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HORMONAL AND NUTRITIONAL REGULATION OF MOLTING, METAMORPHOSIS, AND REPRODUCTION IN BED BUGS, *Cimex lectularius*

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HORMONAL AND NUTRITIONAL REGULATION OF MOLTING, METAMORPHOSIS,
AND REPRODUCTION IN BED BUGS, *Cimex lectularius*

DISSERTATION

A dissertation submitted in the partial fulfilment of the
requirements for the degree of Doctor of Philosophy in the
college of Agriculture, Food and Environment
at the University of Kentucky

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ABSTRACT OF DISSERTATION

HORMONAL AND NUTRITIONAL REGULATION OF MOLTING,
METAMORPHOSIS, AND REPRODUCTION IN BED BUGS, *Cimex lectularius*

The bed bug, *Cimex lectularius* is an obligate hematophagous ectoparasite that feeds on humans. The increase in travel and development of insecticide resistance to commercially available insecticides have enabled the bed bug population to resurge, causing economical and psychological trauma to the human population. Lack of knowledge about the basic molecular biology of bed bugs has motivated us to study the key aspects of molting, metamorphosis, and reproduction. A blood meal triggers expression of various genes that enable bed bugs to molt or undergo metamorphosis. Molting and metamorphosis in bed bugs are regulated by two key hormones: 20-hydroxyecdysone (20E) and juvenile hormone (JH). JH induces expression of Krüppel homologue 1 (Kr-h1) gene. Higher expression of Kr-h1 in the penultimate nymphal instar represses ecdysone inducible gene E93 and the development of adult characteristics in the insect. E93 is expressed during the last instar stage in the absence of Kr-h1. E93 promotes the development of adult structures and metamorphosis to the adult stage. Studies on reproduction have also shown that blood meal and mating are essential for vitellogenin gene expression and oocyte maturation in bed bugs. JH and 20E regulate reproduction in bed bugs. Detailed studies on the involvement of juvenile hormone in reproduction using the next generation sequencing technology identified genes that regulate reproduction in bed bugs. V-Maf, avian musculoaponeurotic fibrosarcoma oncogene homolog B (MafB), forkhead box protein (Foxl2) and heparanase were found to play key roles in regulation of reproduction. The role of ABC transporters was also studied using RNA interference. ABC transporters (ATP-binding cassette) are involved in active transport of various molecules including steroid hormones, cuticle lipids, and other molecules. ABC transporters were also shown to be involved regulation of reproduction, molting and metamorphosis in bed bugs. This study lays a foundation for future research aimed at the development of novel methods for controlling bed bugs.

KEYWORDS: Bed bugs, reproduction, molting, metamorphosis.

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27 April 2016
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(DEDICATION)

Dedicated to the development of the world.

Acknowledgements:

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Chapter 1: Introduction

Bed bug Biology:

The bed bug, *Cimex lectularius* is an obligate hematophagous ectoparasite that feeds on humans. It belongs to the family Cimicidae and order Hemiptera. The female adult lays eggs that hatch into first instar nymph stage after 4-10 days. There are five immature stages of nymphs in bed bugs. Each stage requires a blood meal to molt into next stage. The nymphs from the first stage (of about 1.5 mm) to the fourth instar stage (of about 3 mm) do not have a wing pad and can be differentiated on the basis of their size. Wing pads start developing during the fifth instar stage nymph which is about 4.5 mm. At this stage the wing pads are fused and form a concave line on the thorax. The last instar nymphs then metamorphose into adults. The adult stage can be identified with darker sclerotization of the cuticle. Adults are about 5-7 mm long, brown and flat oval. On blood feeding, adults become engorged and balloon-like reddish-brown. Adults can live for many months without food. Sometime, depending upon food and environmental conditions, they can even survive for a few years (Reinhardt and Siva-Jothy, 2007).

Bed bugs reach their sexual maturity at the adult stage. The adult bed bugs can be morphologically differentiated between male and female. The males have a smaller abdomen and are smaller in size as compared to the females which have a more rounded abdomen. The male aedeagus is visible with the naked eye. The females have a specialized organ known as the ectospermalege on the ventral side of their abdomen. The bed bug female undergoes traumatic insemination. Bed bugs are nocturnal and come out seeking a resting host for a blood meal (Johnson, 1941). Mating is polygamous and occurs throughout the adult life. The male adult pierces the abdomen of a female adult at the ectospermalege with its intromittent organ which acts as a hypodermic needle and releases the sperms into the hemocoel. The sperms are then collected by the mesospermalege and travel to the seminal conceptacle where they are stored (Usinger, 1966). The male reproductive organ consists of testis, mycetome, vas deferens, seminal vesical, male accessory gland, male accessory gland reservoir, ejaculatory duct, and aedeagus. The testis is made up of 7 lobes. The sperm travels through the vas deferens and is stored in the seminal vesicle. The female reproductive system consists of a pair of ovaries, with seven ovarioles. The ovariole consists of the terminal filament, the germarium, and the vitellarium. Oogenesis in the bed

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bug is defined as meroistic telotrophic (Usinger, 1966). The female bed bug continues to lay eggs after every blood feeding. They lay approximately 200-300 eggs in their lifetime (Reinhardt and Siva-Jothy, 2007). Multiple mating reduces the life span of the female (Stutt and Siva-Jothy, 2001).

Economic Impact of Bed bugs:

Bed bugs are generally found in human dwellings, where they have an easy access to human blood. They are generally active during the night. After the development of DDT in 1939, the bed bugs were virtually eradicated with its extensive use during the 2nd World War and later during the 1940s and 1950s. However, a resurgence in bed bug population has taken place in the USA and other countries in recent times (Potter, 2006; Wang et al., 2015). The resurgence of the pest is attributed to an unhygienic conditions in the human dwellings and travels that provided a suitable ecosystem to grow and spread. Moreover, bed bugs have a long life span and ability to survive months without food. Bed bugs are extremely cold tolerant surviving a temperature of as low as -15°C (Olson et al., 2013). Furthermore, they have developed resistance to some of the commonly used insecticides like pyrethroid and neonicotinoid (Romero and Anderson, 2016; Zhu et al., 2010). Resistance is often correlated with a higher expression of detoxifying enzymes like hydrolytic esterase, microsomal oxidases, kdr mutations, and ABC transporters (Dang et al., 2015a; Dang et al., 2015b; Lilly et al., 2016; Mamidala et al., 2012; Palenchar et al., 2015; Romero and Anderson, 2016).

In humans, they generally cause allergic reactions which may be characterized as a mild rash. In severe case psychological disorder or anemia has also been reported (Goddard and de Shazo, 2012; Goddard and deShazo, 2009; Pritchard and Hwang, 2009). Psychological disorders consist of anxiety, impaired quality of life and psychosocial functioning and suicide in extreme cases (Burrows et al., 2013; Rieder et al., 2012). They have been shown to be a potential vector of *Trypanosoma cruzi* (Salazar et al., 2015), an etiological organism for Chagas fever. Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE) were recovered from bed bugs in British Columbia (Lowe and Romney, 2011). Most importantly, they are considered a social stigma (Usinger, 1966). They cost a lot for their control (Hwang et al., 2005). In a study published in American Journal of emergency medicine the direct cost of

control of bed bug in the emergency unit after finding a single bed bug was estimated to be US$ 12,500 (Totten et al., 2016).

In the light of the resurgence of bed bugs as parasites in human dwellings, there has been a recent increase in the study of these insects. Bed bug genome was recently determined by two groups (Benoit et al., 2016; Rosenfeld et al., 2016). Differential expression analysis of genes have shown that the first blood feeding leads to the most pronounced change in gene expression including the genes from the endosymbiont *Wolbachia* (Rosenfeld et al., 2016). Sequencing of bed bug genome identified 650 Mb of sequence with 14,220 predicted protein coding genes. These sequences would be useful in the study of bed bug resistance, development, reproduction, growth and behavior (Benoit et al., 2016). As bed bugs are hematophagous insects and depend exclusively on blood as a source of nutrient and water they have adapted through the course of evolution to this lifestyle as is revealed by a high number of genes associated with blood digestion and pesticide resistance. Multiple putative lateral gene transfer events from various bacteria including *Wolbachia* and *Arsenophonus* were also identified (Benoit et al., 2016). Lateral gene transfer events are useful as they provide novel abilities including insecticide resistance, digestion, resistance to pathogens and facilitating obligate symbiont relationship with other microorganisms in the recipient organisms (Nakabachi, 2015). Phylogenetic classification on the basis of the genome has revealed bed bugs to be closely related to *Rhodnius prolixus* (Rosenfeld et al., 2016).

Recent studies have helped understand bed bug biology to develop new strategies for their control. Studies on response to human odorants have shown the involvement of D-type olfactory sensilla which detects aldehyde and alcohols that help in the host-seeking behavior of bed bugs (Liu and Liu, 2015). After feeding bed bug tends to aggregate in crevices. Bed bug aggregation pheromone comprises of five volatile components dimethyl disulfide, dimethyl trisulfide, (E)-2-hexenal, (E)-2-octenal, 2-hexanone, and histamine (Gries et al., 2015; Olson et al., 2014). These recent discoveries have shown a potential to develop new strategies for their control (Gries et al., 2015).

Hormones in the Growth and Development of Insects:

Insect's growth and development through the immature stages to the adult requires two major hormones, ecdysteroids and juvenile hormone (Jindra et al., 2013; Riddiford,

1993). Juvenile hormones are a group of sesquiterpenoids that are essential for maintaining the immature stages of the insect. It is also required for reproduction in the adults. Juvenile hormone is synthesized from the acetyl-CoA, by the mevalonate pathway in the corpora allata of the insects (Belles et al., 2005; Marchal et al., 2011). Many different juvenile hormones, JH 0, JH I, JH II, JH III, 4-methyl JH I, JHB III have been identified in insects (Gilbert, 2012). Recently a new form of juvenile hormone called juvenile hormone III skipped bisepoxide (JHSB3) (or methyl (2R,3S,10R)-2,3;10,11-bisepoxyfarnesoate) has been reported in the hemipteran stink bug *Plautia stali* (Kotaki, 1995; Kotaki et al., 2009). Sequence analysis of the active site of JH receptor Methoprene-tolerant protein (Met) from different insects has shown amino acid substitution of Y252W in hemipteran bugs like *P. stali*, *Pyrrhocoris apterus*, and *R. prolixus* (Charles et al., 2011). It is possible that bed bugs also utilize JHSB3 as its juvenile hormone as the bed bug carries Y252W substitution. Ecdysteroids are steroid hormones that regulate molting, metamorphosis and reproduction. Ecdysteroids are synthesized in the prothoracic glands, also called ecdysial glands in response to the prothoracicotropic hormone or brain hormone released from the neurosecretory cells in the brain. Ecdysteroids are converted in the cells to 20E, also called ecdysterone, which is the most active form.

Regulation of titers of juvenile hormone and ecdysteroids have been well studied in both holometabolous and hemimetabolous insects. In holometabolous insect, the process involves a complete change in the switching of the genetic program from the immature larval and pupal to the adult stage, whereas, in the hemimetabolous insects, genetic change in morphology occurs from the immature nymphal stage to the mature adult stage. Irrespective of these differences, all insects use ecdysteroids and juvenile hormone to regulate molting and metamorphosis. In both hemimetabolous and holometabolous insects, ecdysteroids titers peak before molting or metamorphosis to the next stage. Juvenile hormone titers are high in the penultimate instar nymphs of the hemimetabolous insects. Similar juvenile hormone titers have been observed in the holometabolous insects. During the prepupal stage, juvenile hormone reappears at the time of ecdysteroid rise for larval-pupal transition and is required to prevent premature development of adult characters. Exogenous application of juvenile hormone in the pupae is known to induce second pupae as found in the *Cecropia* silkmoth (Riddford and Ajami, 1973). Thus, the juvenile hormone

is an anti-metamorphic hormone, whose absence in pupae allows the transition to the adult stage.

Molecular Action of Hormones:

20E functions through a heterodimer of nuclear receptors, ecdysone receptor (EcR) and ultraspiracle (USP). The 20E-EcR-USP complex regulates expression of early genes coding for transcription factors including E75B, E74, E93 and Broad-Complex (*BR-C*), HR3 and HR39 (Bernardo et al., 2014; Fletcher and Thummel, 1995; Horner et al., 1995)). These proteins, in turn, regulate expression of early-late and late genes. E93 is a helix-turn-helix transcription factor containing a Pip-squeak (Psq) motif. E93 was shown to be involved in regulation of metamorphosis and programmed cell death (Lee et al., 2000). Juvenile hormone binds to its receptor Met and its co-activator steroid receptor co-activator (SRC) or Cycle (CYC) (Shin et al., 2012; Zhang et al., 2011). These proteins are members of the basic helix–loop–helix Per-ARNT-Sim (bHLH–PAS) gene family (Charles et al., 2011; Shin et al., 2012). Met protein contains three domains bHLH, PASA and PASB. The bHLH domain is known to interact with DNA. PASA domain forms heterodimer with SRC and PASB domain is required for juvenile hormone binding (Charles et al., 2011). Binding of JH to Met/SRC complex on the promoter region induces expression of downstream genes. These downstream genes include Krüppel homolog 1 (Kr-h1) (Kayukawa et al., 2012), hairy (Shin et al., 2012) and trypsin (Noriega et al., 1997). Kr-h1 is a zinc finger domain containing transcription factor and plays an important role in molting, metamorphosis and reproduction in insects (Lozano and Belles, 2011; Song et al., 2014). Studies on *Bombyx mori* cell lines have identified juvenile hormone response element (JHRE) located in the promoter of Kr-h1 gene. Met protein binds to the core region of JHRE (GGCCTCCACGTG) known as the E-box. BmMet interacts with its ligand juvenile hormone, which then forms a heterodimer with BmSRC. This heterodimer can then interact with the promoter sequence of DNA inducing the expression of Kr-h1 (Kayukawa et al., 2012). Studies on *B. mori* have suggested that Kr-h1, an early gene in juvenile hormone action pathway expressed both by corpora allata and other tissues that are involved in repression of metamorphosis (Kayukawa et al., 2014).

E93, which is an ecdysone inducible gene, is suppressed in the presence of Kr-h1. Its expression starts in the prepupal stage and reaches a maximum in the pupal stage of the

holometabolous insects. However, in the hemimetabolous insects, E93 expression starts only in the last instar nymphs. E93 promotes adult characteristics in both hemimetabolous and holometabolous insects (Belles and Santos, 2014; Konopova et al., 2011; Lozano and Belles, 2011; Urena et al., 2014) .

