THE EFFICACY OF INSECT GROWTH REGULATORS ON TROPICAL BED BUGS, *Cimex hemipterus* (Fabricus) AND ITS MICROBIAL FAUNA USING PCR BASED CULTURE DEPENDENT AND 16S rRNA

NURHIDAYAH BINTI TAIBUKAHN

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by

NURHIDAYAH BINTI TAIBUKAHN

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LIST OF SYMBOLS

β	Beta
%	Percentage
^{o}C	Degree Celsius

LIST OF ABBREVIATIONS

CFU	Colony forming unit
CSI	Chitin synthesis inhibitor
IGR	Insect growth regulators
JHA	Juvenile hormone analogue
LC	Lethal concentration
LT	Lethal time
PPM	Parts per million
TVC	Total viable count

LIST OF APPENDICES

KEBERKESANAN PENGAWAL PERTUMBUHAN SERANGGA TERHADAP PEPIJAT TROPIKA, *Cimex hemipterus* (Fabricus) DAN FAUNA MIKROBNYA MENGGUNAKAN PENDEKATAN KULTUR BERASASKAN PCR DAN PENDEKATAN BEBAS KULTUR 16S rRNA

ABSTRAK

Pepijat merupakan ektoparasit yang khususnya menjadikan manusia sebagai sumber makanan utama manakala organisma berdarah panas yang lain sebagai sumber alternatif. Terdapat dua spesies yang mempunyai kaitan perubatan iaitu pepijat biasa, *Cimex lectularius* L. dan pepijat tropika, *Cimex hemipterus* (F.). Formulasi racun serangga novel sangat dituntut, disebabkan oleh kesukaran dalam memerangi populasi pepijat. Bagi pembasmian menyeluruh, adalah penting untuk mengaplikasi rawatan jangka panjang menggunakan racun serangga merangkumi bahan aktif kimia dan bukan kimia kerana pepijat mampu memodulasi mekanisme kerintangannya. Penyelidikan ini mengeksploitasi pengawalseliaan endosimbion mikrob pepijat tropika, *Cimex hemipterus* kawalan dan dirawat dengan racun serangga yang bergantung kepada analisis kultur berasaskan PCR dan analisis bebas kultur berasaskan metagenomik 16S rRNA. Pada awalnya, nimfa peringkat kelima jenis lapangan dan pepijat tropika dewasa dikumpul dari kawasan perumahan di sekitar Pulau Pinang dan kemudiannya dirawat dengan lima kelas racun serangga iaitu chlorfluazuron yang telah dikilangkan, chlorfluazuron, pyriproxyfen, methoprene, tebufenozide dan β-cyfluthrin + imidacloprid bertindak sebagai kawalan positif. Racun serangga digunakan mempunyai mod tindak balas berbeza yang terdiri daripada perencat sintesis kitin, analog hormon juvana, agonis reseptor ekdisteroid dan

campuran piretroid dan neonicotinoid yang digunakan pada empat kepekatan berbeza (100, 500, 1000 dan 10 000ppm). Antara kesemua kelas racun serangga yang diuji, chlorfluazuron yang telah dikilangkan dan β-cyfluthrin + imidacloprid menunjukkan prestasi mortaliti yang paling ketara dalam dua minggu tempoh bioasai sentuhan permukaan berbanding racun serangga lain yang menawarkan purata kematian kurang daripada 50%. Bersamaan dengan itu, racun serangga chlorfluazuron pada kepekatan yang tinggi menunjukkan kecekapan dalam merencat tumbesaran nimfa peringkat kelima, manakala β-cyfluthrin + imidacloprid mempersembahkan keberkesanan ovisidal yang lebih baik berbanding kesemua racun serangga yang diuji. Pepijat yang telah mati dihomogenkan dalam larutan garam penimbal fosfat dan diinokulasi ke dalam medium dan diinkubasi. DNA daripada koloni bakteria telah diekstrak dan dilakukan penjujukan untuk pengecaman spesies. Teknik bergantung kepada kultur ini mendedahkan unit pembentuk koloni bakteria mempunyai perbezaan yang ketara antara pepijat kawalan dan rawatan ($p < 0.001$). Ini membuktikan peraturan simbion bakteria mungkin ditindas oleh penggunaan racun serangga. Disebabkan limitasi pada teknik ini, komposisi mikrob pepijat kawalan dan rawatan telah ditentukan menggunakan gen 16S rRNA, dan menyusunnya menggunakan penjujukan MiSeq Illumina. Proteobacteria merangkumi lebih daripada 99% populasi mikrob dalam semua sampel dengan *Wolbachia* dan *Burkholderia* sebagai unit taksonomi operasi (OTU) peringkat genus yang paling lazim. Oleh itu, penyelidikan ini membuka peluang untuk kajian tambahan yang memfokuskan untuk memahami peranan dan akauntabiliti bakteria yang mendiami serangga perumah.

THE EFFICACY OF INSECT GROWTH REGULATORS ON TROPICAL BED BUGS, *Cimex hemipterus* (Fabricus) AND ITS MICROBIAL FAUNA USING PCR BASED CULTURE DEPENDENT AND 16S rRNA

ABSTRACT

Bed bugs are ectoparasites that primarily feed on humans and secondarily on other warm-blooded organisms. Two widespread species with medical relevance are the common bed bugs, *Cimex lectularius* L. and the tropical bed bugs, *Cimex hemipterus* (F.). Novel insecticides formulation are highly demanded due to the difficulties in battling bed bugs. For total eradication, it is important to administer long-term treatment using chemical and non-chemical active ingredients since bed bugs are capable in modulating their resistance mechanisms. This research exploits the regulation of microbial endosymbiont between insecticides treated and untreated tropical bed bugs, *Cimex hemipterus* employing PCR based culture dependent and 16S rRNA metagenomic based culture independent analysis. Prior to this study, field strain fifth stage nymph and adult bed bugs collected from residential area around Penang Island were treated with five classes of insecticides comprised of manufactured brand chlorfluazuron, chlorfluazuron, pyriproxyfen, methoprene, tebufenozide and β-cyfluthrin + imidacloprid act as positive control. The treated insecticide poses different mode of action in which chitin synthesis inhibitor, juvenile hormone analogue, ecdysteroid receptor agonist and mixture of pyrethroid and neonicotinoids that were applied at four different concentrations (100, 500, 1000 and 10 000ppm). Among the insecticides classes, manufactured brand chlorfluazuron and β-cyfluthrin + imidacloprid demonstrated the prominent mortality performance within the two weeks

