once every four blood meals intake compared to the normal rate with 20 times optimum rate that tend to reduce the longevity of females. In general, an adult female produces approximately 541 eggs in her lifetime and is capable to lay up to five eggs each day in sheltered locations (McNeill *et al.*, 2017; Usinger, 1966).

According to Rosenfeld *et al.* (2016), the bed bugs are hemimetabolous insect which have no pupal stage, by undergoing five developmental life stages from eggs to adult. In this case, every immature life stages require blood meal to allow them to develop into the next life stage. The eggs hatch and a first-instar nymph emerge which will then feed and moult to successive stages. As shown in Figure 2.1, the bed bug will become an adult after moulted through five instar nymphs. All five nymph stages share similar behaviour and appearance to the adults, but they are smaller, sexually immature, and lack of wings. Fifth-instar nymphs are distinguishable from the younger stages based on the presence of prominent wing-pads on either side of the thorax. In addition, an approximate total of 37 days are required to ensure the total development process of the bed bug from an egg to an adult at optimal temperatures which is 26 ± 2°C (McNeill *et al.*, 2017).

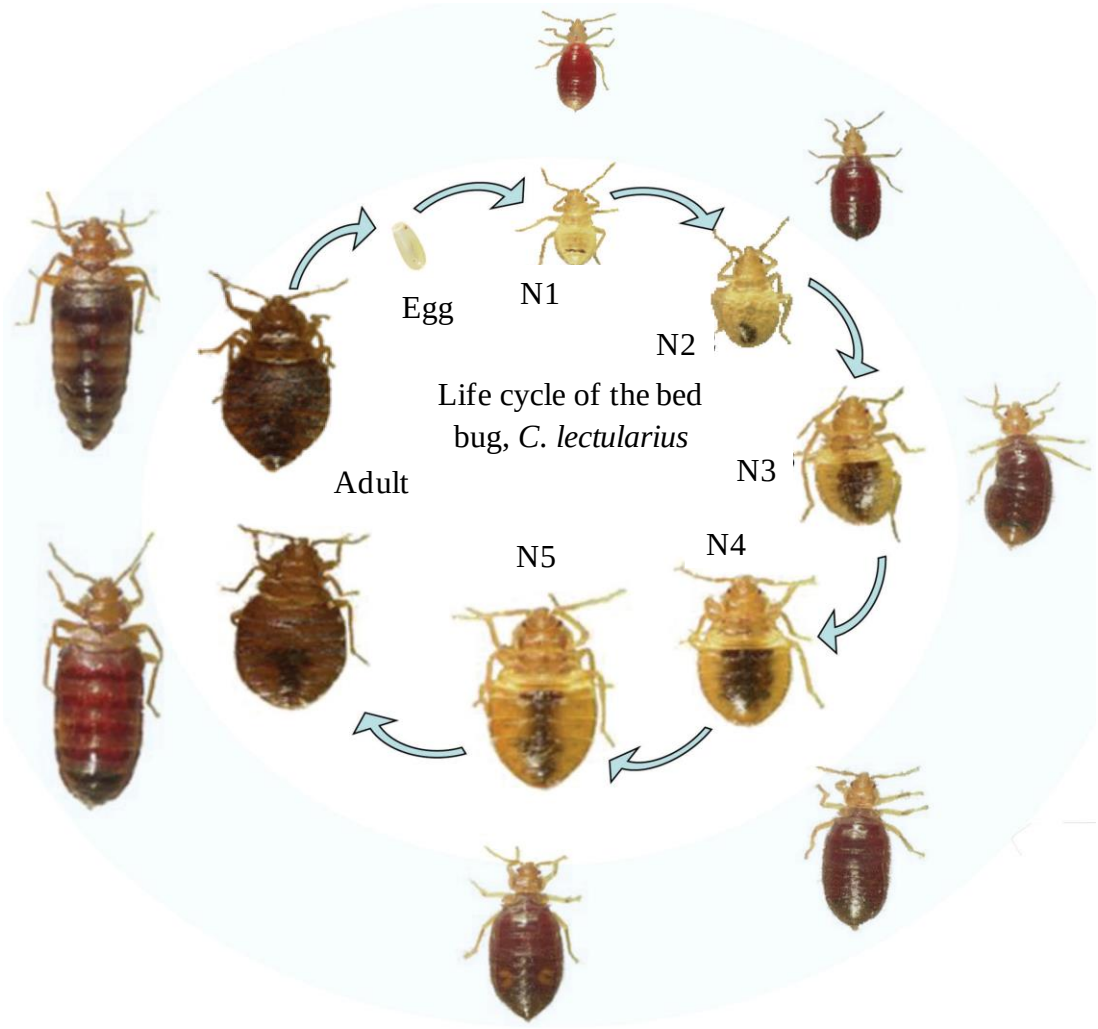

Figure 2.1 The life cycle and different body size of bed bugs (Reis, 2009).