In *Blattella germanica* and *R. prolixus* Kr-h1 functions as an anti metamorphic factor (Konopova et al., 2011; Lozano and Belles, 2011). In these insects, Kr-h1 levels drop during the final nymphal stage which allows development of adult structures. E93 is highly expressed during the last nymphal stage and promotes nymph to adult transition (Urena et al., 2014). E93 cross-talks with the juvenile hormone pathway by down-regulating Kr-h1 and *BR-C* expression during the last nymphal stage of *B. germanica*. Knockdown of E93 during the pupal stage of *Tribolium castaneum* leads to development of supernumerary pupae (Urena et al., 2014). In *P. apterus*, Met and Kr-h1 but not BR-C are involved in antimetamorphic action (Konopova et al., 2011). Knockdown of these genes causes the development of adult color pattern, wings and genitalia. In *Manduca sexta* and *Drosophila melanogaster*, BR-C is a pupal specifier (Zhou and Riddiford, 2002) in the holometabolous insects. Its expression in *M. sexta* at pupal to adult molt results in the formation of the second pupal cuticle. BR-C specifies the immature stage in some hemimetabolous insects such as *Oncopeltus fasciatus.* In this insect, *BR-C* disappears during adult development (Erezyilmaz et al., 2006). Knockdown of *BR-C* in *O. fasciatus* causes the premature appearance of adult characteristics.

Hormones in Reproduction:

Mating initiates molecular changes through hormones and signaling molecules important for vitellogenesis, development of oocyte and embryo. Vitellogenin (Vg) is a female specific protein that is synthesized in the fat body and deposited in the developing oocyte in a form of egg yolk protein called vitelline (Raikhel, 2005). Two major hormones, 20E and juvenile hormone regulate reproduction in insects and might have different downstream genes in different organisms.

In the mosquito, a blood meal triggers ovaries to produce ecdysone which is converted into 20E to take part in egg development (Hagedorn et al., 1975). 20E induces its effect by binding on to a heterodimer of its receptor Ecdysone receptor (EcR) and heterodimeric partner, ultraspiracle (USP). This activates downstream early genes E74,

E75, broad (BR-C) (Chen et al., 2004). The product of these genes along with EcR-USP-20E complex (Kokoza et al., 2001; Martin et al., 2001) then activates *Vg* gene transcription and regulates reproduction. Ecdysone induces Vg expression in *Bombyx mori* fat body through BmBrC-Z2 (Yang et al., 2014). Ecdysone plays a major role in oocyte maturation in *T. castaneum* (Parthasarathy et al., 2010a). Juvenile hormone pathway regulates Vg expression in *Locusta migratoria* (Song et al., 2014) and *T. castaneum* (Parthasarathy et al., 2010b). In *T. castaneum*, EcR knockdown also reduced *Vg* expression. However, ecdysone injections did not induce *Vg* expression, suggesting an indirect role of ecdysone in *Vg* expression possibly through corpora allata (Elliott et al., 2006).

Nutrition regulation of Reproduction:

Nutrition also plays an important role in regulating reproduction (Attardo et al., 2005; Hansen et al., 2014; Shiao et al., 2008). In the mosquito, blood meal triggers various hormones and peptides to regulate reproduction. Signaling molecules present in the blood of the mosquito-like the vertebrate insulin peptide (ILP) promote insulin signaling and phosphorylation of ribosomal S6 kinase (S6K) (a key protein in the target of rapamycin (TOR) signaling pathway). ILP and amino acid through Insulin Receptor (InR) and TOR activate Vg expression in the presence of ecdysone (Hansen et al., 2004; Roy et al., 2007). ILP interacts with InR and activates the phosphoinositide-3 kinase (PI3K) which then activates protein kinase B (PKB/AKT) to phosphorylate S6 kinase to initiate Vg expression (Roy et al., 2007). The phosphorylation is carried out through the involvement of serine-threonine protein kinase TOR (Roy et al., 2007). The juvenile hormone has also been shown to regulate the nutritional pathway through TOR and insect cationic amino acid transporter 2 (iCAT2) expression and phosphorylation of S6 kinase in fat body for the production of *Vg* (Shiao et al., 2008). In *Aedes aegypti*, two cationic amino-acid transporters *Aaslimfast* and *iCAT2* play an important role in inducing an amino acid response to *Vg* expression (Attardo et al., 2006). Blood meals trigger the synthesis of transcription factor AaGATAa through TOR signaling and S6 kinase which directly binds to the *Vg* promoter region to enhance its expression (Park et al., 2006). RNAi-mediated knockdown of AaGATAa reduces Vg expression (Park et al., 2006). Knockdown of tuberous sclerosis complex 2 (TSC2) a negative regulator of TOR signaling enhances *Vg* expression (Attardo et al., 2005; Hansen et al., 2004). In *T. castaneum*, nutrition signaling

controls Vg expression and egg laying through InR, Chico, PI3K, AKT, forkhead transcription factor 1 (FOXO), GATA, TSC, TOR and S6K as was shown by knockdown studies (Parthasarathy and Palli, 2011). Hormonal and nutritional pathways interact during reproduction in *T. castaneum*. Juvenile hormone induces ILP2 in fat body and ILP2/3 in the brain to induce Insulin signaling in the fat body. Phosphorylation status of FOXO a negative regulator of Vg then determines the expression of *Vg* (Sheng et al., 2011).

Goal of this Study:

Due to lack of scientific data available on molecular aspects of molting, metamorphosis and reproduction in bed bugs, we have decided to study these processes. Chapter 2 discusses the role of JH inducible gene Kr-h1 in molting, metamorphosis and repression of adult characteristics in bed bugs, and its interaction with ecdysone inducible gene E93. Chapter 3 identified genes involved in reproduction in bed bugs and studies the role of JH receptor Met in detail. Chapter 4 studied the role of ABC transporters in molting, metamorphosis and reproduction in bedbugs. The role of ABC transporters in insecticide resistance is also studied. Chapter 5 includes the conclusion of this study and the future directions required to develop novel insecticides against bed bug infestation. This research will set a stage for an in-depth research on growth and development of bed bug. These studies will help us understand the basic processes of growth and reproduction in insects which are essential for their survival. This knowledge can be applied on other insects for their control as well. RNAi based insecticides and designing new inhibitor molecules would add to the existing control strategies for these insect pests.

Chapter 2. Krüppel homolog 1 and E93 mediate Juvenile hormone regulation of metamorphosis in the common bed bug, *Cimex lectularius*

Introduction

The resurgence of bed bugs in recent years in the USA and other countries around the world have brought bed bugs in the limelight again (Potter, 2006). They are known to cause an allergic reaction in people, in the form of a mild rash. In extreme case they may cause psychological disorder and anemia (Goddard and de Shazo, 2012; Goddard and deShazo, 2009; Pritchard and Hwang, 2009). They also cause anxiety, impaired quality of life and psychosocial functioning and suicide in rare cases (Burrows et al., 2013; Rieder et al., 2012). It has been suggested that they might be a possible vector of methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* (Lowe and Romney, 2011).

Bed bugs go through five nymphal stages prior to becoming an adult. Nutrition is the major driver of molting and metamorphosis since bed bugs undergo molting or metamorphosis only after a blood meal. JH are sesquiterpenoids that are synthesized in the corpora allata (Belles et al., 2005; Marchal et al., 2011). JH functions through its receptor, Methoprene-tolerant protein (Met) and steroid receptor co-activator (SRC) or Cycle (CYC) (Shin et al., 2012; Zhang et al., 2011). Met is a member of the basic helix–loop–helix Per-ARNT-Sim (bHLH–PAS) gene family (Charles et al., 2011). Krüppel homolog 1 (Kr-h1) (Kayukawa et al., 2012), hairy (Shin et al., 2012) and trypsin (Noriega et al., 1997) are among the genes that are directly induced by JH. Kr-h1 is a zinc finger domain containing transcription factor and plays an important role in JH regulation of molting, metamorphosis and reproduction in insects (Lozano and Belles, 2011; Song et al., 2014). 20E binds to EcR-USP complex and directly regulates expression early genes coding for transcription factors including E75, E74, E93 and Broad-Complex (*BR-C*). These proteins, in turn, regulate expression of early-late and late genes including nuclear receptors HR3 and HR4. E93 is a helix-turn-helix transcription factor containing a Pip-squeak motif. E93 was shown to be involved in regulation of metamorphosis and programmed cell death (Lee et al., 2000). Bed bug biology is unique; these insects can stay alive without a blood meal for months. However, initiation of molting and metamorphosis in bed bug, *C. lectularius* requires blood

feeding (Reinhardt and Siva-Jothy, 2007). In this chapter, we report on the identification of key players that regulate molting and metamorphosis in the bed bugs.

Materials and methods

Insects: *C. lectularius* NY-1 colony was used in this study. The insects were collected from an infested apartment in Plainview, New York in April, 2007. Insects were maintained at 26.7ºC, 65 ± 5% RH and a photoperiod of 14: 10 h (L: D). Insects were maintained on defibrinated rabbit blood (at 37ºC) by the method developed by Montes *et al* 2002 (Montes et al., 2002). Blood was purchased from Quad Five Company. The nymphs were identified on the basis of size and morphology of the wing pads. The N5 wing pads fuse together forming a concave structure, whereas N4 show a straight line in between wing pads.

Gene identification: De novo assembly of 454 sequences was performed. The files used in the assembly are as follows: NCBI Accession number 1) SRX028107; 2) SRX013985; 3) SRX013984; 4) EST *C. lectularius* (#7131) University of Kentucky CIN-1 strain. All the contigs and singletons thus obtained were analyzed using Blast2go. BLASTX was then performed against NCBI NR database. Blast2go software was used to predict the function of assembled genes. GO ID, Enzyme ID and Interpro accession numbers were obtained for all the sequences. Maker annotated genes from the i5k project were also used for identification of some genes.

RNA Isolation, cDNA Synthesis, and Quantitative Real-Time PCR: Total RNA was isolated from three insects for each replicate using the TRI Reagent (Molecular Research Center Inc., Cincinnati, OH). The RNA was treated with DNase I (Ambion Inc., Austin, TX). cDNA was synthesized using Promega kit (Promega, Madison, WI). qRT-PCR was performed using Applied Biosystems Step One Plus TM (Life Technologies TM Real-Time PCR System, Carlsbad, CA). FastStart SYBR Green Master mix (Roche Diagnostics, Indianapolis, IN) and.2 µl of 10 µM primers were used in a ten µl qRT-PCR reaction. Primers used are shown in Table 1S. The mRNA levels were normalized using the internal control RPL8 (ribosomal protein L8).

Double-stranded RNA synthesis and Injection: Fragments of genes coding for select genes were PCR amplified using the primers reported in Table 1S, and these DNA fragments were used to prepare dsRNA, as described by MEGA script RNAi synthesis Kit

(Ambion Inc., Austin, TX). Newly molted N4 and N5 were anesthetized with ethyl ether vapor for 2 min and lined on a glass slide covered with double-sided tape. About one µg (0.1 µl) of dsRNAs were injected into the ventral side between the first and second abdominal segment using Nanojet and an injection needle made using the needle puller (Idaho Technology, Salt Lake City, Utah). The dsRNA prepared using a fragment of *Escherichia coli* maltase gene (*malE*) or green fluorescent protein (*GFP*) was used as a control. Injected nymphs were removed from the slide after recovery and kept in an incubator for four days before feeding them with rabbit blood.

Methoprene treatment: 10 µg Methoprene in cyclohexane was applied on the abdomen of blood fed N5. Methoprene application was repeated on alternating days. The Same volume of cyclohexane was applied to control insects. The bugs were then kept in an incubator and allowed to molt.

Light and Confocal Microscopy: Insect pictures were taken using DinoCapture2.0 software under white light. The tissues were dissected in 0.01 M phosphate buffer saline and fixed in 4% paraformaldehyde. Then the fixed tissues were rinsed with PBS and stained with DAPI. Pictures were then taken using confocal microscope under illumination with light at 405 nm wavelength.

Statistical Analysis: Statistical analysis was performed using Statistix software. One way ANOVA was performed for comparison of expression data in different stages of the insect. Post hoc test consists of Tukey HSD. The level of significance was set at $P = 0.05$. Student t-test (unpaired t-test) was performed for comparing significance in knockdown and induction of gene expression at 95% confidence.

Results

Expression of genes involved in molting and metamorphosis: Expression of genes known to be involved in molting and metamorphosis in other insects was determined during the third (N3), fourth (N4, penultimate) and fifth (N5, final) nymphal stages. Homologs of genes known to regulate molting and metamorphosis in other insects were identified in the bed bug and qRT-PCR primers were designed. The mRNA levels of these genes were quantified in insects collected at 24 h intervals beginning at blood feeding until they molt to the next stage. Expression of JH receptor Methoprene tolerant protein (Met) showed an increase after blood feeding until they molt to the next stage (Fig. 2.1a). Higher

levels of steroid receptor co-activator (SRC) mRNA were detected soon after feeding and then the mRNA levels decrease by 24-48 h after feeding (Fig. 2.1a). The SRC mRNA levels increase again and higher levels of this mRNA were detected at 72 h after feeding. The Kr-h1 mRNA levels did not show significant changes during N3 and N4, however during N5, the Kr-h1 mRNA levels decreased by 16-fold when compared to their levels in N3 and N4 (Fig. 2.1a). JHAMT (JH acid methyl transferase, an enzyme involved in JH biosynthesis) mRNA were detected during N3 and N4 but decreased to undetectable levels during N5 (Fig. 2.1a). Broad-Complex (BR-C) mRNA levels increased from 0 h to 72 h after feeding in N3 and N4. In contrast, BR-C mRNA levels decreased from 0 h to 96 h after feeding in N5 (Fig. 2.1a).

Ecdysone receptor (EcR) mRNA levels did not show significant differences among the stages tested suggesting that EcR gene is expressed most of the times during N3, N4 and N5. The mRNA levels of ecdysone delayed-early genes, hormone receptor 3 (HR3) and hormone receptor 4 (HR4) increased during each molt (Fig. 2.1a). E93 mRNA was not detected during N3; the E93 mRNA levels started to increase at 24 h after feeding during N4 and reached the maximum levels after a molt to N5 and these higher levels were maintained throughout N5 (Fig. 2.1a). The mRNA of phantom and shade (the enzymes involved in ecdysteroid biosynthesis) were detected in N3, N4 and N5 and did not show significant differences among the stages tested (Fig. 2.1a).