of surface contact bioassay compared to other insecticides which offers less than 50% of mortality percentage. In conjunction, chlorfluazuron insecticide at higher concentration resulted in an efficient nymphal restraining capacity against the fifth stage nymph, while β-cyfluthrin + imidacloprid presented with a better ovicidal properties compare to all tested insecticides. The dead bed bugs were homogenized in a phosphate buffer saline solution and inoculated into an agar medium then incubated. The DNA from bacterial colony were extracted and sequenced for species identification. This culture dependent technique reveals the bacterial colony forming unit dictate a significant difference between control and insecticides treated bed bugs (p <0.001), indicating regulation of bacterial symbionts may been suppressed by the insecticide application. Due to limitation in this culture method, the microbial composition of both control and insecticide treated bed bugs was determined by amplifying the 16S rRNA gene, and sequenced by employing MiSeq Illumina sequencing. Proteobacteria accounted for over 99% of the microbial population in all samples with *Wolbachia* and *Burkholderia* as the most prevalent genus-level operational taxonomic units (OTUs). Hence, this research paves the opportunities for additional study focusing at comprehending the role and accountability of the bacteria that inhabit host insects

CHAPTER 1

INTRODUCTION

1.1 Research Background

Cimex spp. have been a major global concern for ages. The first documented instance occurred in Greece around 400 BC (Giorda et al., 2013). Bed bugs have been pestering people for at least 3550 years, when they first parasitized bats and then transferred to humans who shared caves in the Mediterranean and Middle East region, according to experts (Delaunay et al., 2011; Koganemaru & Miller, 2013). Humans begins to move out from caves as they adapt advancement in their living hood via civilization, urbanization and globalization (Potter 2011). Interestingly, bed bugs tag along with them entering their new habitats and continues to haunt them. As modernization took place, people began to travel, migrate and emigrate while concomitantly transfers these bugs from infected areas to uninfected areas. The global widespread of bed bugs infestation are solely due to passive transferral of small population that may hides in between clothing, baggage, furniture or vehicle and they will began infesting their new premises (Reinhardt & Siva-jothy, 2007). Bed bug infestations were a huge problem in the 18th century, especially in underdeveloped countries due to poverty and lack of sanitation, but with the discovery of pesticides and control techniques in the 19th century, the infestation began to reduce. Nonetheless, the infestation resurfaced in the mid-nineteenth century and becomes ubiquitous, notably after World War II until today (Potter, 2011).

The World Health Organization (WHO) has proposed a few approaches to combat these raging infestations, including detecting bugs by inspecting possible hiding places, performing simple household measures, using total release forger, insect repellent, impregnated mosquito nets, smoke generators, and residual insecticides (Chavasse et al., 1997). Although these procedures have been employed for decades, no overwhelming results have been achieved because bed bugs have learned to adapt to the control measures enforced, there is a lack of public information concerning bed bugs, poor hygienic conditions in the home, and labor-intensive and costly measures (Hwang et al., 2005). As a result, experts are working to develop a viable bed bug extermination approach that includes the use of chemical insecticides and insect growth regulators (Davies et al., 2012).

1.2 Problem Statement

Bed bug infestations are a nuisance to humans, who have been battling this insect for centuries. As international travel, migration, and emigration increased, bed bug infestation had became a global dilemma (Punchihewa et al., 2019). Insecticide resistance may be a crucial predictor of bed bug resurgence, particularly after World War II. Recently, scholars identified endosymbionts could be a plausible resistance modulator in bed bugs as endosymbionts has a pivotal function in host metabolism, digestion, providing nutrients, stimulate immune system and degrading insecticides/pesticides. Hence, the purpose of this study is to vindicate the efficacy of insecticides of different class beta-cyfluthrin + imidacloprid (nicotinic acetylcholine receptor + sodium channel modulator), chlorfluazuron (chitin synthesis inhibitors), tebufenozide (ecdysone receptor agonist), methoprene and pyriproxyfen (juvenile hormone analogue) on survival competence of tropical bed bugs based on the regulation of their microbial symbionts. A PCR based culture dependent method used

to observe the differentiation in bacterial growth between control and insecticide treated bed bugs together with 16S rRNA metagenomic approach, which indicates exposure to these insecticides could either upregulate or downregulate the proliferation rate of their microbial symbionts.

1.3 Research Hypothesis

Surface contact bioassay application of insecticides like beta-cyfluthrin + imidacloprid, chlorfluazuron, tebufenozide, methoprene and pyriproxyfen on tropical bed bud, *Cimex hemipterus* would propose a noteworthy finding. Because insect growth regulators (IGR) have an unusual mode of action that involves chitin synthesis inhibitors and substances like juvenile hormone analogue and ecdysteroids receptor agonist. The effect of IGR could be defined from the early stages of bed bugs through adulthood. Insect cuticle production is suppressed by chitin synthesis inhibitors, whereas juvenile hormone analogue and ecdysteroids agonist interfere with insect hormonal function. Chlorfluazuron, a chitin synthesis inhibitor utilized in this study triggers abortive molting, egg hatching defects, cuticle deformity, and eventually infection in the treated bed bugs. Ecdysone receptor agonist; tebufenozide disrupts molting and metamorphosis. Meanwhile, juvenile hormone analogues like pyriproxyfen and methoprene are more effective during the metamorphosis and embryogenesis phases. This leads to supernumerary instars, aberrant and delayed development, inability to reproduce, and sterile bed bug species. On the other hand, beta-cyfluthrin and imidacloprid are pyrethroid + neonicotinoid compounds that have shown potential results when tested against tropical bed bugs. It demonstrates a decrease in bed bug population growth, along with changes in behavior and fecundity where bed bugs experience uncontrolled nerve firing and overstimulation of nervous system. The oviposition of adult females is delayed, male mating behavior is reduced,

and egg hatching capacity is reduced. When examined using a surface contact bioassay approach, insecticides were able to prevent bed bug development and subsequently result in mortality. Furthermore, DNA sequencing of the polymerase chain reaction bacterial culture reveals the sequence of bacterial DNA between the control and treatment groups that can be identified and distinguished. In conjunction to this, metagenomic analysis reveals differences in regulation of bed bugs bacterial symbionts between control and treated bed bugs.