2.1.2 Resurgence of Bed Bug

The common bed bug, *C. lectularius* has been associated with human since the ancient time (Usinger, 1966) and has been widely documented for the last 3000 years (Panagiotakopulu and Buckland, 1999). As mentioned by Usinger (1966), bed bug may have evolved from their ancestor which were cave-dwelling ectoparasites of mammals (especially bats) and turns to human as the alternative hosts. Urbanization and human social evolution are believed to lead to the widespread of bed bugs throughout the world as a result of human movement from the caves in the effort of building better settlement for the community (Potter, 2011).

The bed bug was almost completely eradicated during World War II due to the extensive use of pesticide such organophosphate and DDT in developed countries (Doggett *et al.*, 2012). After a decade, a study conducted by Krueger (2000) showed increase of incidences of bed bug infestations in major cities of United States between the year 1998 and 2000. More importantly, the resurgence was not only reported in the United States but all over the world (Boase, 2001; Doggett *et al.*, 2012; Faúndez and Carvajal, 2014; Hirao, 2010; How and Lee, 2010; Hwang *et al.*, 2005; Zulaikha *et al.*, 2016). In relation to this, various factors have been postulated to be responsible for the resurgence of bed bugs which include the improvement of transportation that promotes human movement and migration (Eddy and Jones, 2011), lack of information on pest management policies and practices (Boase, 2001), and lack of effective residual insecticide in the current market as well as the development of resistance to pyrethroid insecticides in bed bugs (Davies *et al.*, 2012; Myamba *et al.*, 2002). However, Romero *et al.* (2007) pointed out the main reason that contribute to the resurgence of bed bug refers to the increase of insecticide resistance of this pest.

According to the World Health Organization (WHO), insecticide resistance can be defined as the inherited ability of the organism to survive in certain doses of toxic or insecticide which could kill majority of the population of the same organism. A considerable amount of published studies show that bed bugs have developed resistance towards various chemicals through four different modes, namely target site resistance (Dang *et al.*, 2015a, 2015b; Yoon *et al.*, 2008; Zhu *et al.*, 2010), behavioral resistance (Tawatsin *et al.*, 2011), penetration resistance (Koganemaru *et al.*, 2013), and metabolic resistance (Karunaratne *et al.*, 2007; Lilly *et al.*, 2016).

2.1.3 Medical and Economical Impact of Bed Bug in Human Daily Life

Bed bugs tend to produce big impact to human life in terms of health issues and economy. This nocturnal animal can cause itchiness, anemia, inflammation, and reduced quality of sleep to those who are bitten (Pritchard and Hwang, 2009). Hosts immune system will respond to the insect saliva protein and cause itchy welts on the host bitten place (Romero *et al.*, 2007). More importantly, this insect can cause secondary infection to the host when the feeding wound caused by the bed bug is not treated well which will further infect the host (Ter Poorten and Prose, 2005). In most cases, some pathogen can be detected in bed bugs; however, a study conducted by Silverman *et al.* (2001) showed the association between virus Hepatitis B and HIV with the bed bug where both virus can persist in bed bugs guts but with no viral replication. Hence, it can be concluded that the bed bug is not able to transmit virus or diseases to the host, especially human.

According to Hwang *et al.* (2005), bed bug infestation has caused multimillion dollar lost for the spending on bed bug treatments, changes of furniture, reputation

damages, and legal charges for court cases. In addition, this does not only occur in the hospitality industry but also in other industries such as poultry and household.

2.1.4 Chemical Control of Bed Bugs

Insecticides are generally used to control bed bugs infestations as part of bed bug management. The right product type and proper formulation are critical to successful eradication (Doggett *et al.*, 2012). According to Potter (2011), DDT has proved to be used worldwide as insecticide against bed bugs in the beginning of 1942 and had caused a major decrease in infestations of bed bugs. However, it was reported that bed bugs developed resistance within 5 years due to excessive and continuous insecticide use (Johnson and Hill, 1948).