Insulin receptor (InR), protein kinase B (Akt3), insulin-like peptide 1 (ILP1), insulin-like peptide 2 (ILP2) and target of rapamycin (TOR) mRNAs were detected during N3, N4 and N5 stages and did not show significant differences among the stages tested (Fig. 2.1b). Cationic amino acid transporter iCAT2 expression showed a significant increase at 48 h after a blood meal in N3 as well as at 72 and 96 h after a blood meal in N5 (Fig. 2.1b). Similarly, Slimfast (Slif), another cationic amino-acid transporter showed an increase in expression at 0 and 72 h after a blood meal in N3 and soon after a blood meal in both N4 and N5. Whereas the Na (+)-coupled neutral amino acid transporter 6 (NAT1) showed a significant increase in expression prior to molting to N4, N5 and adult (Fig. 2.1b).

Expression pattern of these key genes involved in JH and 20E action suggest that these two hormones play important roles in molting and metamorphosis of *C. lectularius*. Decrease in the expression of Kr-h1 and increase in the expression of E93 during N5 stage

suggest that these two genes may play important roles in cross-talk between JH and 20E in the regulation of metamorphosis of *C. lectularius*. Therefore, we concentrated on determining the function of these two genes in the regulation of *C. lectularius* metamorphosis.

Role of Juvenile hormone in regulation of metamorphosis: To study the role of JH in the prevention of metamorphosis in the bed bugs, ten μg of JH analog, methoprene was applied on the abdomen of blood fed N5. An equal volume of cyclohexane was applied to control insects. Eighty-five percent of control cyclohexane treated insects molted into adults (Table 1, Fig. 2.2a). Methoprene application to N5 induced development of supernumerary nymphs (Fig. 2.2b). Eighteen percent of methoprene treated nymphs molted into the supernumerary nymphal stage and the rest of them died (Table 1). When compared to N5 is shown in Figure 2.2c and adult shown in Figure 2.2a, the supernumerary nymphs showed nymphal characters including lighter sclerotization of the cuticle especially the first three segments in the anterior region of the abdomen (blue arrowhead in Fig. 2.2b), the presence of ecdysial lines on the head (red arrowhead in Fig. 2.2b) and partially developed wing pads (yellow arrowhead in Fig. 2.2b).

Kr-h1 plays a key role in JH regulation of metamorphosis: To determine the role of Kr-h1 in the regulation of metamorphosis, we prepared dsRNA targeting Kr-h1 and injected it into N4. The control insects injected with *malE* dsRNA molted into N5 (Fig. 2.3a) and showed typical characters of N5 consisting of first three less sclerotized segments at the anterior region of the abdomen (blue arrow head), lighter sclerotization of the cuticle, presence of ecdysial lines on the head (red arrowhead) and fused wing pads forming a concave line (yellow arrowhead). In contrast, 31% of the Kr-h1 dsRNA injected insects developed precociously into adults exhibiting external features including developed wing pads (Fig. 2.3b, yellow arrowheads), absence of first three less sclerotized abdominal segments, absence of ecdysial lines, and darker sclerotization of the cuticle as compared to that in control N5 (Table 2 and Fig. 2.3b). External morphology of adult male (Fig. 2.3c) and female (Fig. 2.3d) are shown for comparison.

malE and Kr-h1 dsRNA injected nymphs were dissected after molting to the next stage and the development of reproductive organs was recorded using a confocal microscope. The reproductive organs were not well developed in the control insets (Fig. 2.4a-c) when

compared to those in Kr-h1 dsRNA injected insects (Fig. 2.4d-f). In control insects injected with malE dsRNA, the ovaries are smaller in size (Fig. 2.4a) as compared to the Kr-h1 dsRNA injected insects (Fig. 2.4d). The germarium or the vitellarium did not develop well in the control insects (Fig. 2.4b). The control insects did not show the presence of oviduct or seminal conceptacle (Fig. 2.4c). In contrast, Kr-h1 dsRNA injected showed developed ovaries (Fig. 2.4d-f). The ovaries are larger in size, germarium is clearly visible (Fig. 2.4e green arrowhead), lateral and common oviducts are developed (Fig. 2.4f). The ovaries dissected from adults are shown in Figures 2.4g-i for comparison. The ovaries dissected from newly emerged adults are larger in size, the germarium is well developed (green arrowhead) and some of them showed the presence of vitellarium (orange arrowhead) (Fig. 2.4h). The lateral and common oviducts and seminal conceptacle are well developed in these ovaries (Fig. 2.4i).

The N4 insects injected with malE dsRNA molted into N5 and contained testis and mycetome (Fig. 2.5a). Figure 5b shows an enlarged view of a testicular lobe. However, the vas-deferens, seminal vesicle, ejaculatory duct, male accessory gland reservoir and male accessory glands were absent in these insects (Fig. 2.5a and 2.5c). In contrast, Kr-h1 dsRNA injected nymphs showed well-developed testis (Fig. 2.5d-e), vas-deferens, seminal vesicles, male accessory gland reservoirs and male accessory glands (Fig. 2.5f). Ejaculatory pump and aedeagus were not clearly defined as seen in the control adult males. Reproductive system in control adults is well developed and showed seven testicular lobes, mycetome, seminal vesicle, ejaculatory duct, male accessory gland reservoir and male accessory gland (Fig. 2.5g-i).

Role of E93 in regulation of metamorphosis: RNAi-mediated knockdown of E93 was carried out in the N5. About 50% of E93 dsRNA injected insects molted into supernumerary nymphs, 25% developed into adults and the rest of the 25% died (Table 2). The supernumerary nymphs showed nymphal characters including the presence of first three less sclerotized segments at the anterior region of the abdomen (blue arrowhead in Fig. 2.6a), lighter sclerotization of the cuticle, underdeveloped wing pads (yellow arrowhead in Fig. 2.6a) and the presence of ecdysial lines on the head (red arrowhead, Fig. 2.6a). All control N5 injected with *GFP* dsRNA developed into adults (Fig. 2.6b). Fifth instar nymph is shown in Figure 6c for comparison.

Cross-talk between Kr-h1 and E93: To study the cross-talk between Kr-h1 and E93, we quantified mRNA levels of Kr-h1, E93 in insects injected with Kr-h1 or E93 dsRNA or treated with methoprene. Application of methoprene to day 3 N5 induced Kr-h1 mRNA levels by about 15-fold when compared to its expression in the control insects treated with acetone (Fig. 2.7a). In contrast, application of methoprene resulted in a seven-fold reduction in E93 mRNA levels when compared to its expression in control insects treated with acetone (Fig. 2.7b). Injection of Kr-h1 dsRNA caused 50% knockdown in Kr-h1 mRNA levels in N4 (Fig. 2.7c) and the knockdown in Kr-h1 resulted in 15-fold increase in E93 mRNA levels when compared to its expression in control insects injected with malE dsRNA (Fig. 2.7d). Injection of E93 dsRNA into N5 caused >80% knockdown in the expression of this gene (Fig. 2.7e) and a six-fold increase in Kr-h1 mRNA levels, when compared to its expression in control insects, injected with *GFP* dsRNA (Fig. 2.7f). These results suggest that Kr-h1 and E93 regulate each other and both of them are involved in JH suppression of metamorphosis (Fig. 2.7g).

Discussion

Bed bug populations and problems caused by these insects are increasing in the USA and around the world. One of the reasons attributed to the resurgence of bed bugs is the development of insecticide resistance in these insects making them difficult to control using currently available insecticides (Romero and Anderson, 2016; Romero et al., 2007; Zhu et al., 2013; Zhu et al., 2010). New insecticides with a novel mode of action are urgently needed. The studies included in this paper have been conducted to understand the hormonal regulation of molting and metamorphosis in bed bugs with a goal to identify key genes involved in regulation of these processes.

Molting and metamorphosis have been studied in both hemimetabolous and holometabolous insects. In all insects JH and ecdysone both play an important role during this process. Ecdysone titers peak before every molt or metamorphosis (Riddiford et al., 2003). In hemimetabolous insects JH titers are high in the immature stage but falls in the last instar stage (Treiblmayr et al., 2006). Similarly in holometabolous insect *Bombyx mori* JH titers fall in the last instar larvae but peaks before entering the pupae stage (Furuta et al., 2013). mRNA expression levels of Kr-h1 a JH inducible gene has been shown to correlate well with JH hormone titers (Lozano and Belles, 2011). Kr-h1 mRNA expression

profiles have been studied in other hemimetabolous and holometabolous insects which include *P. apterus* (Konopova et al., 2011; Smykal et al., 2014), *B. germanica* (Lozano and Belles, 2011), *Tribolium* (Minakuchi et al., 2009), *Bombyx mori* (Kayukawa et al., 2014) and *Manduca sexta* (Belles and Santos, 2014).

qRT-PCR studies on the expression levels of homologs of genes identified as key players in JH and 20E biosynthesis and action suggested that these two hormones regulate molting and metamorphosis in bed bugs. Significant reduction in the expression of Kr-h1 and increase in the expression of E93 during N5 stage suggested that these two genes might mediate cross-talk between JH and 20E action in regulation of molting and metamorphosis. The negative interaction between the two genes was confirmed by topical application of JH analog, methoprene, on fifth instar nymphs. Topical application of methoprene in fifth instar nymphs induced expression of Kr-h1 mRNA and inhibited E93 mRNA expression while inducing a supernumerary molt. Knockdown of E93 mRNA in the fifth instar stage also induced Kr-h1 expression while inducing a supernumerary molt. E93 mRNA was induced with the knockdown of Kr-h1 mRNA in the fourth instar stage. These data suggest that the presence of JH and Kr-h1 during N1-N4 promote nymphal molt and prevent metamorphosis. While E93 promotes metamorphosis. The function of Kr-h1 as a repressor of adult characteristics was more evident when it's knockdown in the fourth instar stage resulted in the precocious development of ovaries and testis.

Injection of dsRNA targeting two regions of Met gene in *C. lectularius* resulted in 60-80% reduction in Met mRNA levels (Fig. S1.1). However, no detectable phenotype was observed (Table 2S) . This may be because very low levels of Met protein remained in the RNAi insects is sufficient for its function. In silkworm, *Bombyx mori* knockdown of CYP15C1 and JHAMT (which are involved in later steps of JH synthesis pathway) results in the precocious development of adults (Daimon et al., 2012). Met and Kr-h1 knockdown also induce development of adult characters in holometabolous insect *T. castaneum* (Minakuchi et al., 2009; Parthasarathy et al., 2008). In other hemimetabolous insects including *B. germanica*, *P. apterus* and *R. prolixus* blocking JH action by knocking down Met and Kr-h1 results in the precocious development of adults from the penultimate stage nymphs (Konopova et al., 2011; Lozano and Belles, 2011). Met knockdown in *B. germanica* during the penultimate stage causes precocious adult development. Whereas,

its knowledge during the final nymphal stage causes developmental defects in the adults which include shortened wings and decrease in the expression of EcR, RXR, E75 and ILP-1 (Lozano and Belles, 2014). In *B. germanica* E93 defines nymphal to adult molt (Belles and Santos, 2014). E93 expression increases in the last stage nymphs and E93 induces the formation of adult characteristics. Similarly in holometabolous insects E93 expression increases in the pupal stage promotes the development of the insect to adult. Knockdown of E93 in the pupae prevents the formation of adults and results in the formation of the second pupa (Urena et al., 2014).

The research reported here showed that JH and 20E inducible gene Kr-h1 and E93 regulate molting and metamorphosis in *C. lectularius*. Based on our data and data reported from other hemimetabolous and holometabolous insects, we conclude that higher levels of JH during the penultimate nymphal stages induce Kr-h1 expression and suppress E93 expression to prevent metamorphosis. Whereas, a decrease in JH titers as depicted by a drop in Kr-h1 mRNA levels allows the expression of E93 and development of promotes the development of adult structures. Thus, we see that role of Kr-h1 and E93 is conserved throughout the hemimetabolous and holometabolous insects. However, molecular mechanisms of the cross-talk between Kr-h1 and E93 in transduction of JH signals that regulate molting and metamorphosis as well as the function of 20E in these interactions remain unknown and will be the focus of future studies.

Table 2.1. Effect of methoprene application on metamorphosis.