1.4 Research Objectives

1. To evaluate the effectiveness of four classes of insecticide; chitin synthesis inhibitor (chlorfluazuron), juvenile hormone analogue (methoprene and pyriproxyfen), ecdysone receptor agonist (tebufenozide) and pyrethroid + neonicotinoid (beta-cyfluthrin + imidacloprid) against the mortality rate, nymphal and egg development rate of tropical bed bugs.

2. To observe the differentiation in bacterial growth between control and insecticide treated bed bugs employing polymerase chain reaction culture dependent technique.

3. To demonstrate the regulation of bacterial symbionts between control and insecticide treated *Cimex hemipterus* using 16S rRNA metagenomic culture independent approach.

CHAPTER 2

LITERATURE REVIEW

2.1 Biology of Bed Bugs

2.1.1 General Biology

Pests, especially those in the phylum Arthropoda, have long been associated with humans. These arthropods transmit diseases to humans in addition to being a nuisance when they bite, feed on, live on, or reproduce on human skin (Diaz, 2015; Potter, 2011; Russell et al., 2013). As a result, many methods have been established over many centuries for addressing the aforementioned threats. There are over 110 species of arthropods in the family Cimicidae and all of them are obligatory hematophagous that solely feed on vertebrates (Heaton, 2013; Pinto et al., 2007). *Cimex* species is one of the most renowned hematophagous where *Cimex lectularius* and *Cimex hemipterus* are the principal species linked with humans. Both species impose significant similarities in their biology and characteristics that impact healthcare (Delaunay et al., 2011; Heaton, 2013). *Cimex lectularius* commonly infests temperate regions, while *Cimex hemipterus* is abundant within 30 degrees north and south of the equator (Zorrilla-Vaca et al., 2015).

Over the years, humans have been a sort of transportation for bed bugs since these bugs have dispersed all over the world, particularly from the advancement of urbanization, globalization, and civilization, in addition to migration from infected

areas to uninfected areas, travel, clothing, baggage, furniture, and vehicles like car, bus, train, ship, and airline (Reinhardt & Siva-jothy, 2007). Fascinatingly, as a hematophagous ectoparasite, bed bugs congregate in cracks and crevices. They demonstrate nocturnal and cryptic activity by emerging during the wee hours to feed on their host (Ab Majid & Zahran, 2015). Mankind is the dominant host of these bugs as it yields the maximum proportion of oviposited eggs, but they can also feed on other species, such as mice, rabbits, guinea pigs, birds, and chickens (Abd Rahim et al., 2016). This Cimicidae requires blood meal exclusively for survival, growth and reproduction (Akhoundi et al., 2020). Bed bugs can travel up to 5-20 feet from their refuge to locate their victim via their olfactory organs that alert to the presence of a potential host by the emanation of body odour, heat and carbon dioxide from their sleeping hosts (Doggett et al., 2012; Goddard & DeShazo, 2009).

2.1.2 Life Cycle

As a member of the hemimetabolous metamorphosis insect, bed bugs' life cycle comprises of seven stages (Okwa & Omoniyi, 2016). The evolution of each stage requires a blood meal and the complete life cycle of bed bugs from egg to egg takes roughly 30 to 70 days, depending on the environmental condition and host availability (Harlan, 2006; Delaunay et al., 2011). Nevertheless, according to Benoit et al., (2009), the optimal conditions for bed bugs to undergo full development in a little over one month consist of a temperature ranging from 25-32°C and a relative humidity of 40-75%. Throughout bed bugs' development, their body size expands and blood meal triggers an increase in body length by 30% – 50% and weight by 150% – 200% (Goddard & DeShazo, 2009). Remarkably, these hematophagous organisms have the ability to survive for 12 months without engaging in feeding activities, especially in

older nymphs and adults. In colder conditions, their survival period can extend to 1.5-2 years, allowing them to sustain their population (Delaunay et al., 2011).

This cimicid requires unique copulation between male and female, known as traumatic insemination to begin its life cycle (Kamimura et al., 2014) (Figure 2.1). From the copulation, within 3 days, the female produces a batch of 1-12 eggs per day (Mullen and Durden 2009; Khan and Rahman 2012). The eggs take around a week to hatch into 1st stage nymph and upon receiving blood meal, the nymph will subsequently complete its nymphal stage, which is divided into five stages before emerging into adult (Miller et al., 2013).

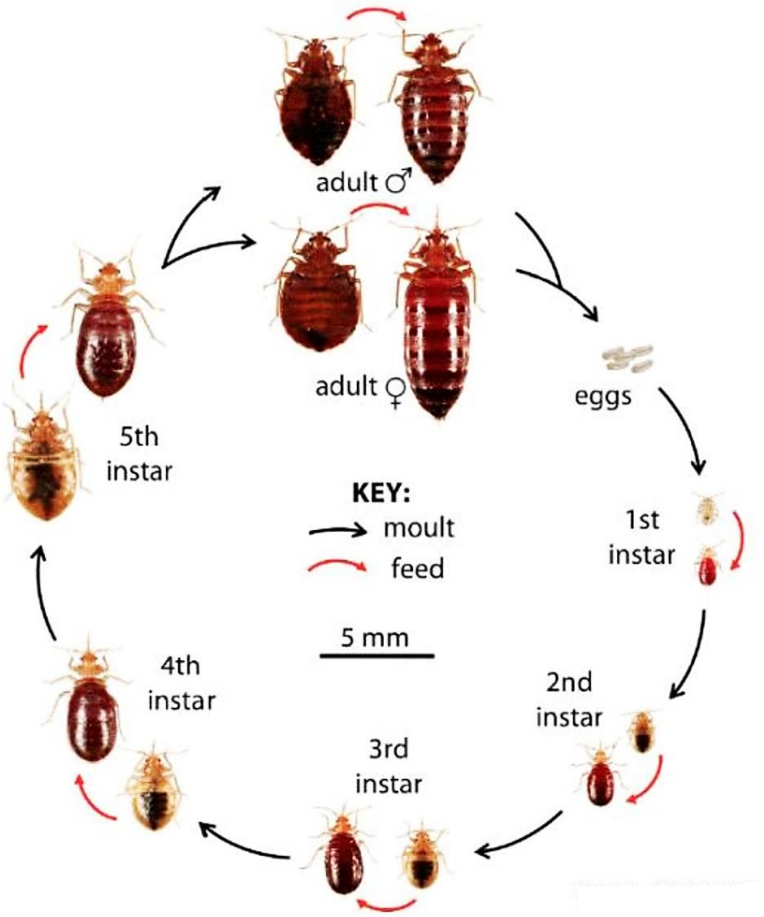

Figure 2.1 Life cycle of bed bugs (Palencia, 2012).