By the mid-1950s, National Pest Control Association has started recommending malathion (organophosphate insecticide) as another option to control bed bugs infestations (Feroz, 1971). During the 1980s and early 1990s, organophosphates continued to be successful against bed bugs, almost four decades after the compounds were first used to kill bedbugs. Most of organophosphates were extremely toxic and non-specific, affecting all humans, birds, amphibians and mammals, and had a high potential for delayed additive toxicity (Roberts and Reigart, 2013). Organophosphates are no longer used for bed bug control in Europe and the United States except in impregnated strips, though they are used in many other countries (Doggett *et al.*, 2012).

Pyrethroids have become one of the most important and widely-used insecticide groups during the last half century Due to the highly favorable characteristics of low mammalian toxicity, low volatility, high photostability, and increased residual efficacy (Potter *et al.*, 2010). To date, pyrethroids are still widely

used for the control of bed bugs. However, pyrethroids are generally poorly effective against modern, resistant bed bug strains, particularly when applied as a residual (Romero *et al.*, 2009).

In 1991, imidacloprid (neonicotinoid insecticide) was introduced into the market (Jeschke and Nauen, 2008). Imidacloprid contains nicotine neurotoxin found in tobacco, which causes inhibition in the transmission of stimuli, which may paralyze insects and eventually kill them. Bed bug treatments containing neonicotinoids are often combined with other active ingredients, including pyrethroids and diatomaceous earth which possess dual mode of action (Romero and Anderson, 2016).

2.2 Biological Rhythm

Biological rhythms are internal rhythms of biological activity that involve cyclic changes of chemical or function the organism body. Biological rhythm can either be controlled by the internal biological clock (endogenous) or controlled by synchronizing internal cycles with external stimuli (Hamby *et al.*, 2013).

2.2.1 Basic Concept of Biological Rhythm

Biological rhythm can be grouped into four major groups which are diurnal rhythm, circadian rhythm, ultradian rhythm, and infradian rhythm. Circadian rhythms oscillate for approximately 24 hours ("circadian" is translated from Latin as "about one day"). Most organisms in this world have endogenous biological clock that influences their daily activity, particularly in the aspect of biology and physiological activities. On top of that, the endogenous biological clock also develops the rhythmic activity pattern that can synchronize with the daily changes caused by the environmental conditions. In relation to this, Vitaterna *et al.* (2001) state that this

rhythm can be defined as circadian rhythms that possess several properties which include the ability to be entrained by external time cues for example light-dark cycle which have a period time of approximately 24 hours. Apart from that, another property is described as the pattern of rhythm under constant environmental conditions (constant darkness or constant light) that will persist under normal environmental condition (light-dark cycle). They also are resistant to temperature changes (Dunlap *et al.*, 2004). On the other hand, the properties of circadian rhythm are fulfilled and driven by endogenous circadian clock

Nevertheless, diurnal rhythm is only observed under normal condition (light-dark cycle) which may or may not be driven by endogenous circadian clock (Rund *et al.*, 2016; Zeng *et al.*, 2017). A biological rhythm is called ultradian if its period is shorter than 24 hour. Ultradian rhythms have been observed in physiological functions, like cellular processes, respiration, circulation, hormonal release and sleep stages, as well as in behavioral functions, often related to feeding patterns (Gerkema, 2002).

According to Bell-Pedersen *et al.* (2005), nearly all physiological and behavioral functions in almost all organisms, ranging from bacterial single – cell species to insects, birds, mammals and humans occur on a rhythmic basis. The predictable daily changes allow the organism to organize their daily activities based on their needs such as foraging, egg hatching, oviposition, and locomotor activity. The specificity of circadian rhythms across different branches of phylogenetic tree demonstrates the primary contribution of these rhythms to the sustaining of life on Earth. Study conducted by Ouyang *et al.* (1998) showed that the synchronization of endogenous rhythms and environmental cycles might contribute to prediction of reproductive fitness in competing cyanobacteria. Another study by Beaver *et al.* (2002) showed *Drosophila* strains with a disrupted circadian clock gene exhibited significant

declines in fertility. In addition, ground squirrels with disrupted the circadian rhythmicity by brain lesion were much more likely to be killed in naturalistic environments by predators (DeCoursey and Krulas, 1998).