Percent	Mortality	Supernumerary nymph	Adult
Cyclohexane	15	0	85
Methoprene	82	18	0

Table 2.2. Effect of Kr-h1 and E93 knockdown on metamorphosis

	Knockdown in 4th instar nymph			Knockdown in 5th instar nymph		
Gene	Mortality (%)	N5 (%)	Precocious adults	Mortality (%)	Adult (%)	Supernumerary nymphs
malE	0	100	N5 nymphs	0	100	Adult
E93	-	-	-	25	25	50
Kr-h1	2	67	31	-	-	-
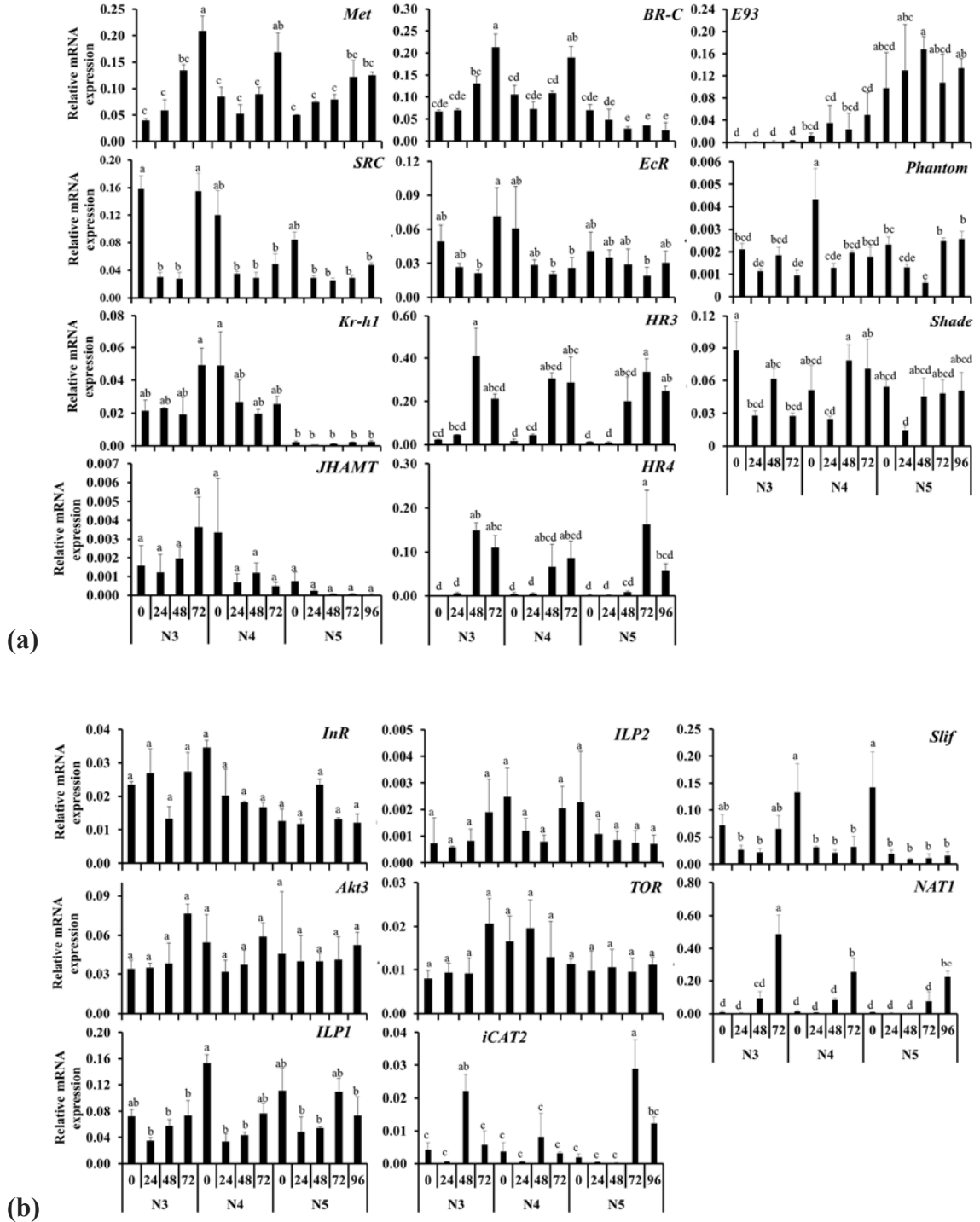
Figure 2.1. Expression profile of developmental genes in *Cimex lectularius* nymphs. RNA from third (N3), fourth (N4) and fifth (N5) nymphal stages were collected at <u>24 h</u> intervals beginning at the time of feeding until they enter next stage. (a) Relative mRNA levels of genes regulating hormonal pathways: Relative mRNA levels of Methoprene-

tolerant protein (Met), Steroid receptor co-activator (SRC), Krupple homologue 1 (Kr-h1), Ecdysone receptor (EcR), Hormone receptor 3 (HR3), Hormone receptor 4 (HR4), Ecdysone-induced protein 93F (E93), Phantom, Shade, and Broad-Complex (*BR-C*). (b) Relative mRNA levels of genes regulating nutritional pathways: Insulin Receptor (InR), RAC-gamma serine/threonine-protein kinase (Akt3), Insulin-like peptide 1 (ILP1), Insulin-like peptide 2 (ILP2), Target of Rapamycin (TOR), insect cationic amino acid transporter 2 (iCAT2), Slimfast (Slif), and probable sodium-coupled neutral amino acid transporter 6 (NAT-1). mRNA expression was normalized using expression levels of ribosomal protein 8 (rpl8). Data shown are mean + SD (n=3). (Alphabets represent significance at 95% CI).
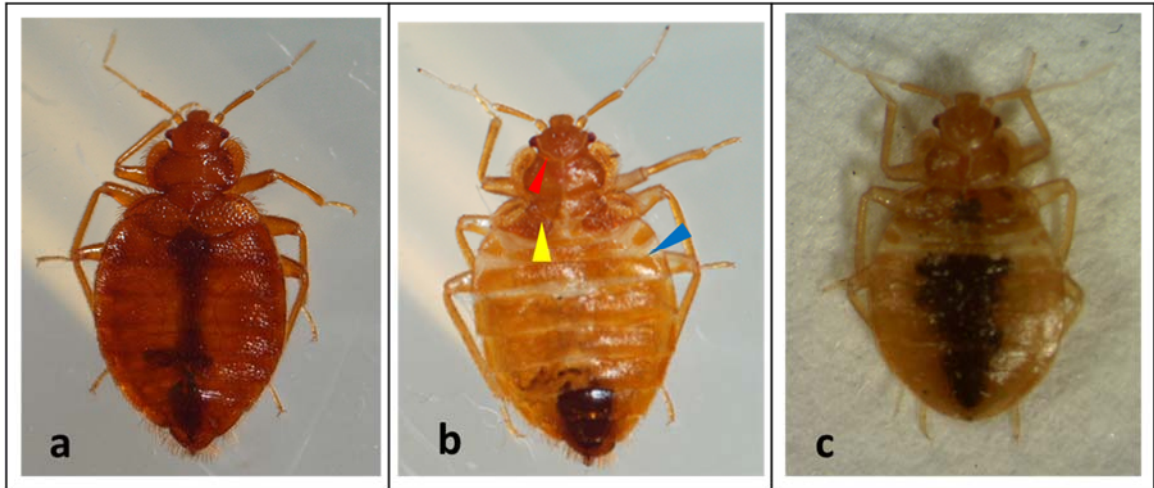
Figure 2.2. Methoprene induces a supernumerary molt. Ten μg of methoprene in cyclohexane was topically applied on the abdomen of N5 after blood feeding. Methoprene application was repeated every alternating day until molt. The control insects were applied with cyclohexane, these insects developed into adults (a). 18% of methoprene treated insects molted to N6 (b). 82% mortality was observed in methoprene treated insects. The N6 showed the presence of nymphal characters including the presence of first three less sclerotized segments at the anterior end of the abdomen (blue arrowhead), lighter sclerotization of the cuticle as compared to the adult, presence of ecdysial lines towards the head (red arrowhead) and partially developed wing pads (yellow arrowhead). The experiment was repeated three times and similar phenotypes shown in photographs have been observed in all experiments. Untreated N5 is shown in Figure 2C for comparison.
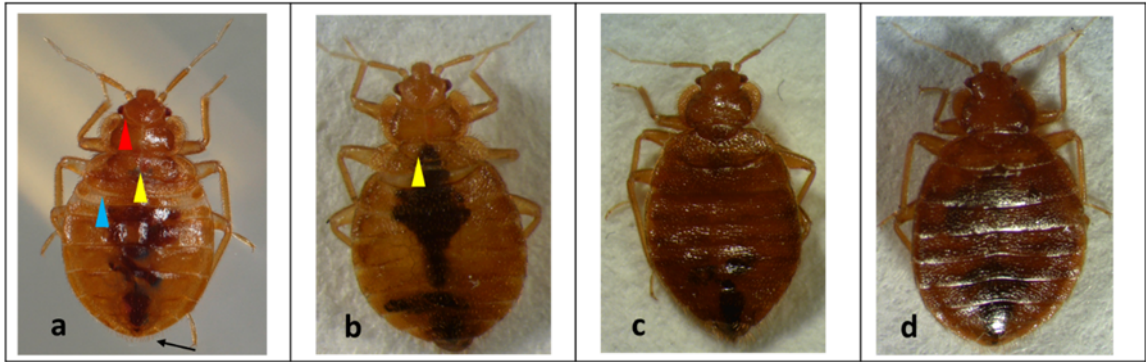
Figure 2.3. RNA interference (RNAi) mediated knockdown of Kr-h1 in N4. Kr-h1 dsRNA was injected into fourth instar nymphs. The insects were incubated for four days before blood feeding. malE injected bugs were used as a control. 100% of the control bugs molted into fifth instar nymphs (a). 31.11% of dsKr-h1 injected bugs molted into a precocious adult (b), the remaining bugs molted to N5. Control adults male (c) and female (d) are also shown for comparison. The precociously developed insects showed the development of wing pads (yellow arrowhead). Wing pads were fused in fifth instar nymphs. Three less sclerotized segments in the anterior region of the abdomen, which is normally seen in fifth instar nymph (blue arrowhead in Fig. 2.3a) are absent in the preciously developed adults. Ecdysial lines (red arrowhead in Fig. 2.3a) which are a characteristic feature of nymphs were also absent in the precociously developed adult and control adults. The experiment was repeated three times.
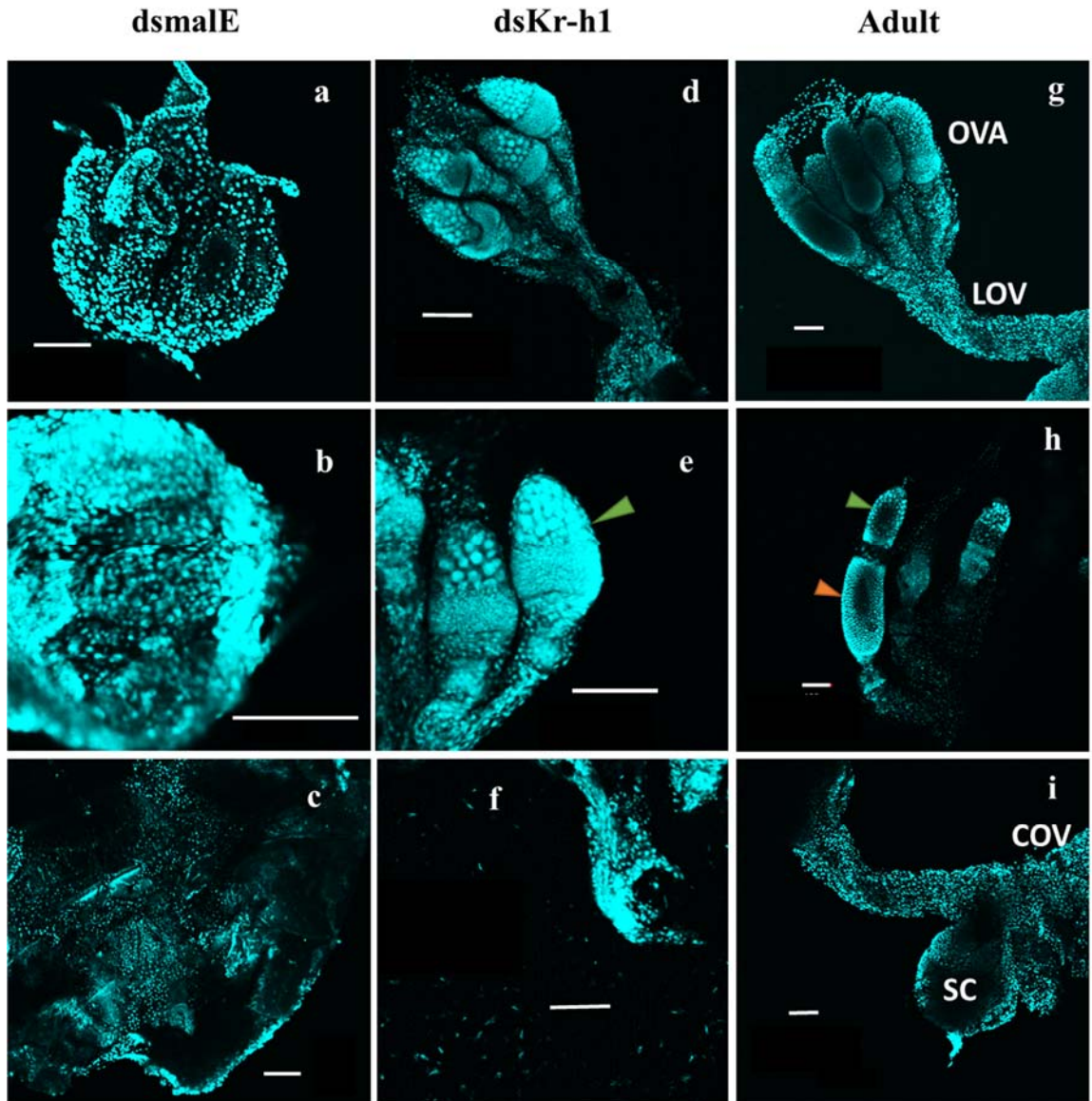
Figure 2.4. Precocious development of ovaries in Kr-h1 knockdown insects. RNAi-mediated knockdown of Kr-h1 was carried out in N4. Control insects injected with malE dsRNA molted into fifth instar nymphs. Ovary development was reduced in control N5 (a-c). Ovaries (d-f) form Kr-h1 dsRNA injected insects showed the precocious development of ovaries (d-f). These ovaries showed well-developed ovarioles (e) where the germarium (green arrowhead) is detected. Lateral oviduct and common oviduct are also developed (f). These structures including the germarium (green arrowhead) and oocytes (red arrowhead) are well developed in control adult (g-i). OVA, ovarioles; LOV, lateral oviduct; COV, common oviduct and SC, seminal conceptacle. Bar represents 100µm.
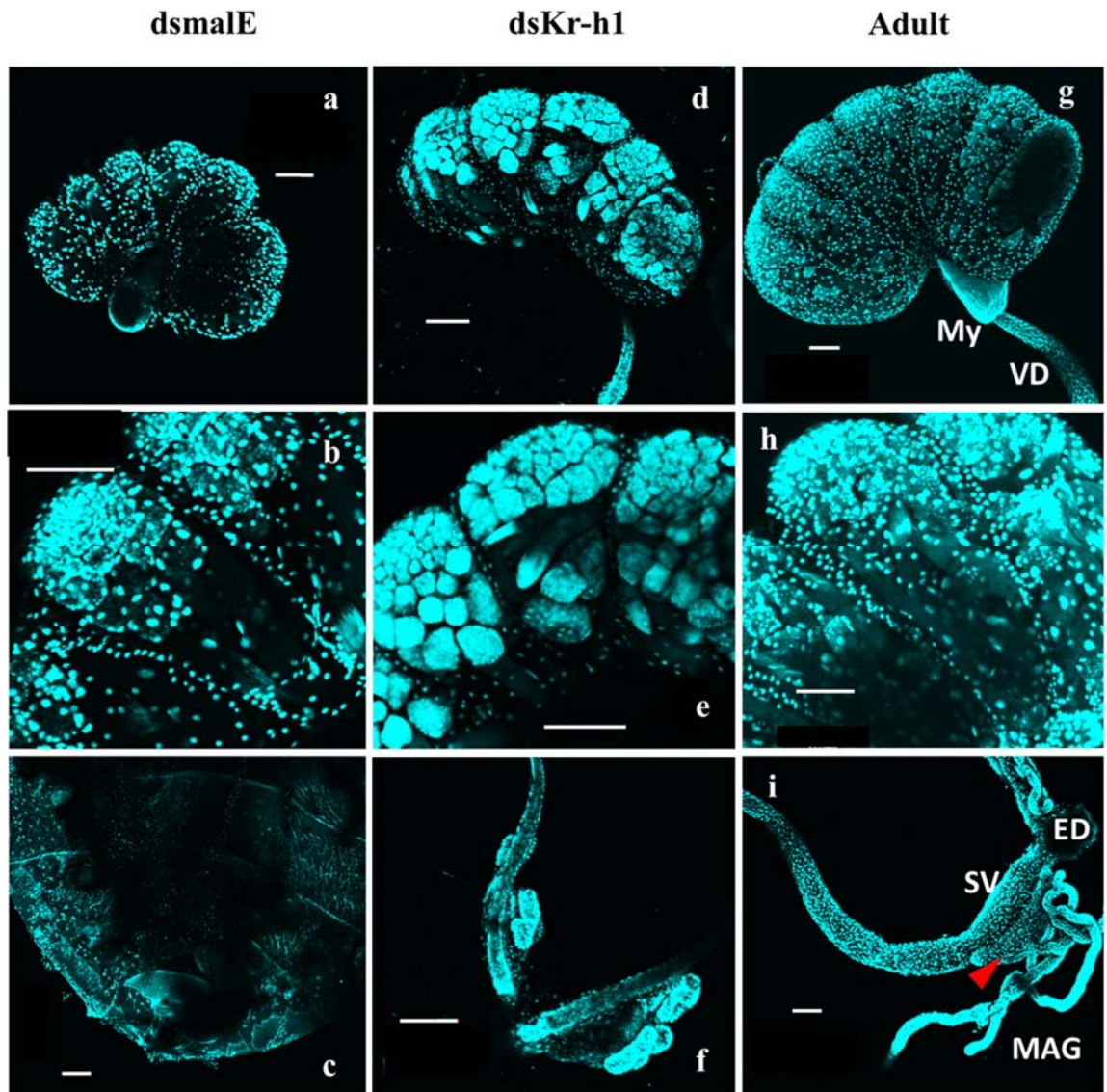
Figure 2.5. Precocious development of testis in Kr-h1 knockdown insects. RNAi-mediated knock down of Kr-h1 was carried out in N4. Control insects injected with malE dsRNA were dissected to observe the development of testis (a-c). Kr-h1 knockdown insects showed the development of male reproductive organ (d-f). Vas-deferens (VD), seminal vesicle (SV), male accessory gland reservoir (Red arrow head) and male accessory glands (MAG) were observed in kr-h1 knockdown males. Male reproductive organs dissected from control adults are shown in g-i for comparison. Ejaculatory duct (ED) was not clearly defined in kr-h1 knockdown insects. Mycetome (My) is shown in adults. Mycetome was present in kr-h1 knockdown insects also but not shown in the figure. Bar represents 100μm.