2.1.3 Morphology

The two cimicids associated with human poses morphological differences beside their geographical distribution but the differentiation requires experts and entomologist to pinpoint the structural dissimilarities. Throughout their lives bed bug tend to develop into next stage via shedding their exoskeleton called exuviae. Undoubtedly, the morphology of these cimicid bugs has played a significant role in their successful adaptation and survival. Their diminutive stature and dorsoventral flattening body allow them to clandestinely scavenge for sustenance onto their host. Among the hemimetabolous development stages of bed bug, there is a few distinct dissimilarities in their morphological appearance that can be distinguished.

Theoretically, the global range of *C. lectularius* encompasses several regions, including Australia, the United States, Denmark, Nigeria, and Iran, contradicting *C. hemipterus* which exhibits a more restricted distribution mostly within tropical areas, such as Southeast Asia (Seidel and Reinhardt, 2013). Common bed bug; *C. lectularius* exhibited a more rounded bodily morphology compare to *C. hemipterus* that displays a little smaller and darker body (Khan and Rahman 2012). One notable characteristic that sets apart the two species is the presence of an upturned lateral flange on the pronotum of adult *C. lectularius*. This feature contributes to a broader thorax in comparison to adult *C. hemipterus*, as documented by Doggett et al. (2012) (Figure 2.2). Consequently, the neck of the tropical bed bugs seemed elongated, since a significant portion of it remained exposed due to the lack of coverage provided by the pronotum. *Cimex hemipterus* exhibits a somewhat elongated and slender abdomen in comparison to other bed bug species. Furthermore, the coxae of *Cimex hemipterus* demonstrate a proximal end with a zigzag structure, facilitating its attachment to the insect's body (Usinger

1966). However, errors in identification may occur due of the physical resemblance between these two *Cimex* species, perhaps leading to an underestimation of actual infestation rates (Lewis et al., 2020). In addition to the criteria set by experts, one alternative approach to distinguishing between them is through the utilization of phylogenetic analysis (Tawatsin et al., 2013). The application of molecular methodologies, such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), enables efficient species comparison within a limited timeframe (Hemingway and Ranson, 2000).

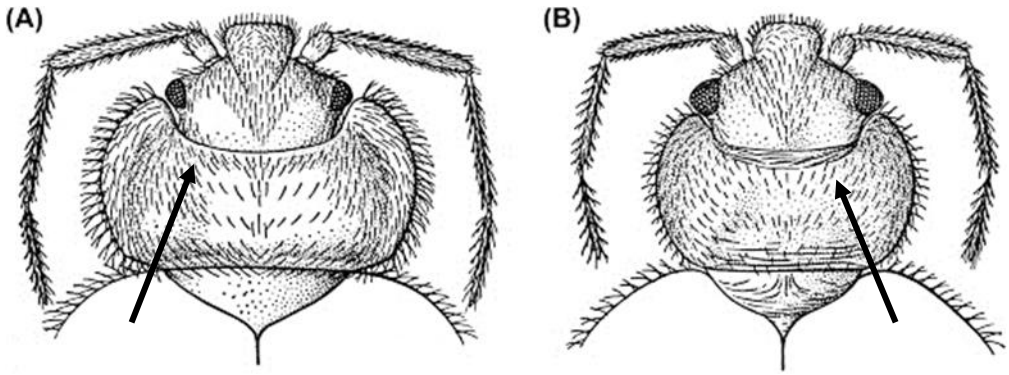

Figure 2.2 Differentiation between common bed bugs (A) and tropical bed bugs (B) at the pronotum (arrow) (Krinsky 2019).

Despite, having dissimilarity in their pronotum extension, bed bugs still exhibit similarities in their morphological appearance. Adults bed bugs have a dull reddish-brown colour, narrower than 3 mm and resembles an apple seed (Krinsky 2019; Pinto et al 2007). When a bed bug is not feeding, its three-segmented rostrum is stored in a groove between the bug's head and thorax (Pinto et al. 2007). The rostrum consists of the labium, which can be bent in two places, the mandibular stylets, and the maxillary stylets, each of which can be bent in two places as well (Krinsky 2019). The antennae of a bed bug have four segments, and the bug's head may be seen above the pronotum, as similar with other Hemipterans. The two forewings of a bed bug are reduced to scale-

like pads, while the bug's rear wings are absent (Cheng, 1969; McNeill 2016). Thus, bed bug can't fly or jump due to its little wings, yet it can move quite swiftly (0.12 m/min) when it crawls. There are a few distinguishing features between the sexes of the bed bug (Figure 2.3). Adult female bed bugs are often a bit larger in size when compared to males and tend to be more rounded at the end of their abdomen. Female bed bugs have a rounded tip to their abdomen, contrarily males' is pointed because of their paramere (Harlan 2006; Cooper & Harlan 2011; Todd 2023). Publication by Cooper & Harlan (2011) also disclose the existence of a prominent spermalege groove on the ventral side of the abdomen is another physical characteristic for female identification.