The circadian rhythms are vital to synchronize crucial life process with environmental changes that increase their fitness for survival. To date, daily rhythms in insecticide susceptibility have been investigated in insect groups such as mosquitoes (Yang *et al.*, 2010), flies (Hamby *et al.*, 2013), honeybees (Piechowicz *et al.*, 2016), locusts (Onyeocha and Fuzeau, 1991) and cockroaches (Lin *et al.*, 2014). However, a few taxa had been studied due to long history of circadian rhythms discovery including its importance and the number of pest species.

2.2.2 Daily Rhythms in Insecticide Susceptibility

Beck (1963) mentioned that the first daily rhythms of insecticide susceptibility of insect managed to be recorded on German cockroach, *B. germanica* that underwent the experiment on normal light-dark cycle (12L: 12D photoperiod). The cockroach was most susceptible at the middle of the night when using carbamate insecticide. A similar finding obtained in a recent study by Lin *et al.* (2014) that was conducted on the same species of cockroach when using pyrethroid insecticide.

Meanwhile, a study conducted by Balmert *et al.* (2014) on malaria mosquito, *An. gambiae* showed that the mosquito were most resistant during late afternoon when using DDT as insecticide, while they were shown to be resistant at early and late of the day when using pyrethroid that produces bimodal rhythms. This finding is in agreement with the result of another study that investigated dengue fever mosquito, *Ae. aegypti* whereby higher resistance was observed during the day when pyrethroid insecticide is applied (Yang *et al.*, 2010).

A similar study was also conducted to determine the daily rhythms of insecticide susceptibility on lepidopteran insect using pyrethroid insecticides (Egi and Sakamoto, 2016;Karthi and Shivakumar, 2016), which revealed that the lepidopteran insect are more resistant towards pyrethroid at the end stage of photophase.

This daily efficacy variation observation was supported by Polcik *et al.* (1964) and further proposed that daily changes in the sensitivity towards insecticide treatments are dependent on the properties of the insecticide as well as endogenous rhythmic changes in pest physiology. However, this chronotoxicity not only depends on the specific time but also depends on the species, mode of treatment, and the insecticide tested (Halberg *et al.,* 1974).

The advancement of technology has allowed the daily rhythm of insecticide susceptibility to be further investigated in order to gain more detailed understanding in using metabolic enzymatic activity and the detoxification gene. Overall, it is hoped to provide a clear picture on the mechanism of the insecticide susceptibility in terms of the molecular aspects as well as an added advantage to the pest management in manipulating the daily rhythm to enhance the result of the pest control.

2.3 Biochemical Metabolic Enzyme Activity

The three major metabolic detoxification enzymes that play an important role in insecticide detoxification by insects which are Glutathione S-tranferase (GST), CYP450, and general esterase (Yin *et al.*, 2008; Kim *et al.*, 2004).

2.3.1 Glutathione S-Tranferase (GSTs) Metabolic Enzyme Activity

Glutathione S-transferases (GSTs) are a broad family of enzymes involved in a variety of biological processes. They play a central role in the detoxification of both

endogenous and xenobiotic compounds, as well as in physiological processes such as intracellular transport, hormone biosynthesis and oxidative stress protection (Enayati *et al.*, 2005). GSTs are categorized under phase II considering that the metabolic enzymes are capable of conjugating the reduced glutathione to the electrophilic centers of exogenous and endogenous compounds (Fang, 2012). On top of that, GSTs play an important role in xenobiotic metabolism through the catalysis of redox and conjugation reactions, which in turns facilitate the solubility of compounds to further excrete the toxic compounds from the system.

2.3.2 CYP450 Metabolic Enzyme Activity

CYP450s metabolic enzyme is grouped under phase I based on its capability to oxidize endogenous and exogenous compound by oxidation or other methods. Generally, phase I involves oxidation, reduction, and hydrolysis of the compound which include the detoxification of insecticide, especially in the metabolism of pyrethroids (Mamidala *et al.*, 2011a; Jones *et al.*, 2011). CYP450s possess xenobiotic resistance to insects by degrading more soluble and less toxic forms of compounds. They are mainly found in the food canal, fat, endocrine, exocrine and olfactory organs and epidermal tissues (Wang *et al.*, 2014). This protein has the absorption rate of 450nm when reduced and saturated with carbon monoxide. In general, CYP450 enzymes bind molecular oxygen [$S+(NADPH+H^+)+O_2 – S(O)+ NADP^+ + H_2O +$; wherein S is the substrate] and receive electrons from NADPH, which then leads to the formation of water (Bergé *et al.*, 1998) for the purpose of making the insecticide to be less toxic to the insects.