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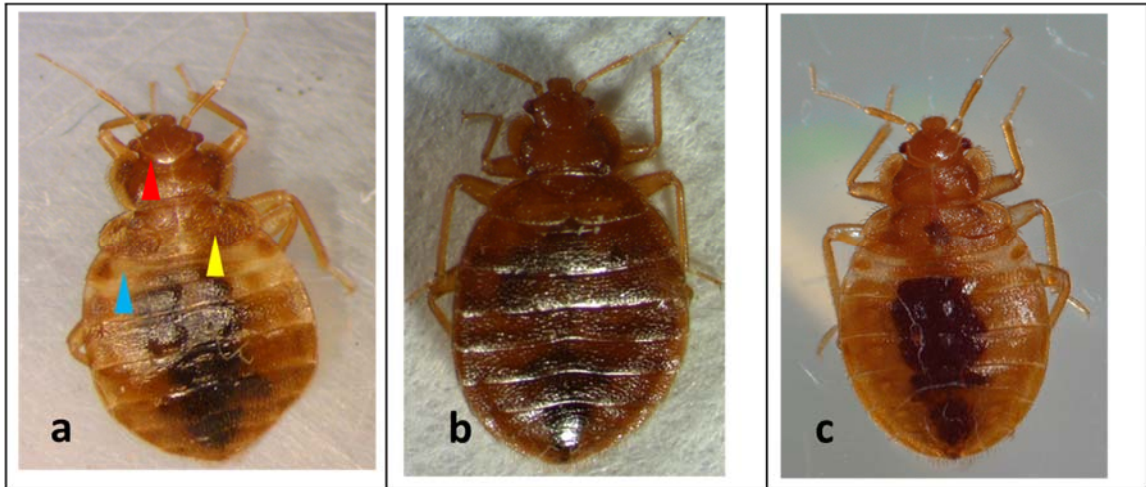
Figure 2.6. RNA interference (RNAi) mediated knockdown of E93 in N5. E93 dsRNA was injected into N5, *GFP* was used as a control. The control insects metamorphosed into adults (b). Whereas 50% of E93 injected insects molted into N6 (a). N6 showed first three less sclerotized abdominal segments (blue arrowhead) in the anterior region of the abdomen, partially developed wing pads (yellow arrowhead), ecdysial lines on the head (red arrowhead) and lighter sclerotization of the cuticle. 25% mortality was observed in this group, whereas 25% developed into adults. Control N5 is shown for comparison (c). The experiment was repeated three times with similar results.
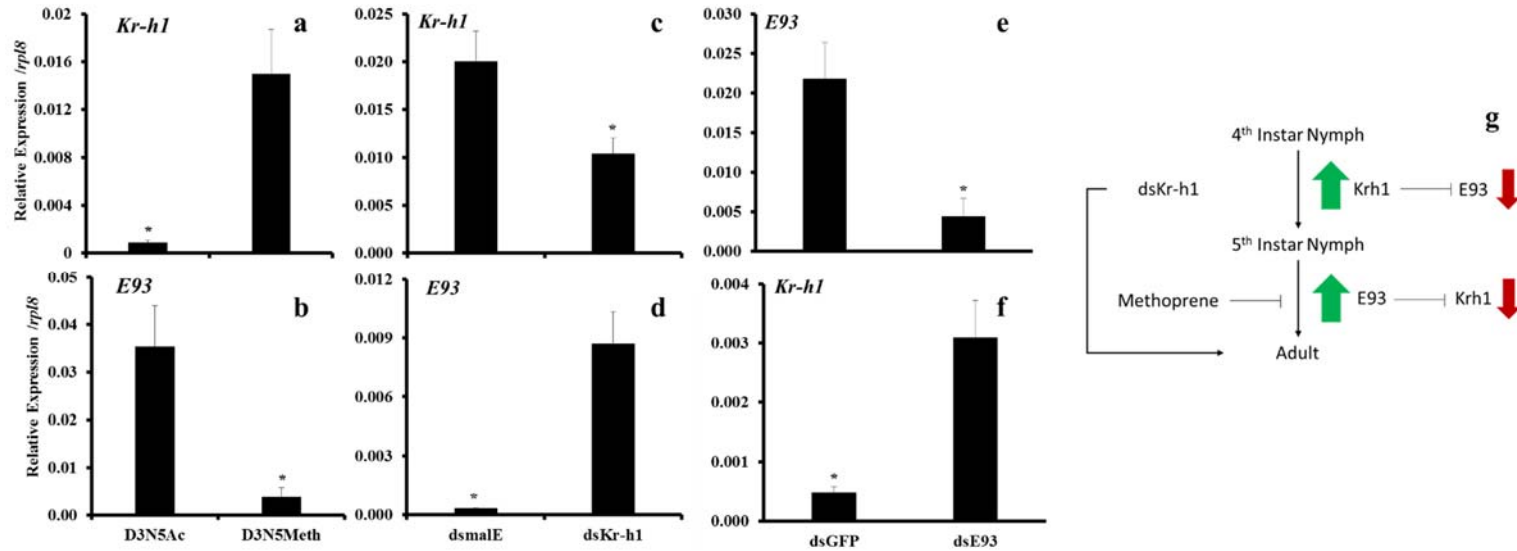
Figure 2.7. Interaction of Kr-h1 and E93 pathway in the control of molting and metamorphosis. After blood feeding of N5, 10 ug of methoprene was topically applied every day. RNA was extracted on the third day at 6 hours after methoprene application. qRT-PCR analysis of RNA isolated from these insects showed an increase in Kr-h1 mRNA levels (a) and suppression of E93 mRNA levels (b). One μg of Kr-h1 or malE dsRNA was injected into N4. The insects were fed after days and total RNA was extracted two days after feeding. Knockdown of Kr-h1 in N4 (c) caused a decrease in Kr-h1 mRNA levels and an increase in *E93* mRNA levels (d). Knockdown of E93 in N5 (c) caused a decrease in E93 mRNA levels (e) and an increase in Kr-h1 mRNA levels (f). Proposed model for cross-talk between Kr-h1 and E93 is shown in Figure g.

Chapter 3: Ovarian Development in the bed bug, *Cimex lectularius*

Introduction

In bed bugs, mating initiates molecular changes mediated by hormones and signaling molecules important in the regulation of vitellogenesis, development of oocyte and embryo. Two major hormones 20E and JH are known to regulate reproduction in insects (Hansen et al., 2014; Raikhel, 2005; Riddiford, 2012). In the mosquito *Aedes aegypti*, a blood meal triggers ovaries to synthesize ecdysteroids, which then regulate egg development in *A. aegypti* (Hagedorn et al., 1975). 20-hydroxyecdysone, binds to its receptor Ecdysone receptor (EcR) and ultraspiracle (USP) and induce expression of the downstream early genes E74, E75, broad (BR-C) (Chen et al., 2004). The products of these genes along with EcR-USP-20E complex (Kokoza et al., 2001; Martin et al., 2001) then activate *Vg* gene transcription and regulate reproduction. 20E plays a major role in oocyte maturation in *T. castaneum* (Parthasarathy et al., 2010a). On the contrary, JH, a sesquiterpenoid hormone, induces its effect through its receptor Methoprene-tolerant protein (Met) and co-activator, steroid receptor co-activator (SRC) (Zhang et al., 2011) or Cycle (CYC) (Shin et al., 2012). JH regulates Vg expression in *Locusta migratoria* (Song et al., 2014) and *T. castaneum* (Parthasarathy et al., 2010b).

Nutrition also plays an important role in the regulation of reproduction. In *A. aegypti*, blood meal increase amino acids and insulin peptides in the hemocoel and triggers the synthesis of hormones that regulate reproduction through the insulin and TOR pathway. In a study on the regulation of reproduction in the yellow fever mosquito, *A. aegypti;* sequencing of RNA isolated from pre- and post-blood meal mosquitoes showed that 35% transcripts in the post-blood meal animal encode the yolk protein. The other most highly expressed transcripts are cathepsin b, carboxypeptidase and vitelline membrane protein family (Price et al., 2011). Two Vg proteins are highly expressed in the bed bug during reproduction. In this study, we identified transcription factors, MafB and Foxl2 as the important players for the regulation of reproduction in the bed bug. MafB is a member of large Maf family of transcription factors involved in development (Yang and Cvekl, 2007). Foxl2 is a transcription factor containing the forkhead domain. In the female mice it represses formation of the testis (Uhlenhaut et al., 2009). Its role in insects has not been studied. Other factors affecting embryonic development were also identified. These include

HPSE, cuticular proteins like unclassified cuticle protein 1 (UCP1; CLEC006545), krotzkopf verkehrt (kkv) and laccase 2, and zinc finger-containing proteins CLEC005028 and stripe (Sr). HPSE is an endoglycosidase that cleaves heparin sulfate from heparin sulfate proteoglycans (HSPG). HSPG is found in the extracellular matrix where it interacts with various ligand molecules to regulate growth factor signaling. In *Drosophila* mutations in genes, synthesizing HSPG glycosaminoglycan reduces heparin sulfate and cause developmental defects (Bornemann et al., 2004). Cuticle provides structural stability to the insect. Knockdown of enzymes involved in cuticle synthesis has been shown to produce embryonic lethality (Arakane et al., 2005; Araujo et al., 2005). Zinc finger proteins interact with DNA, RNA and proteins to regulate various genes and cells function (Laity et al., 2001). This study would enable further detailed analysis of reproduction in the bed bug to help find new molecular targets for their control.

Materials and methods

Insects: The bed bugs used in the present study are derived from the colony designated as NY1 raised from those collected in the infested apartment in Plainview, New York in April 2007. Insects were maintained at 26.7 ºC, 65 ± 5% RH and a photoperiod of 14: 10 h (L: D) and were fed defibrinated rabbit blood, supplied by the Quad Five Company, Montana, USA based on the method proposed by Montes et al. (2002) (Montes et al., 2002). Insects were fed once in a week. Fifth instar nymphs were collected after feeding. These nymphs develop into virgin adults in about 5-6 days, which were then used in the experiments described here.

RNA Isolation, cDNA Synthesis, and Quantitative Real-Time PCR: Total RNA was isolated from three insects for each replicate using the TRI Reagent (Molecular Research Center Inc., Cincinnati, OH). The RNA was treated with DNase I (Ambion Inc., Austin, TX). cDNA synthesis (Promega) and qRT-PCR was performed using Applied Biosystems Step One Plus TM (Life Technologies TM Real-Time PCR System, Carlsbad, CA). FastStart SYBR Green Master (Roche Diagnostics, Indianapolis, IN) was used in a qRT-PCR reaction, with 0.2 µl of primers of 10 µM concentration in a 10 µl qRT-PCR reaction. Primer sequences are shown in the **Table S1**. The mRNA levels were normalized using the internal control, ribosomal protein L8 (RPL8).

Quantitative Real-Time PCR analysis of gene expression in whole body and tissue: Relative expression of selected genes was performed in whole body, ovaries, and fat body tissue samples. Fat body tissue also contained cuticle from abdomen attached to it. Whole insects were collected at day 0 to day 4 at 24 h interval after treatment. Insects were dissected at day 0, day 2 and day 3. The female bed bugs were provided with four treatments to study the effect of feeding and mating. The four groups were as follows: - blood fed and mated (F+M+), only blood fed (F+M-), only mated (F-M+), and without blood fed and not mated (F-M-). Total RNA was extracted and qRT-PCR was performed.