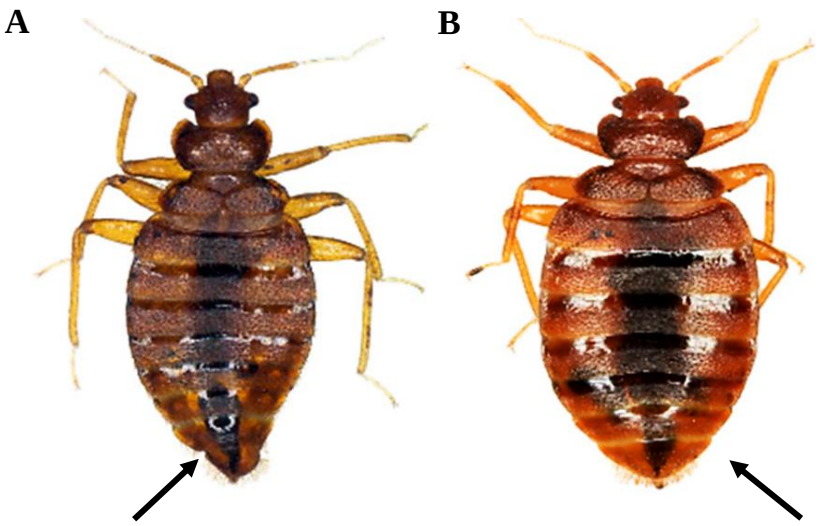

Figure 2.3 Morphological difference of *Cimex hemipterus*, tropical bed bug (A) male with pointed end and (B) female with rounded end (Hamlili 2023).

Nymphs are commonly recognized as the juvenile stage of adult that mimic adult's feature. But they deploy characteristics like decreased in body size, reduced hairiness, underdeveloped reproductive organs, and vestigial hemelytral wing-pads, which can be attributed to their hemimetabolous development (Usinger, 1966; Krinsky,

2019). According to Pinto et al. (2007); McNeill (2016) and Krinsky (2019), the nymphs of these organisms emerge from their eggs within a span of 3 to 10 days. These nymphs exhibit a size range of 1 to 6 mm and display a translucent coloration (Todd 2023).

Studies reveals fed females usually begin their oviposition upon mating and keep going until their blood supply runs out (Reinhardt and Siva-Jothy, 2007). Morphologically, bed bug eggs are about 1 mm in length, 0.44 mm in width and either white or yellowish white in colour (Mullen and Durden, 2009; McNeill, 2016). Bed bug female lay their eggs singly or in clusters between tiny cracks, crevices, or on rough surfaces close to their hiding places, and uses a translucent sticky-like substance to secure the eggs to the surface (How and Lee 2010; Kahn and Rahman 2012). At around the five-day mark, the developing embryo's red eye patches become visible in the egg signifying viable egg (Todd 2023). Next, the formation of rim (operculum) around one end of the egg allows the first stage nymph to push off when it emerges from the egg in 4–12 days (Krinsky 2019, Pinto et al. 2007).

2.1.4 Feeding

A blood meal is required for the nymphal stage of the bed bug to molt to the next life stage, while an adult requires a blood meal to reproduce their progney and sustain their living hood. Every 3-7 days, bed bugs travel in search of host to sate their hunger by feding for approxiamately 10 minutes, and the remaining days are occupied for digesting (Lehnert, 2013). The feeding mechanism of these Cimicidae are rather fascinating from the discovery of Usinger et al. (1966) and Krinsky (2019) who had thoroughly explored them. The authors disclose when feeding time approaches bed bug will search for their host by protruding their antennae and press their rostrum against

host skin then with thrusting motion, bed bugs' stylets will be pierced into the skin, and the labium will be flexed for stability as they are injected beneath the skin's surface. Subsequently, the maxillary stylets begin to find a vein or artery by threading through the route formed by the mandibular stylets, which alternately pierce the skin. If a vein or artery isn't reached on the first try, the bed bug may shift its position slightly and try again leading to additional injury to the host (Usinger 1966).

In total, bed bug takes approximately 5 to 20 minutes to complete their feeding process depending on their body size and ability to find vein to withdraw blood (Usinger 1966). In order to fulfil their hunger, bed bug pierce several times into host skin to locate vein and sometimes their proboscis may detached from host due to movement from host or they shift for a comfortable position to withdraw blood (Usinger 1966; Potter 2011) Following a successful feeding, the Cimicidae return to their hideout via an airborne aggregation scent emitted by other bedbugs breeding within the hideout (Reinhardt et a., 2009). Host victimized during the feeding period may suffer medical reaction like cutaneous, skin lesions, systemic hypersensitivity, severe haemorrhage, excoriation of the skin, and neurological collapse from salivary protein injection during piercing of their mouthpart (Todd, 2023; How & Lee, 2010; Okwa & Omoniyi, 2016). These protein has numerous function like vasodilating, anticoagulation and platlet suppressor that facilitate bed bugs' feeding competence where they enhance bed bugs to withdraw blood without interuption of host blood function (Todd, 2023). In addition, bed bug bites also have the potential to produce a secondary infections if they are scratched and then accidently exposed to an infectious disease such as cellulitis, folliculitis, and eczematoid dermatitis (Goddard & deShazo 2009). Howerver, bed bug bite does not transmit any pathogen to their host although they haul various pathogen like *Tryponema*

cruzi, Rickettsia parkeri, hepatitis B virus and more because bed bugs may exclusively serve as a phoretic vector for these infections (Lai et al., 2016).

Apart from skin reaction, host also may potentially encounter psychological stress, which can be characterized by several symptoms like sleep disturbances, emotional turmoil, heightened state of alertness, anxiety, and certain manifestations of psychosis such as formication and insomnia (Heukelbach & Hengge 2009; Potter 2011). During this blood-feeding process, bed bugs face various hurdles such as the host's innate and acquired immunological responses, significant fluctuations in bodily water content, and exposure to thermal radiation resulting from the recently ingested bloodmeal (Lehane, 2005). Although blood is the prominent food source of these *Cimex spp* yet they are still inadequate in the nutrients suppliment required by bed bugs for their longetivity, fecundity and survival (Rio et al., 2016). Thus, this will be atone by their endosymbionts via mutualistis association.