2.3.3 Carboxylesterases (CEs) Metabolic Enzyme Activity

A wide range of insecticides contain ester bonds and are therefore susceptible to hydrolysis by esterase activity (Sogorb and Vilanova, 2002). The group of hydrolase enzyme is capable of hydrolyzing compounds that contain ester bonds (Figure 2.2). Moreover, these enzymes are involved in the degradation of organophosphates, carbamates, and pyrethroids by mutant esterases in target site (Hotelier et al., 2010).

Organophosphates and carbamate target site resistance is mediated by mutations in acetylcholinesterase that make it less susceptible to insecticide inhibition. Meanwhile, this metabolic resistance includes either insecticide hydrolysis or mutant carboxylesterase sequestration. On another note, esterase enzyme activity has been widely studied in insect pest such as *C. lectularius* (Yoon *et al.*, 2008), *Bombyx mori* (Yu *et al.*, 2009), *Anopheles gambiae* (Perera *et al.*, 2008), and *Drosophila suzukii* (Hamby *et al.*, 2013). This esterase enzyme activity were found elevated in the insecticide susceptibility and suggested that the esterases causes resistant by a combination of sequestration and enhanced hydrolytic detoxification.

2.4 Metabolic Detoxification Gene

Within the last decades, a considerable amount of genome studies revealed and identified the genes that are associated with metabolic resistance (Liu, 2012). A genome study on malaria mosquito, *An. gambiae* conducted by Rund *et al.* (2011) showed that a total of 60 genes are predicted to be associated with metabolic detoxification, which are further represented by 35 genes of Cyctochrome P450 mono-oxygenases (CYP450s), 16 genes of Glutathione S-tranferases (GSTs), and two genes of carboxylesterases (CEs).

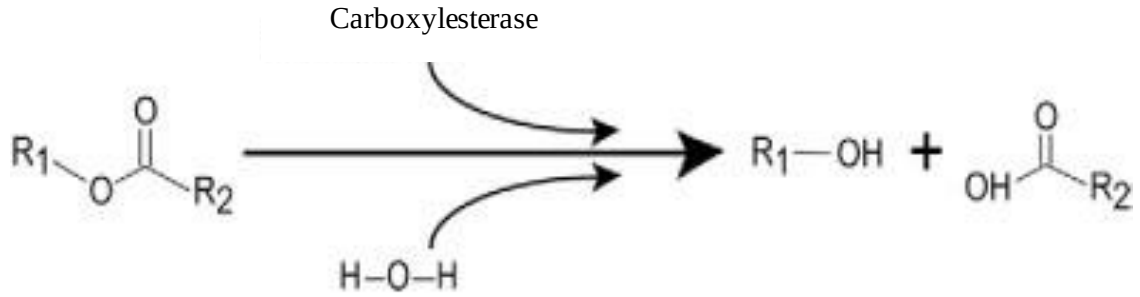

Figure 2.2 Basic carboxylesterase hydrolysis reaction. Carboxylesters are cleaved by carboxylesterases via the addition of water to form an alcohol and an acid metabolite (Montela *et al.*, 2012).

However, genome research by Adelman *et al.* (2011) on the common bed bug, *C. lectularius* revealed about 51 candidate genes that can be potentially associated with metabolic detoxification. Specifically, the 51 candidate genes are further divided whereby a total of 34 genes are from CYP450s, 10 genes from GSTs, and another 7 genes from CE genes. However, further investigation on the candidates only managed to identify three genes of CYPs, one gene of GSTs, and two genes of CEs that were upregulated expression in real-time PCR analysis of pyrethroid-resistant bed bugs (Adelman *et al.*, 2011).

2.4.1 The Expression of Glutathione S-Tranferases (GSTs) Metabolic Detoxification Genes

GSTs are classified into three groups based on their cellular localization, namely cytosolic, microsomal, and mitochondrial. In addition, blood-feeding insects are thought to possess both cytosolic and microsomal GSTs (Mamidala *et al.*, 2011). Generally, the GST family in insects encodes for a diverse set of proteins which include Delta, Epsilon, Sigma, Theta, Omega, and Zeta. However, only Delta and Epsilon are highly expressed in insects that are encountered with insecticides.