Sequencing, sequence analysis, and transcription factor analysis: The virgin adult female bugs were microinjected with 0.2 µl of dsRNA targeting methoprene-tolerant protein (Met) (1 µg) through the ectospermalege. Same amount of dsRNA of green fluorescent protein (GFP) was injected in the control bugs. The insects were incubated for five days. The insects were allowed to feed and mate with un-injected virgin adult males only once. After mating, the males were removed. The dsGFP injected insects were collected at 0 h, 6 h, 12 h and 24 h after feeding and mating. The dsMet injected insects were collected at 6 h, 12 h and 24 h after feeding and mating. Three biological replicates were used for each treatment. Total RNA from the whole body was isolated and used for the preparation of mRNA libraries as per the Illumina TruSeq RNA Sample Preparation v2 guide. The libraries were pooled in a group of six to a final concentration of 10 nM in 10µl and samples were shipped in dry ice for sequencing to DNA core facility, University of Missouri, Columbia, USA. All raw sequences were deposited to NCBI SRA submission under SRA accession: SRP072368. Quality control of libraries was performed using FASTQC and printseq_lite.pl. Reads less than 100 bp were removed. The base calling accuracy is measured by Phred quality score (Q score), which was set at a value greater than 30; as a Q score of 30 corresponded to 99.9% accuracy or 1 in 1000 probability of incorrect base call. Reads were trimmed and filtered to remove the low-quality bases. Bowtie index of the genome was prepared using bowtie2. The reference genome was obtained from i5k database (Benoit et al., 2016). Tophat was used to map sequence reads to the genome scaffold. Differential expression analysis was done using default settings of cufflinks software-cuffdiff (Trapnell et al., 2013). Raw reads were provided as input. Cuffdiff calculates the number of reads that are aligned on the transcript and provides

results as FPKM (fragment per kilobase of exon per a million fragments mapped) value of expression data for differential analysis. Cuffdiff normalizes data using the geometric method. FPKMs and fragment counts are scaled via the median of the geometric means of fragment counts across all libraries (Anders and Huber, 2010). Transcripts were also obtained from the i5k data base. They were identified using NCBI blast plus software blastx using nr database (Altschul et al., 1990). The cut-off e-value was kept at < 0.0001. The results were then uploaded on blast2Go software to obtain Gene ontology (GO) terms, Enzyme Classification (EC) number and Interpro IDs wherever possible (Gotz et al., 2008). PfamScan ver 1.5 was used to determine the Pfam ids for all the proteins in bed bug genome (Finn et al., 2014). Pfam is a database of protein families each represented by multiple sequence alignment and hidden Markov models. There are 147 Pfam motifs in TF database. We were able to identify 413 genes containing Pfam motifs (e-value ≤ 0.001) from the i5K generated bed bug transcripts. GO IDs were obtained using Blast2go software. Sequences with GO:0003676-nucleic acid binding and GO:0046872-metal binding were selected. We obtained 138 such sequences.

Double-stranded RNA synthesis, injection and mating experiments: Fragments of genes coding for target genes were PCR amplified using the primers reported in **Table S2**, and these DNA fragments were used to prepare dsRNA, as described by MEGA script RNAi Kit (Ambion Inc., Austin, TX). Day 1-2 old virgin adult females were used for the experiment. The insects were anesthetized with ethyl ether vapor for 2 min and lined on a glass slide covered with double-sided tape. One µg of (0.2 µl) dsRNA was injected into the ectospermalege using nanojet. The glass needles were prepared by using needle puller (Idaho Technology, Salt Lake City, Utah). The dsRNA targeting GFP gene was injected as a control. Injected adults were removed from the slide after recovery and kept in an incubator for five days before feeding them with rabbit blood. The uninjected virgin adult males were also blood fed. Engorged male and female insects were kept in a 48-well plate for two days. The males were then discarded.

Methoprene application: 10 µg methoprene in 1µl acetone was applied on the abdomen of each adult virgin female after blood feeding. Methoprene application was repeated at 24 h and 48 h after first treatment. The control insects were treated with the

same volume of acetone. The bugs were then kept in an incubator and the samples were collected at 6 h after the final application.

Dissection and confocal microscopy: The adult females were dissected in 0.01 M phosphate buffer saline (PBS) on third day after feeding and mating to study the development of their primary oocytes. The ovaries were then fixed in 4% paraformaldehyde (in 1x PBS at pH 7.2) overnight. The tissue was rinsed with PBS and stained with DAPI. Pictures were then taken with a confocal microscope under illumination with light at 405 nm wave length.

Protein extraction, SDS-PAGE and sequencing: Total protein from the hemolymph was extracted three days after treatment. Holes were made in the abdomen of the insect using a needle. The insect was kept in 20 µl PBS containing proteinase inhibitor and centrifuged at 1000 rcf for 1 minute. Total protein content in the supernatant was quantified using Bradford assay. After denaturing the protein samples, 10 µg of the sample was loaded on a 6% SDS-PAGE. The gel was stained using Coomassie brilliant blue dye. The gel image was recorded using a gel documentation system. The gel was submitted to Proteomics Core Facility, the University of Kentucky for the identification of ~220 KDa band. The samples were digested with trypsin. The extracted peptides were desalted and analyzed by MALDI TOF/TOF.

Statistical analysis: Statistical analysis was performed using Statistx 10.0. Student t-test (unpaired t test) was performed for comparing significance in knockdown and induction of gene expression. One way ANOVA followed by Tukey HSD test was performed for determining the significance of expression profiles of genes.

Results

Sequence analysis: The mRNA libraries sequenced from the female bed bugs injected with GFP or Met dsRNA and collected at 0, 6, 12 and 24 h after feeding and mating were analyzed. An average number of reads in all the samples were 24 million of which 17 million (about 67%) were mapped onto the bed bug reference genome obtained from the i5k database.

*Differential expression of genes***:** The differential expression of genes in the GFP (0 , 6 , 12 and 24 h samples) and Met (6 , 12 and 24) dsRNA injected insects samples were calculated using Cuffdiff. Compared to the expression in unfed and unmated females, in

RNA collected at 6 h after feeding and mating, 351 genes increased by 2-fold in control insects injected with GFP dsRNA. Interestingly, this increase in expression was prevented for 62 of these genes in insects injected with Met dsRNA. By 12 h after feeding and mating 411 genes showed a two-fold or more increase in their expression in control insects and this increase was blocked for 79 of these genes in insects injected with Met dsRNA. By 24 h after feeding and mating, 310 genes showed a two-fold or more increase in their expression in the control insects, of which increase in expression of 30 genes were blocked in insects injected with Met dsRNA (Fig. 3.1a).

Differential expression analysis of transcription factor (TF) domain containing genes, nucleic acid (NA) and metal binding genes: We identified a total of 533 genes in this group. These genes contain 147 Pfam motifs included in the transcription factor database and/ or nucleic acid and metal binding properties in the bed bug genome. These genes may bind to DNA in the promoter regions and regulate expression of downstream genes. Metal binding proteins are often associated with transcription factors and act as cofactors. Differential expression analysis of the sequencing data of these genes showed that expression of only 28 of these genes increased by a two-fold or more from 0-6 h in the control insects. Only five genes showed a decrease in expression in the Met knockdown insects. Thirteen genes showed a two-fold or more increase in expression from 0-12 h, in control insects. Out of these, only two genes showed a decrease in expression in Met knockdown insects. Eleven genes showed two-fold or more increase in expression 0-12 h in control insects, out of which only one gene Forkhead binding domain (Foxl2) (CLEC004651) showed a decrease in expression in Met knockdown insects (Fig. 3.1a).

QRT-PCR confirmation of select differentially expressed genes: Based on their expression levels, the difference in expression among treatments and their putative function in reproduction 21 genes were selected for qRT-PCR verification (Fig. 3.1b). qRT-PCR data showed that the expression pattern of all genes tested is similar to that obtained by differential analysis of expression of RNA-seq data. 11 genes were selected from hi-seq expression analysis of genes containing transcription factor domain (Pfam), and GO terms for nucleic acid (GO:0003676) and metal binding (GO:0046872) for confirmation by qRT-PCR. As shown in Figure 3.1c, similar expression patterns were observed in transcriptome and qRT-PCR analysis for most of the genes tested.

Vitellogenin gene in the bed bug: Three vitellogenin mRNAs have been identified in the genome and transcriptome of *C. lectularius*. The ~200KDa protein band was identified as Vg2 and Vg3 using MALDI TOF/TOF (Fig. S 2.1). Vg1 is 1321aa long, contains the DUF1943 and the VWD domain and is similar to that found in other hemipteran insects (Fig. 3.2a and b). Vg2 (1854 aa long) and Vg3 (1868 aa long) also contain Vitellogenin_N, DUF1943 and the VWD domain and are closer to Vg2 of *Triatoma infestans* (Fig. 3.2a and b). The Vg in the bed bugs is closely related to those in the other hemipterans as shown by the phylogenetic tree (Fig. 3.2b).

Protein expression levels of individual Vg isoforms were estimated (Fig. 3.2c). 10 µg of total hemolymph protein from the dsGFP injected insects were loaded in lane 1. The ~200KDa band contains all the three Vg isoforms. Knockdown of Vg1 and Vg2 mRNA was performed, and the protein was loaded in Lane 2. Lane 2 shows Vg3 protein. Lane 3 shows Vg2 protein which was obtained by knockdown of Vg1 and Vg3. Lane 4 shows protein from Vg2 and Vg3 knockdown insects and lane 5 shows protein from Vg1, Vg 2 and Vg3 knockdown insects. No Vg protein band was detected after knockdown of Vg2 and Vg3 or Vg1, Vg2 and Vg3 (lanes 4 and 5) suggesting that Vg2 and Vg3 are expressed higher when compared to Vg1. Vg protein levels increase beginning on after adult emergence (Fig. 3.2d). No Vg protein was detected in 4th instar (N4), 5th instar (N5) and in newly emerged day 0 adults. To study the effect of nutrition and mating on Vg expression, total protein from the hemolymph was collected after three days from the four treatments. The treatments consist of blood feeding and mating (F+M+), only feeding (F+M-), only mating (F-M+) and no feeding and no mating (F-M-). Fifteen µg of total protein from hemolymph was loaded on SDS-PAGE. All the groups showed the presence of Vg protein. However, Vg was more abundant in the first two groups (F+M+ and F+M-) as compared to the last two groups (F-M+ and F-M-) (Fig. 3.2e). Similar to protein levels Vg2 and Vg3 mRNA levels are higher when compared to Vg1 mRNA levels (Fig. 3.2f). The maximum levels of Vg mRNA levels were detected in females at three days after emergence.

RNAi-mediated knockdown of selected genes in insects

*Knockdown of genes***:** Based on the change in expression levels, of the selected genes with time as recorded by qRT-PCR in dsGFP and dsMet treated female adults during reproduction, 11 genes were selected for studying their function using RNAi. Significant

knockdown of genes was observed in the adult female bed bug each injected with dsRNA targeting a specific gene. All the genes showed 50-99% knockdown efficiency (Fig. S2.2). Genes known to affect reproduction through hormonal, and nutritional pathways were also selected.

Vg gene expression in the knockdown insects: Relative expression of *Vg* mRNA was determined in the insects treated with dsRNA specific for the Vg isoform. Injection of Vg isoform-specific dsRNA reduced the expression of Vg1, Vg2 and Vg3 to 9.2%, 1.6%, and 2% respectively (Fig. 3.3a-c). Out of the 11 genes selected from sequence analysis, knockdown of transcription factor MafB reduced Vg1 expression to 5.7%, Vg2 to 4.5% and Vg3 to 2%. Knockdown of all other 10 genes, stripe, stubble, unclassified cuticle protein (UCP1), Foxl2, HPSE, kkv, CLEC005028, hexamerin, CLEC000870, laccase 2 and Cyp6a20 did not affect Vg gene expression. Knockdown of all other genes In addition to the 11 genes selected from the sequencing data, we also selected 18 genes known to play important roles in the regulation of reproduction in other insects. Knockdown of JH receptor Met reduced expression of Vg1 to 26%, Vg2 to 33% and Vg3 to 13%. Similarly, knockdown of SRC, the co-activator of Met reduced mRNA levels of Vg1 to 22%, Vg2 to 32% and Vg3 to 5.2%. Knockdown of transcription factor GATAa also reduced mRNA levels of Vg1 to 21%, Vg2 to 31% and Vg3 to 22%. Knockdown of early gene Kr-h1 in the JH pathway did not decrease the expression of *Vg*. Knockdown of genes coding for enzymes involved in ecdysteroid biosynthesis or action including Phantom, Shade, EcR, USP and BR-C or genes involved in nutritional signaling, InR1, InR2, ILP1, ILP2, Akt3, mTOR and cTOR did not have any effect on *Vg* expression. Knockdown of Ago1 and Dicer1 from the RNAi pathway also did not reduce the expression of *Vg* (Fig. 3.3d-f).

Fecundity in the knockdown insects: Knockdown of JH pathway genes, Foxl2, Met and SRC reduced fecundity to 0%, 0.7% and 0.5%, respectively (Fig. 3.4a and b). Knockdown of Kr-h1 had no effect on fecundity. Knockdown of ecdysone pathway genes, EcR, USP and BR-C reduced fecundity to 5.7%, 59% and 57%, respectively. However, knockdown of Phantom, shade and had no effect on egg laying. Knockdown of mTOR, cTOR, InR2 and GATAa reduced egg laying by 56%, 76%, 45% and 0% respectively (Fig. 3.4b). However InR1, ILP1 and ILP2 had no effect on fecundity. Transcription factor MafB knockdown reduced fecundity to 0%. However, knockdown of the stripe, stubble,

unclassified cuticle protein 1, HPSE, kkv, CLEC005028, hexamerin, CLEC000870, laccase 2, Cyp6a20 had no effect on egg laying. Knockdown of RNAi pathway gene Ago1 reduced fecundity to 25%. However, Dicer1 knockdown had no effect on fecundity (Fig. 3.4a and 4b).

Hatching of eggs laid by the knockdown insects: No hatching of first instar nymph was observed in the eggs laid by females injected with Stripe, unclassified cuticle protein 1, HPSE and kkv dsRNA. Knockdown of CLEC005028 and laccase 2 reduced hatching to 16% and 3.6%, respectively (Fig. 3.3c). Knockdown of ecdysone pathway genes, phantom, shade, USP and BR-C reduced the hatching of eggs to 15%, 53%, 0% and 20%, respectively. The eggs laid by Kr-h1 knockdown insects showed 0% hatching. Knockdown of mTOR, cTOR and InR2 reduced hatching to 7%, 26% and 18%, respectively. InR1, ILP1 and ILP2 knockdown did not show any effect on egg hatching. Knockdown of RNAi pathway genes Ago1 and Dicer1 reduced egg hatching to 0% and 22%, respectively (Fig. 3d). Hatching was not affected in eggs laid by females injected with stubble, hexamerin, CLEC000870 and Cyp6a20. (Fig. 3.3c and 3.3d).