2.1.5 Reproduction

Bed bugs, scientifically classified as Hemiptera: Cimicidae, engage in a copulation method known as traumatic insemination. This manner of insemination has led to the development of specific adaptations in female bed bugs, as highlighted by Siva-Jothy et al. (2019). The process of copulation entails the male's aedeagus penetrates the female's reproductive tract wall during copulation, injecting sperms into her hemocoel (Stutt & Siva-Jothy, 2001; Kamimura et al., 2014). Although female bed bugs have a comparable reproductive system to other insects, Brittany (2018) finds that male bed bugs never use the genital tract for insemination. To accommodate this act, female bed bugs employ a specialized reproductive structure known as the spermalege, sub divide into ectospermalege and the mesospermalege in order to minimize the

potential harm caused by the penetration of the integument during copulation. The mesopermalege is also accountable for the process of sperm selection, as it houses phagocytic haemocytes that possess the ability to eliminate sperm of inferior quality, additionally the female reproductive organ (exclude spermalege) serves as the exclusive purpose of oviposition (Morrow & Arnqvist, 2003;Siva-Jothy, 2006).

It is noteworthy where females features a distinctive paragenital reproductive system, through which sperm traverse in order to reach the ovaries (Baker 2020). Once the male has transferred the sperm into the female mesospermalege, the sperm will subsequently undergo migration towards the seminal conceptacles, where they will be deposited and ultimately traverse the oviducts with the purpose of fertilizing any eggs that undergo development within the ovaries (Baker 2020). In accordance with Reinhardt and Siva-Jothy (2007), male exhibit preference for copulating with females that have just fed. Additionally, males bed bugs mates with any bed bug that is approximately the same size as recently fed female, including males and larger nymphs (Brittany 2018). In reaction to the perceived danger, bed bugs exhibit a defensive behaviour by emitting alarm pheromones with a pleasant scent (Saari et al., 2019). The warning pheromones have experienced evolutionary changes, leading males to utilize them as a means of discerning sexual expression to prevent engaging in gay intercourse (Ryne, 2009).

Based on findings of Reinhardt and Siva-Jothy (2007), on average each female experiences approximately about five instances of traumatic insemination following each feeding event leading to the likelihood of female bed bugs survival during copulation is minimal. Regardless, every fertilized adult female has the capacity to generate a range of 200 to 500 eggs throughout her lifespan (Miller 2014). The process

of traumatic insemination has been found to have adverse effects on the female bed bugs. These effects include the introduction of pathogens and a decrease in the female's ability to regulate water balance (Goodman 2016). The trauma also results in the allocation of nutritional energy towards the process of wound repair, as well as the activation of the immune response to facilitate the healing of the lesion inflicted upon the female (Reinhardt et al., 2009). The process of mating recovery depletes the female's energy reserves, so diverting resources that could otherwise be allocated towards egg formation. According to Polanco et al. (2011), empirical evidence suggests that virgin adult female bed bugs exhibit a 25% increase in egg production compared to females that engage in frequent mating by the male bed bugs.

2.1.6 Pheromones

Pheromones are a category of semiochemicals emitted by insects in response to alterations in their behaviour and physiology. Although specialized secretory glands typically produce pheromones, they can also be made by other bodily parts, including the digestive tract, the genitalia, the ovaries, and the testicles (Wertheim et al., 2005). Insects are associated with pheromones to sustain their living hood. In the case of bed bugs, they are commonly linked with two pheromones in which are alarm and aggregation pheromones.

Alarm pheromones are often recognized for their advantageous function in facilitating individuals' evasion of possible dangers. The musty scent frequently linked with bed bug invasions can be attributed to the bed bug alarm pheromone (Baker 2020). When bed bugs are in danger or alarmed, they secrete trans-oct-2-enal and trans-hex-2-ena from metathoracic, which prompts movement among individuals of the same species (Liedtke et al., 2011; Baker 2020). Early research into the makeup of scent

glands found that the two aldehydes were presented significantly high, along with acetaldehyde, butanone, and a few other small parts (Liedtke et al., 2011; Ulrich 2015). In accordance with Ryne's (2009) proposition, a combination of both aldehydes also been suggested as a potent signal for sex recognition, as well as a stimulant for locomotion. Liedtke et al. (2011) reported release of these pheromones from the dorsal abdominal region in nymphs may serve as a mechanism to deter males from engaging in painful extragenital insemination against older nymphs. The research of Akhoundi et al. (2023b), proclaimed that the alarm pheromone of bed bugs also exhibits anti-fungal capabilities since it has been found to effectively suppress the survival of conidia, thus suggesting that the alarm pheromone could potentially show efficacy in combating infections that can propagate bed bug aggregations.

In contrast, the dispersion of bed bugs is accommodated by their aggregation pheromone. Wertheim et al. (2005) imply that aggregation pheromones play a role in facilitating the establishment of aggregations by attracting and crippling all individuals of the same species towards the source of pheromone release. The individuals that exhibit a response to the release of an aggregation pheromone can belong to either the same sex or the opposite sex as the organism emitting the pheromone. According to Ulrich (2015), there was a prevailing belief that nest odours played a significant role in facilitating the social behaviour of bed bugs. This initial endeavour is to elucidate the volatile aggregation pheromone emitted by these cimicids which yielded in discovering of essential components namely dimethyl disulfide, dimethyl trisulfide, trans-oct-2-enal, trans-hex-2-enal, and methyl butyl ketone (Gries et al. 2015).

Interestingly, the formation of aggregations offers several advantages, such as improved resource utilization, better mate-finding opportunities, and heightened

defensive mechanisms against predatory and unfavourable environmental conditions (Wertheim et al., 2005). It is widely acknowledged this demeanour exhibited by bed bugs confers benefits to their group since aggregation promotes preservation of water and mitigate the risk of desiccation, as Benoit et al. (2007) demonstrated. Additionally, juvenile individuals also experience advantage from this pheromone in which nymphs raised within the colony exhibit accelerated development compared to nymphs raised in solitary conditions (Saenz et al., 2014). Moreover, first instar nymphs demonstrate enhanced host detection capabilities when they are in aggregations accompanied by females, as opposed to aggregations without female presence. It is a common occurrence for adult insects to employ aggregation pheromones in order to augment the feeding efficiency of juvenile insects amongst the aggregations colony (Wong et al., 2013; Baker 2020). The aforementioned relationship can be classified as a manifestation of subsociality, which refers to the parental care behaviour that occurs after oviposition and enhances juvenile organisms' advancement and survival (Ulrich 2015).