The daily expression of Glutathione S-tranferase (GST) gene was investigated in German cockroach, *B. germanica* by Lin *et al.* (2014). The result of the study showed that the expression decreases at the beginning of photophase, but increases in the middle of scotophase. Meanwhile, deep sequencing study of bed bug by Adelman *et al.* (2011) identified ten GSTs gene candidates from the classes of sigma, omega, zeta, theta, and delta. However, the finding showed that only one candidate from sigma classes (*GSTs1*) is significantly expressed in pyrethroid-resistant strain.

2.4.2 The Expression of Cytochrome P450 (CYP450) Metabolic Detoxification Genes

Over 600 insects of CYP450s gene managed to be identifed in genome sequencing research which include the families and subfamilies of CYP4, CYP6, CYP12, CYP15A, CYP18A, CYP28A, CYP29A, and others (Ranson *et al.*, 2002). Hence, several approaches including the expression level of the gene, cross strain comparison for gene sequence, and RNAi studies were performed to determine the molecular mechanism for CYP450-mediated resistance.

In insects, the Cytochrome P450 plays an important role in detoxifying insecticides. For example, in the case of *An. gambaie*, the genes associated with pyrethroid metabolism are highly expressed around dusk, particularly the Cytochrome P450 gene (*CYP6M2*, *CYP6P3*, and *CYP6Z1*) (Balmert *et al.*, 2014; Rund *et al.*, 2011b). Moreover, the *CYP9M9* in *Ae. aegypti* is shown to be rhythmic with the highest expression observed during the beginning of night (Yang *et al.*, 2010). On another note, bed bugs research on metabolic detoxification revealed that *CYP6DM2*, *CYP397A1*, *CYP400A1*, *CYP398A1*, and *CYP4CM1* were overly expressed in pyrethroid-resistant strain (Adelman *et al.*, 2011; Zhu *et al.*, 2013).

2.4.3 The Expression of Carboxylesterases (CEs) Metabolic Detoxification Genes

The gene amplification, upregulation of gene expression, or the combination of the two mechanisms managed to reveal the role of esterases in the detoxification of insecticides which include organophosphate, carbamates, and pyrethroids which are found in many insect pests (Egi and Sakamoto, 2016; Hardstone *et al.*, 2015; Hemingway *et al.*, 2004).

According to Labbé *et al.* (2011), a total of 76 putative carboxylesterases genes managed to be identified in the silkworm genomes. However, only 55 genes were found to be potentially involved in the detoxification of insecticides in silkworm out of the 76 identified CE genes. Moreover, esterase gene is also implicated in other insects such as the tarnished plant bug, the green peach aphid, and the parasitoid wasps (Claridge-Chang *et al.*, 2001; Li *et al.*, 2016).

A recent study by Adelman *et al.* (2011) showed that only two CEs gene (*CE3959* and *CE21331*) were significantly up-regulated in the bed bug pyrethroid-resistant strain, *C. lectularius*. In regard to this, the genes were further investigated by Zhu *et al.* (2013) on another population of bed bug pyretroid-resistant strain. However, only *CE21331* gene showed significantly increased expression in the resistant strain.

2.4.4 The Expression of ATP-binding cassette (ABC) transporters Metabolic Detoxification Genes

ATP-binding cassette (ABC) proteins are found in all living organisms comprise a large and variable transporter superfamily across the membranes (Dassa and Bouige, 2001). Most of these ABC proteins function as primary active transporters, requiring ATP binding and hydrolysis to transport substrates through lipid membranes. The substrates covering amino acids, sugars, heavy metal ions and conjugates, peptides, lipids, polysaccharides, xenobiotics and chemotherapeutic drugs across cellular membranes (Rees *et al.*, 2009). Due to its ability to transport toxins and drugs across membrane, ABC transporters are important player with resistance to major insecticide although not directly involved in detoxification of the compounds (Dang *et al.*, 2017).