Oocyte development in control and knockdown insects: To study the effect of feeding and mating on oocyte growth, ovaries were dissected from the female bed bugs at 24 h interval after feeding and mating. Only the most mature primary oocyte per ovariole was observed. The increase in the size of primary oocyte was observed from day 0 to day 3 (Fig. 3.5a-d). The maximum size of primary oocyte was observed on day 3 after feeding and mating. Primary oocyte length in the group of female adults which were not fed and not mated or only mated did not show any development with time. These oocytes appeared to be similar to the day 0 oocyte in the insects which were blood fed and mated (Fig. 3.5a). Only feeding also increased growth in the primary oocyte, however to a smaller size compared to fed and mated insects (Fig. 3.5e). Development of primary oocyte was also recorded on the third day after feeding and mating in the knockdown insects. Vitellogenin knockdown reduced oocyte length (Fig. 3.5f). Knockdown of JH pathway genes, Met, SRC and Foxl2 (Fig. 3.5g-i) reduced the development of primary oocyte as compared to the oocytes in control insects (Fig. 3.5d). However, knockdown of Kr-h1 an early gene in JH pathway and HPSE had no effect on oocyte growth, oocyte length was observed to be similar to the control (Fig. 3.5d). Knockdown of EcR and BR-C also decreased the length

of the primary oocyte (Fig. 3.5j and k). However, knockdown of ultraspiracle a partner of EcR as well as knockdown of Shade and Phantom showed the development of primary oocyte similar to that observed in control insects. Knockdown of transcription factor MafB a homolog of *Drosophila* Traffic jam (Tj) reduced the development of primary oocyte (Fig. 3.5l). Knockdown of nutritional signaling genes, transcription factor GATAa reduced oocyte length (Fig. 3.5m). However, InR2 (Fig. 3.5n), mTOR, cTOR, InR1, ILP 1 and ILP 2 knockdown insects showed primary oocyte development similar to that in control. Primary oocyte development in insects showing knockdown of Stripe or Stubble mRNA, was similar to that observed in control. Member of RNAi pathway Ago1 knockdown reduced the length of the primary oocyte (Fig. 3.5o) whereas Dicer1 knockdown had no effect.

Expression analysis of genes in the fat body and ovary: Bed bug fat body produces three Vg mRNAs namely *Vg1*, *Vg2* and *Vg3*. The expression patterns of all the *Vg* mRNAs were found to be similar (Fig. 3.6). *Vg* mRNA was detected only in the fat body. Feeding and mating of the female adult bugs induced expression of *Vg* gene. *Vg* mRNA reached a peak on day 2. Only feeding also induced *Vg* mRNA but the levels are lower as compared to that in fed and mated insects. No increase in expression of Vg mRNA was detected in the insects only mated but not fed.

The mRNA of JH receptor Met was detected in both fat body and ovary. Met mRNA levels increased from day 0 to the maxim levels on day 3 in the fat body as well as in the ovary of the female bed bugs that were fed and mated (Fig. 3.6). Only feeding also increase Met expression in the fat body (Fig. 3.6). Only mating or no feeding and no mating did not show much difference in the expression of Met mRNA over time. Kr-h1 is expressed mainly in the ovaries. Kr-h1 expression increased on day two after feeding and mating. In ovaries, Kr-h1 expression peaked on day two. Only feeding and no mating as well as no mating groups did not show an increase in Kr-h1 expression in fat body or ovaries. Only mating group induced Kr-h1 gene expression in the ovaries but not in the fat body (Fig. 3.6). Expression of forkhead binding protein L2 was detected in the ovaries only. Foxl2 expression was induced in day 2 fed and mated insects but not in only fed or only mated insects. (Fig. 3.6). HPSE mRNA is detected only in the ovaries. HPSE expression increased in the ovaries after feeding and mating. No induction of HPSE mRNA

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was observed in only feeding, or only mating, or no feeding and no mating groups (Fig. 3.6).

Ecdysone receptor EcR mRNA showed an average of 6-fold higher expression in the ovaries in all treatment groups as compared to its levels in the fat body (Fig. 3.6). Ovaries constitutively expressed EcR. USP a heterodimer of EcR in the ecdysone action showed expression in both ovaries and fat body (Fig. S2.3). HR3, an early gene in the ecdysone pathway was expressed in all the groups tested at about 7-fold higher in the ovaries as compared to the fat body (Fig. 3.6). HR3 expression increased in the ovaries on day 3 after feeding and mating. HR3 expression remained unchanged in other treatment groups.

Expression analysis of genes involved in nutritional signaling showed that ILP1 is constitutively expressed in both the tissues in all the treatment groups (Fig. 3.6). However, ILP2 showed expression pattern similar to Vg. ILP2 expression peaked on day 2 and day 3 in the fat body dissected from fed and mated bugs. Only feeding was able to induce ILP2 in day 2 fat body. No expression of ILP2 was observed in the ovary (Fig. 3.6).

Unclassified cuticle protein 1 (UCP1) mRNA also showed expression pattern similar to that of Vg. UCP1 was expressed higher in the fat body/epidermis and no expression was observed in the ovaries. UCP1 was later confirmed to be expressed by epidermis only (Fig. S2.4). UCP1 expression increased on day two after feeding and mating. Only feeding was also able to increase UCP1 expression however only mating and no mating and no feeding did not show any increase in UCP1 mRNA expression. Transcription factor MafB was found to be constitutively expressed in all treatment groups in the ovary. However, MafB expression increased in the fat body of the blood-fed and mated insects and reached the maximum levels on day 2 and day 3 (Fig. 3.5). The fat body of other treatment groups expressed MafB mRNA at lower levels as compared to that observed in feeding and mating groups. Expression analysis of other genes in whole body and tissues were also recorded (Fig. S2.5).

JH Regulation of reproduction

Effect of Methoprene on gene expression: Methoprene was applied on blood fed virgin female bugs. Ten µg of methoprene was applied at every 24 h interval and the samples were collected at 48 h and 72 h. Kr-h1, Foxl2 and HPSE were induced by 2.7, 4

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and 685-fold in the whole body of methoprene treated insects. ILP2 and Vg1 showed a three-fold increase in the fat body tissue. An expression of Hairy, a JH-inducible gene in the mosquito,- was unaffected by methoprene application. HR3 an ecdysone-induced early gene showed a two-fold decrease in expression in the ovaries (Fig. 3.7).

*Regulation of Transcription factors downstream to Met/SRC complex***:** To determine the relationship between Foxl2 and Kr-h1, the two transcription factors from the forkhead domain family and the zinc finger transcription factor family, knockdown of Met, SRC, Kr-h1 and Foxl2 was performed, and their effect was studied on the expression of Kr-h1 and Foxl2. Knockdown of Met, SRC and Foxl2 reduced Foxl2 expression to 19%, 44%, and 52%, respectively (Fig. 3.8). A knockdown of Kr-h1 had no effect on Foxl2 expression, suggesting that Foxl2 is expressed downstream to Met/SRC complex. Knockdown of Met and Kr-h1 reduced Kr-h1 expression to 22% and 21%, respectively (Fig. 3.8). However, knockdown of SRC and Foxl2 had no effect on Kr-h1 expression. These data suggest that Foxl2 is directly regulated by Met/SRC and Kr-h1 is not involved in its expression. Also, neither Foxl2 nor SRC are required for Kr-h1 expression.

*Foxl2 regulates oocyte development through methoprene***:** Methoprene applied at 10 μg each on the blood-fed unmated female bugs at 24 h intervals increased oocyte length (Fig. 3.9a) as compared to the control insects treated with acetone (Fig. 3.9b). The increase in the length of the primary oocyte with the methoprene application was similar to that observed in insects that were fed and mated (Fig. 3.9c), suggesting that mating and feeding induce oocyte maturation through JH. Oocyte growth was reduced in the blood-fed unmated females injected with Foxl2 dsRNA and treated with methoprene (Fig. 3.9d) suggesting that Foxl2 may mediate methoprene action in oocyte maturation. Knockdown of Kr-h1 in virgin blood fed females showed oocyte development similar to that observed in blood fed females applied treated with methoprene (Fig. 3.9e). Knockdown of Met (Fig. 3.9f) and SRC (Fig. 3.9g) in blood fed virgin adult females applied with methoprene showed a severe reduction in oocyte maturation. This suggests methoprene regulates oocyte development through Foxl2 but independent of Kr-h1.

Regulation of genes downstream to transcription factor MafB: MafB is expressed constitutively in the ovaries (Fig. 3.5). Knockdown of MafB significantly reduced its expression to 13% and reduced Foxl2 expression to 10% and HR3 expression

to 26%. Knockdown also resulted in an increased expression of cell adhesion mRNA, Neurotactin (Nrt) to 236% and DE-cadherin (DECad) to 197% (Fig. 3.10). Knockdown of Foxl2 significantly reduced the expression of Foxl2 and HR3 to 49% and 69%, respectively. Knockdown of Foxl2 did not affect the expression of MafB (Fig. 3.10) or any cell adhesion molecules (Fig. S2.6). EcR/USP knockdown had no effect on Nrt, DECad, MafB, and Foxl2 expression (Fig. S2.7). Neither did MafB knockdown had any effect on expression of EcR/USP (Fig. S2.8).

Expression pattern of genes required for embryonic development: RNAi pathway genes, Dicer1 and Ago1 showed a four-fold increase in expression in the female adults at two days after feeding and mating as compared to its expression in the eggs soon after oviposition (Fig. 3.11). These genes showed a two-fold increase in expression from 0-24 h of embryonic development. Nutritional pathway genes InR2, mTOR and cTOR that effect embryonic development are also expressed three-fold higher in the female adult as compared to that in the eggs soon after oviposition. mRNA levels of InR2 did not change with time during embryogenesis. The mRNA levels of mTOR and cTOR increased by two-fold from 0-24 h in the embryo. Expression these genes did not change with time. Cuticle protein UCP1 is expressed at nine-fold higher in the 0 h embryo as compared to the in the adult. UCP1 expression did not change in 0-24 h embryos. Laccase 2 and kkv genes also are involved in cuticle synthesis showed a two- and four-fold higher expression in the adult as compared to in the eggs soon after oviposition. These genes also did not show a change in expression pattern in 0-24 h embryos. Zinc finger-containing proteins, CLEC005028, Kr-h1 and stripe showed two-, 640- and 13- fold higher expression in the female adult as compared to that in the eggs soon after oviposition. Expression of CLEC005028 showed a 2.5 fold increase from 0–24 h, Kr-h1 mRNA expression did not show any change in expression in 0-24 h embryos. Expression of Stripe mRNA increased 20-fold from 0–24 h in the embryo. HPSE showed a 1000-fold higher expression in the female adults as compared to that in the eggs soon after oviposition. HPSE expression did not change over time in 0-24 h embryos (Fig. 3.11).

Discussion

Reproduction in insects have been studied in many insects including hemipteran insects. Both JH and 20E regulate reproduction. Nutrition and JH play important roles in

regulation of reproduction in the hemipteran insect, brown planthopper, *Nilaparvata lugens*. Vg expression is stimulated by the presence of amino acids and JH. JH is known to bind to its receptor Met to induce Vg expression in the fat body (Lu et al., 2016). Vg is secreted into the hemolymph and deposited into the developing oocyte. JH also regulates the uptake of Vg and development of oocytes as shown in *Diploptera punctata* (Marchal et al., 2014). In this insect, ovaries at a particular stage of development produce a peptidergic ovarian factor that stimulates corpora allata to produce JH (Elliott et al., 2006). Met has been known to transduce its effect through its downstream gene Kr-h1. In the migratory desert locust, *Locusta migratoria.* RNAi-mediated knockdown of Kr-h1 decreased Vg expression and oocyte development (Song et al., 2014). JH also plays an essential role in reproduction in holometabolous insect such as *T. castaneum*. JH and nutrients induce Vg expression (Sheng et al., 2011) through the insulin pathway. JH induces ILP2/3 in these insects which finally effect subcellular localization of FOXO, a negative regulator of Vg. In *T. castaneum* 20E has a more direct effect on oocyte maturation. Knockdown of EcR, USP and ecdysteroid synthesizing enzymes has been shown to effect oocyte development. Met knockdown also showed a decrease in oocyte development in these insects (Parthasarathy and Palli, 2011). In mosquito *Aedes aegypti,* blood meal induces secretion of ecdysone by the ovaries. Blood meal also increases the concentration of amino acids and signaling peptide-like insulin in the mosquito which also triggers Vg expression in the fat body through the TOR pathway (Attardo et al., 2006; Raikhel, 2005; Roy et al., 2007). Transcription factors, GATAa and BR-C have been shown to directly interact with the Vg promoter to induce its expression (Park et al., 2006). The Hi-Seq analysis by Illumina sequencing have revealed many differentially expressed genes including cathepsin b, vitellogenin, and vitellogenin carboxypeptidase (Price et al., 2011). Through knockdown studies, the role of nutrition and hormonal signaling in the regulation of reproduction in bed bugs was identified. A detailed study on the differential expression of genes has shown the role of methoprene-tolerant protein, transcription factors and zinc finger proteins in the reproduction of bed bugs.