2.1.7 Endosymbionts

Symbiotic relationship known as endosymbiosis, where one organism lives inside of another organism (the host). According to Moran et al. (2008), these connections take place along a spectrum, comprising interactions known as mutualism, whereby equally the host and the symbiont gain benefit from the association, or interactions known as parasitism, in which the microbiome gains at the expense of the host (Goodman 2016). Microbial constituents influence numerous aspects of biology, including defence against infection and digestion. Consequently, these bacteria additionally engage in the adaptation process as co-evolutionary associates. Certain insects have a restricted physiological capacity, which renders their survival without their symbiotic partners (Thongprem, 2021). Thus, as previously stated, symbionts have

key functions for their hosts and impact nourishing needs, general growth, reproductive performance, defence against pathogens infections, and regulation of host gene expression, including biochemical detoxification of chemicals and insecticides (Rupawate et al., 2023). Bedbugs host a range of microbial symbionts within them, with varying quantities. *Wolbachia* has been discovered as one of the ubiquitous microbes of bedbugs since they offer nourishment like vitamin B for their host insect (Lim & Ab Majid 2021). Abundance of symbionts within an organisms sub divide them into two classes in which primary and secondary. Primary microbial communities exhibit a mutually beneficial interaction with their host, where both parties are dependent on one other. They are typically confined to specific host cells called bacteriocytes and are transmitted vertically from mother to their progeny and co-evolve with the host (Heaton 2013). Secondary microbes exhibit greater variability and have fewer precise boundaries. These microorganisms have a tendency to be optional for the host, have the ability to penetrate different host cells, and are frequently transmitted horizontally between host species that are not related (Hypša & Nováková, 2008).

2.2 History of Bed Bugs

The process of fossilization recovery of bed bugs artifacts has been documented in archaeological sites stretching back about 3500 years (Panagiotakopulu & Buckland, 1999). According to Potter (2011), archaeologists had the posit that bats were initially served as primary hosts of bed bugs, exhibiting a preference for zoophilic interactions**.** Nevertheless, over the course of history, it is widely theorized that bed bugs underwent a process of adaptation to humans who shared the same cave habitats during that specific era. But due to the rise of civilization, globalization, and urbanization the occurrence of human migration has become more prevalent, as

individuals strive to enhance their living conditions. However, the unintentional migration of individuals has inadvertently promoted the dissemination of bed bugs via the passive dispersion over many regions, such as Europe, Asia, and America (Potter, 2011).

From the course of this modernization, researchers have garnered significant attention in determining the precise historical timeframe of bed bug presence, driven by the development and application of molecular instruments and analysis techniques. Agnarsson et al. (2011) and Roth et al. (2018) recently had conducted a fossil-based phylogenetic study to analyze the evolutionary relationships of bugs. Their findings give compelling evidence that bugs have experienced significant modification over time predating bats and have formed parasitic partnerships with bats on several occasions. Akhoundi et al. (2020) found that the clade of *Cimex* sp. that feed on humans diverged around 5-10 million years ago, which happened before the first *Homo* species was discovered. The simultaneous presence of many hominid lineages in both spatial and temporal dimensions has enabled the occurrence of diverse instances of host shifting. Roth et al. (2018) predicted that Cimicidae had an approximate age of 115 MYA based on phylogenetic tree analysis. The emergence of these cimicids, estimated to have taken place around 93.8 million years ago, predates the oldest documented evidence of bats by a margin of 30-50 million years (Todd 2022). Therefore, irrespective of the precise chronology of hominids' early habitation in caves, it can be deduced that the cimicids, which were already engaging in parasitic relationships with bats and birds, were able to exploit this new ecological niche (Roth et al., 2018). The late Pleistocene age witnessed the emergence of an increased inclination towards specific host preferences, as human populations, bats, and bed bugs cohabitated in the caves of the Northern Mediterranean and Central Asia (Simov et al., 2006; Potter 2011, Akhoundi 2020).

Following this, it is possible that the elevated levels of humidity in the caves had an impact on human populations, leading them to shift from caves to huts. This transition was driven by the desire to seek more advantageous environmental conditions. Therefore, it is probable that this particular species of bug travelled in tandem with human populations to their newly formed towns (Kulzer, 2002). For instance, about 11,500 years ago, as agriculture emerged and sedentary lives were established, *C. lectularius* continued to maintain its symbiotic association with human populations by inhabiting their residences (Panagiotakopulu & Buckland 1999). It is conceivable to suggest that bed bugs may have originated in the Middle East and North Africa, eventually spreading their presence across Europe and Asia. One conceivable rationale for the movement across the Mediterranean Sea during the Bronze Age could be ascribed to the possible infiltration of merchant ships (Potter 2011; Akhoundi et al., 2020; Todd 2022).

Todd (2022) made an insightful observation that renowned authors such as Aristotle and Aristophanes had included concerns with bed bugs infestation into their literary and dramatic works, such as Historia Animalium and The Frogs. Nevertheless, there existed some authors who maintained a more sanguine viewpoint towards the issue of bed bugs. Pliny the Elder and Pedanius Dioscorides are renowned figures during Gregorian calendar eras, they have extensively examined the potential therapeutic benefits associated with bed bugs where they proclaim that bed bugs were potentially utilized in the creation of cures for many maladies, including snake bites, urinary infections, and general health issues. These remedies involved pulverizing bed bugs and combining them with other substances. Moreover, the excavation of archaeological sites has provided substantial proof of the presence of cimicids, complementing the references contained in ancient literary sources.

The presence of bed bug leftovers in the palace lavatory was documented by Bain et al. in 2004. In the 17th century, Pehr Kalm, a Swedish botanist, made the claim that Canada demonstrated a similar incidence of bed bug infestations as those reported in the Old World (Merriam, 2006; Todd 2022). Pehr Kalm conducted his investigations on bed bugs in Quebec during his journey to the northern part of New York state in 1749. According to Kalm's narrative, he conveyed his unease experienced during the nocturnal hours as a result of the existence of bed bugs (Merriam, 2006). Furthermore, Pehr Kalm's observation in 1771 and Bain's findings in 2004 indicate that bed bugs had comparable levels of success in both urban and rural environments. Specimens of *Cimex lectularius* L. were found in Jamestown, a settlement situated in the southern region of the eastern coast of the United States. The fossils were discovered in a colonial well located within James Fort, as recorded by King et al. in 2013.