The *white* gene was first identified insect ABC transporter gene which involved in the transport of eye pigment precursors in *Drosophila melanogaster* (Ewart and Howells, 1998). Upregulation of other ABC transporter genes that associated with exposure to highly efficient insecticides such as pyrethroids also reported in *Ae. aegypti* (Bariami and Jones, 2012), *An. gambiae* (Bonizzoni *et al.*, 2012), and *C. lectularius* (Mamidala *et al.*, 2012).

In bed bugs, ABC transporters were suspected involved in metabolic resistance due to overexpression of the genes encoding ABC transporter as reported by Mamidala *et al.* (2012). RNAi study were conducted by Zhu *et al.* (2013) have confirmed the role of ABC transporters by using *Abc 8* and *Abc 9* gene which mediated metabolic resistance to pyrethroids in *C. lectularius*.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bed Bugs Culture

The common bed bug, *C. lectularius* was obtained from the culture collection at Urban Entomology Laboratory, Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia and were maintained in a programmable climatic chamber (Percival®model CU-41L4) at the Centre for Chemical Biology, Universiti Sains Malaysia under 27°C with 60 ± 5% RH, and a photoperiod of 12:12 (L: D) h. The strain used in this study was the Monheim strain which is an insecticide-susceptible laboratory strain.

The bed bugs were provided human blood meals (self-feeding) once every week. The bed bugs were placed in insect container covered by fine mesh cloth. The insect container was placed upside down on the arm of the volunteer and the bed bugs pierced and sucked human blood through the mesh lid. The last stage of the nymph (5^{th} instar) was isolated from its colony and was separated individually in a Petri dish. This last instar nymph was blood-fed until it moulted into adult bed bugs. According to DeVries *et al.* (2015), an unmated adult male has a greater metabolic rate as compared to unmated adult females, and have stable metabolic rate after feeding between 7 to 10 days. Because of this, the unmated adult male was used in this study and not offered blood meal after moulted from the last nymphal stage.

Time of the day reported in this study was according to Zeitgeber time (ZT), where ZT 0 can be defined as when the lights were on and ZT 12 is when the lights were off. ZT 0 was set at 0700 (UTC+08:00) and ZT 12 was set at 1900 (UTC+08:00)

(Table 3.1). All experiments under the normal 24 h light-dark (LD) cycle were initiated one hour after the lights were switched on (ZT 1). For experiments conducted in 24-hour constant darkness (DD cycle), a Circadian Time (CT) was used to refer the time unit. All bed bugs were acclimatized in the experimental chamber for two LD cycle prior to the experiment day (Romero *et al.*, 2010).

3.2 Insecticide Bioassay

Ten unmated adult male bed bug were collected every four hours interval at each ZT 1, ZT 5, ZT 9, ZT 13, ZT 17 and ZT 21 of the LD cycle and CT 1, CT 5, CT 9, CT 13, CT 17 and CT 21 of the DD cycle to demonstrate insecticide susceptibility fluctuation. Two different classes of insecticides were used in this study. Deltamethrin was used to represent the pyrethroid insecticide whereas imidacloprid was used to represent the neonicotinoid insecticides. The insecticide was prepared according to the baseline susceptible data for deltamethrin and discriminating concentration for imidacloprid (Dang *et al.*, 2017). Stock solution (10X) of deltamethrin was prepared by diluting 0.0153 g of technical grade deltamethrin (97%, Bayer Environmental Science, Kuala Lumpur, Malaysia) in 10 ml of acetone (99.5%, Merck, Kuala Lumpur, Malaysia). Then, the 10X deltamethrin solution was diluted to 1X with acetone. A 0.5 ml of deltamethrin solution (19.2 mg AI m^{-2}) was decanted on a glass PPetri dish (70 x 18 mm). The glass Petri dish was allowed to dry in fume hood one hour prior to the experiments.

A 0.0312 g of technical grade imidacloprid (95%, Bayer CropScience, Hawthorn East, Victoria, Australia) was dissolved in 10 ml acetone (99.9%, Merck, Kuala Lumpur, Malaysia). This stock solution (20X) were diluted into 10X (192 mg AI m^{-2}) and used for the experiment. 0.5 ml of imidacloprid solution were decanted

Table 3.1 Actual time (UTC+08:00) for ZT and CT

Condition	Time point	Actual time (UTC+08:00)
LD (ZT)	1	0800
	5	1200
	9	1600
	13	2000
	17	0000
	21	0400
DD (CT)	1	0800
	5	1200
	9	1600
	13	2000
	17	0000
	21	0400