2.3 Resurge of Bed Bugs

Recorded history prevailed that mankind had conquered the infestation of this robust ectoparasite. However, after a hiatus for almost fifty years, bed bugs had tremendously reemerged and began to haunt humans (Doggett et al., 2012). They became global plague, particularly in developed regions such as the continents of North America, Eastern Asia, Australia, and Europe (Doggett et al., 2004; Potter, 2007). According to Cooper (2011), certain pest control officers have observed a greater than tenfold rise in bed bug treatments since bed bugs have been documented in every state in the United States over a span of 5 years. The aforementioned situation is primarily attributed to the simultaneous increase in international travel. However, it is important to consider a variety of factors that contribute to this phenomenon, such as the reuse of infested furniture, the frequent utilization of bait traps containing pesticide residue, and the emergence of pesticide resistance within bed bug populations (Benoit, 2011).

Unfortunately, a few aspects of lifestyles and insect biology have been instrumental in the countless infestations of bed bugs (Knolhoff & Onstad, 2023). The establishment of an effective approach to combat the substantial experience pertaining biology, epidemiology, and genetic compositions of bed bugs is keenly anticipated with the objective to facilitate the formulation and implementation of efficacious eradication initiatives (Chebbah et al., 2021).

2.4 Traditional Control Method

Bed bugs' feeding nature led to physical, social, mental, and economic effects on the host, including skin reaction. Host are also capable of experiencing psychological strain, discomfort, hypersensitive reaction, secondary infection and pathogen transmission (Abd Rahim et al., 2016; Xie et al., 2019). Thus, people since ancient times had used various traditional methods to eradicate the infestation of bed bugs from becoming severe. In Greek and Roman history (400 B.C), bed bugs were repelled by hanging hare feet, stag, bearskin, and placing a basin of water under the bed, while in the 17th century, inspection and examination by professionals were recommended for the first time (Potter 2011). Based on a bed bug manual called A Treatise of Buggs in 1730, the author suggest using trees with insecticidal property like Quassia and Sassafras as repellent against bed bugs (Potter, 2011). Besides, traps were filled with oil or kerosene, and wood slabs with numerous holes were employed in those days (Koganemaru & Miller, 2013). In the mid-17th century, people fill wall cracks with gun powder and set them on fire. Then, the chrysanthemum flower extract called pyrethrum is widely used in the 18th century (Potter et al., 2008).

As time passes, people try alternative such as utilizing boiling water by pouring at the infested site and apply mercury, arsenic, sulphur, fats from salt pork or bacon to it. At this time, corrosive sublimate such as mercury chloride was also applied,

especially onto infested bedding (Potter, 2011). Subsequent to all these methods, to vanquish the bed bug infestation, chemical insecticide was used in the 19th century where rigorous steam disinfectant, fumigation using hydrogen cyanide were established, and cleaning of furniture and bedding by utilizing coal oil (Potter et al., 2008). The establishment of these method was driven from an incident during World War II where infestation took down the U.S military bases and became a morale issue among the soldiers. This urge politician to come up with this effective but hazardous method (Todd 2022). Before the 20th century, the poor have lack of knowledge regarding bed bugs, do not have access to insecticide as it was expensive, and there was a limited supply of insecticide. Therefore, most of the low-income families live and accept the infestation without any control measures taken. Hence, during the 19th century, they utilize glue traps, double-sided sticky tape, bean leaves, and alternative adhesives that were cheap and easy to access, yet it was not reliable enough against bed bug infestation (Potter, 2011).

2.5 Bed Bugs Resistance

Insects commonly exhibit a response to threats by either emitting or promoting their defence mechanisms. This can include the development of metabolic processes, modification of gene structure, and genetic mutation, leading to the acquisition of resistance (Hemingway and Ranson, 2000). Miller (2014) suggested resistance is the ability of insects to survive upon being subjected to a dosage of insecticide that typically eradicates a population. Bed bugs began to show resistance against insecticide that was widely used since the mid-18th century as it exhibits short life effect, lack residual effect, lack of effectiveness, and require to follow up spray in a week or two (Suwannayod et al., 2010). Apart from resistance towards insecticide, bed bugs also reveal resistance against non-chemical control such as vacuuming, steaming, disposal of an infested item,

heat, and cold treatment since they managed to reinfest (Punchihewa et al., 2019). The primary concern arises when bed bug shows resistance against insecticides such as pyrethroid and malathion that were previously effective (Davies et al., 2012).

In 1940, Dichlorodiphenyltrichloroethane (DDT) was invented with a distinctive bed bug control approach with a long residual effect. However, resistance towards bed bugs was noticed in 1947 as DDT was used as an indoor residual spray for malaria, and cross-contamination occurred (Haynes & Potter 2013). Moreover, DDT was banned in 1972 as it was a possible human carcinogen besides causing a seizure, vomit, and tremor (Costa, 2015). Consequently, The National Pest Control Association proposed malathion as an alternate insecticide to DDT; however, due to cross-contamination, malathion began to demonstrate resistance against bed bugs. (Koganemaru & Miller, 2013). Meanwhile, pyrethroid had been proven as a highly effective, extremely efficient, safe, affordable, reasonable, and longevity residual effect insecticide since the first time introduced compared to others, (Zhu et al., 2013). Although there was a report showing pyrethroid cross-resistance with the use of other insecticides to combat malaria, pyrethroid is still used today as it does give a satisfactory result with the combination of insect growth regulator (Davies et al., 2012).

Most of the control measures implemented in the olden days were harmful to the environment and humankind. In those years, the development of various insecticides had shown the occurrence of resistance and resurged of bed bug infestation. The resistance of bed bug remains until today since resistance mechanism can be primarily linked to three primary factors: behavioural resistance, physiological resistance, and symbiont-mediated resistance. Behavioural resistance can be categorized into two primary groups: stimulus-dependent and stimulus-independent responses (Ranabhat