In bed bugs, Met regulates Kr-h1, Foxl2 and HPSE, which are involved in reproduction. Kr-h1 is a zinc finger transcription factor. Kr-h1 effects embryogenesis in *Drosophila*. Kr-h1 is expressed at 8-10 h after fertilization in the nervous system during

the embryonic development (Beck et al., 2004). Role of Kr-h1 has been well studied in metamorphosis of insects. Kr-h1 is an early gene in JH pathway which is known to be transcribed by Met/SRC complex in the presence of JH (Kayukawa et al., 2012). Transcription factor Foxl2 was identified for the first time to take part in oocyte development through JH in insects. In humans, Foxl2 has emerged as a major player in ovary development in humans (Uhlenhaut et al., 2009). In mice, Foxl2 suppresses Sox9 hence suppressing the male characters. Sox9 belongs to the HMG-box transcription factors located on the autosomal chromosome. Sox9 deletion in bipotential gonad results causes the development of ovaries (Barrionuevo et al., 2006) in XY mice. Deletion of Foxl2 in mice reprograms the granulosa cells to Sertoli cell and theca cell into Leydig-like cells in females and increases testosterone production (Uhlenhaut et al., 2009). HPSE was also identified for the first time as a JH regulated gene. Heparanase is an endoglycosidase, it acts on Heparan sulfate (HS). Heparan sulfate regulate concentration of signaling molecules through changes induced in its fine structure through sulfation of HS (Kleinschmit et al., 2010). Heparan sulfate binds to growth factors hence regulating signaling (Kim et al., 2011; Lin et al., 1999) and also possibly through diffusion. In humans, it confers invasive phenotype to tumor cells (Vlodavsky et al., 2007). In *Drosophila,* mutations in the sister of tout velu (sotv), or Tout velu (Ttv) co-polymerase that synthesizes HSPG glycosaminoglycan (GAG) chains reduces the levels of heparin sulfate. Loss of HSPG impairs Hh, Wg and (Decapentaplegic) Dpp signaling causing developmental defects (Bornemann et al., 2004). Zinc finger proteins Stripe and CLEC005028 were identified to effect embryonic development in bed bugs. Zinc finger protein often interacts with DNA, RNA, regulates protein folding and assembly, apoptosis and lipid binding (Laity et al., 2001). The protein stripe contains three zinc finger domains. The motif is homologous to the DNA-binding domain encoded by members of the early growth response gene family. In *Drosophila* strip gene is responsible for normal muscle development (Lee et al., 1995). Mutation in this gene affects embryonic development and is lethal (Volk and VijayRaghavan, 1994). Mutations in stripe also cause defects in adult muscle development (de la Pompa et al., 1989). Transcription factor MafB was also identified to effect reproduction through various pathways in different tissues. In *Drosophila* Tj homolog of MafB is expressed in somatic gonadal cells (SGCs) independent

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of the germline. MafB is expressed both in testis and ovary of *Drosophila*. In testis MafB mutants show defects in fusome formation (Li et al., 2003). Tj mutant ovaries show few or no germ cells. *Drosophila* traffic jam acts as a transcriptional repressor of several cell adhesion molecules Fasciclin-III (Fas3), DEcad and Nrt in the ovary. Tj knockdown increases expression of these cell adhesion molecules which results in the improper interaction of germ cells with the somatic cells thus repressing the proper development of germ cells (Li et al., 2003). Other genes effecting embryonic development include Stubble and those coding for cuticular proteins. Gene stubble (CLEC002263) also known as *Stubble-stubbloid* (*Sb-sbd*) encodes a type II transmembrane serine protease. In *Drosophila,* it is involved in bristle and leg morphogenesis (Hammonds and Fristrom, 2006) and required for ecdysone-regulated epithelial morphogenesis of imaginal tissue during metamorphosis (Appel et al., 1993; Beaton et al., 1988). Cuticle synthesizing genes that effect embryonic development include kkv, UCP1, and laccase2. Kkv gene encodes a chitin synthase enzyme (CS-1). CS-1 catalyzes the last step in chitin synthesis (Araujo et al., 2005). CS-1 has been shown to be important in tracheal development in *Drosophila* embryo (Araujo et al., 2005; Moussian et al., 2005). Tracheal development starts at stage 11 of embryogenesis in *Drosophila*. Chitin is also essential for procuticle integrity, an intact procuticle stabilizes the chitin less epicuticle. Kkv mutants also show impairment in sclerotization and pigmentation of cuticle (Moussian et al., 2005). Laccase 2 is a phenol oxidase (Arakane et al., 2005) which is involved in tanning renders the cuticle hard, dark and water insoluble. Laccase 2 knockdown in *Tribolium* through RNAi in larvae, pupae and adults inhibited cuticle tanning, subsequently caused deformities and led to eventual death (Arakane et al., 2005).

qRT-PCR analysis have identified the role of nutrition or blood feeding and mating in reproduction. Nutrients induce a cascade of gene expression events that trigger Vg expression in the fat body and the development of primary oocyte (Fig. 3.6 and Fig. 3.5a-e). Blood meal provides the necessary nutrients including amino acids essential to initiate reproduction. Knockdown of TOR or Insulin pathway genes did not affect Vg expression or primary oocyte development. However mTOR, cTOR or InR2 knockdown reduced fecundity and embryonic development. GATAa knockdown reduced the expression of Vg and primary oocyte maturation and hence severely affected fecundity (Fig. 3.4). Both JH

and ecdysone were found to regulate reproduction. High expression of EcR and its early gene HR3 in the ovaries as compared to the fat body showed that these genes have a major role in ovaries (Horner et al., 1995). EcR knockdown suppressed fecundity whereas, knockdown of its heterodimeric partner, USP decreased fecundity to only 60%. Knockdown of ecdysone synthesizing enzymes had no effect on fecundity. No hatching was observed in these eggs. Partial knockdown of ecdysone synthesizing enzymes could explain this effect. EcR had a more sever effect than USP on fecundity showing that EcR might also act independent to USP in regulating genes important for oocyte maturation and fecundity. BR-C knockdown also reduced the fecundity in bed bugs.

JH was found to the major hormone regulating reproduction as its action is required in both fat body and ovaries. JH regulates Vg expression in the fat body through its receptor Met and co-activator, SRC. Transcription factor MafB was also found to regulate Vg expression. JH-inducible genes that affected reproduction include Kr-h1, Vg, Foxl2 and HPSE (Fig. 3.12). Decrease in Vg mRNA through knockdown of genes that effect Vg expression (MafB, Met, SRC, GATAa or Vg) reduced oocyte development and fecundity suggesting that Vg is essential for oocyte development. However JH regulates fecundity through more than one way. Met knockdown might also affected patency (Lu et al., 2016) or uptake of Vg by developing oocyte. Met/SRC complex also affects oocyte development through Foxl2. As application of JH analogue methoprene to Foxl2 knockdown insects failed to rescue oocyte development. Transcription factor Foxl2 also regulates HR3 expression. Hence, Foxl2 provides a link between JH and ecdysone pathway during regulation of ovaries. Expression analysis shows that Foxl2 is expressed predominantly in both female and male reproductive organs (Fig. S2.9). Knockdown studies to elucidate the JH pathway also showed that Foxl2 acts downstream to Met/SRC but independent of Kr-h1 an early gene in the JH pathway. Also, Kr-h1 expression was found to be independent of SRC as opposed to what has been shown in other insects (Zhang et al., 2011). HPSE expression is regulated by both Met and SRC (Fig. S2.10). Ovaries also express HPSE another JH-inducible gene during reproduction of the insect. Both Foxl2 and HPSE were found to be induced during reproduction. Unlike Foxl2, HPSE knockdown did not have any significant effect on fecundity or oocyte development. However, HPSE knockdown completely suppressed embryonic development. HPSE was found to be expressed

maternally as its expression was found to be 1000 fold higher in the adult as compared to the zero hour embryo. HPSE knockdown might have affected signaling in the follicle cells or the developing embryo by regulating heparan sulfate levels hence indirectly suppressing embryonic development. Kr-h1 a zinc finger transcription factor also affected embryonic development. In *Drosophila* Kr-h1 is expressed in the embryonic nervous system. Embryogenesis in bed bugs takes 4-5 days as compared to *Drosophila* which takes 22-23 h. Hence, Kr-h1 might be expressed at a later stage in the bed bug embryo. This experiment also showed that knockdown effect in the mother can be transferred to the progeny. However, tracking of P^{32} labeled radioactive dsGFP, we were not able to detect siRNA in adults or egg possibly due to sensitivity issues (Fig. S2.11). Zinc finger containing protein stripe showed an increase in expression by 20 fold in the embryo from zero to 24 h. This shows it's role in the early stage of the embryonic development. Only one large Maf Transcription factor, MafB was identified in the bed bug genome. Bed bug large Maf is homologous to the large Maf factor, traffic jam in *Drosophila* and MafB in *Homo sapiens*. MafB is constitutively expressed in the ovaries and its expression increased in the fat body during reproduction. MafB regulated reproduction by affecting oocyte development and Vg expression. MafB knockdown induced expression of cell adhesion molecules DECad and Nrt. Hence causing improper interaction of the developing oocyte with the follicle cells (Li et al., 2003). MafB also induces Foxl2 expression as was shown by knockdown studies. Analysis of promoter sequence of Foxl2 and Vg showed MafB binding site in both *Vg* and Foxl2 promoter region. MafB regulates *Vg* expression in the fat body. Lack of Vg protein also reduced the development of primary oocyte in the bed bug. The role of MafB in regulating Vg expression in insects or arthropods has not yet been documented. Stubble knockdown in bed bugs showed no effect. The development of legs in the holometabolous insects starts from an imaginal disc which gives rise to legs and wings. The imaginal disc has a role in the holometabolous insects during metamorphosis. In the hemimetabolous insects, like bed bugs these structures are produced during embryogenesis. Knockdown of Stubble in bed bug during the adult stage did not decrease fecundity or hatching as compared to the control. Leg development takes place during an embryonic stage in the hemimetabolous insects, unlike in the holometabolous insects. This difference in development could explain the lack of phenotype in bed bugs. Knockdown of cuticular

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genes did not affect fecundity but embryonic development was suppressed. Expression analysis of zero-day old embryo and mother two days after feeding and mating using qRT-PCR showed two-fold higher expression in the embryo as compared to the adult. Hence, Kkv transcripts might be synthesized in the embryo unlike the mmy transcripts which are synthesized by the mother and deposited into the embryo. The embryonic lethality could be attributed to defects in procuticle formation. UCP1 (CLEC006545) did not show any homology with *Drosophila* genes. NCBI conserved protein domain database does not show the presence of any conserved domains. UCP1 mRNA expression increases in the whole body from 0 - 24 h after feeding and mating. UCP1 was shown to be expressed in epidermis only (Fig. S2.4). mRNA expression of UCP1 in adult and zero hour embryo showed 9 - fold higher expression in the embryos as compared to the adult suggesting UCP1 like chitin synthesizing gene mummy (Araujo et al., 2005) is deposited in the embryo by the mother. mRNA is deposited in the embryo by the mother. Laccase2 knockdown in adult might have affected the strength of the cuticle which could have resulted in death of the embryo possibly in the late stage of embryonic development.

Through this study we have shown JH action pathway genes regulate processes affecting vitellogenesis, oogenesis and embryogenesis in bed bugs. Figure 3.12 summaries all the genes and the different steps in reproduction they effect. Transcription factor MafB has also emerged as a central factor regulating various aspects of reproduction. This study hence lays a foundation for a detailed study on reproduction in bed bugs for developing new strategies for their control.

Figures:

(a)
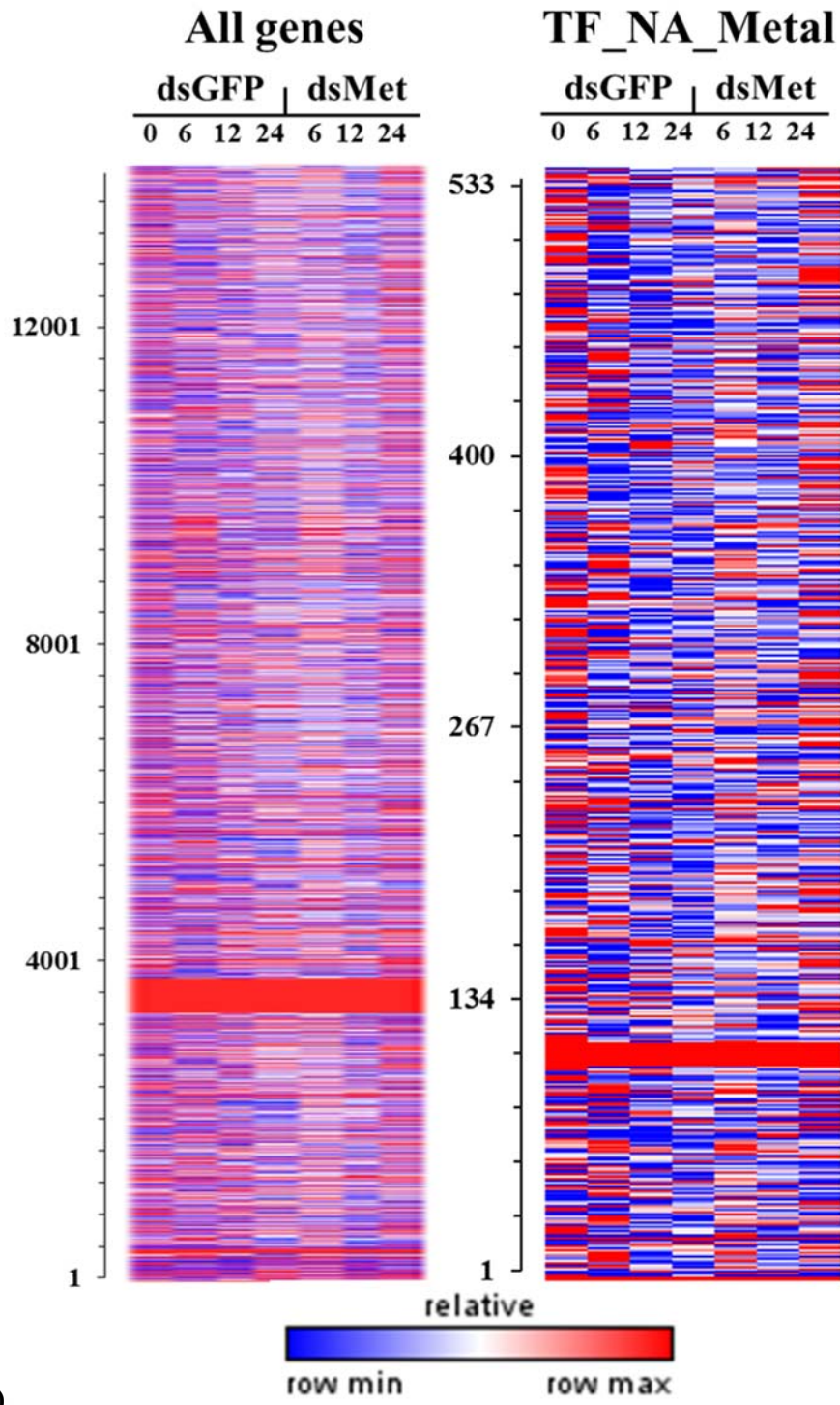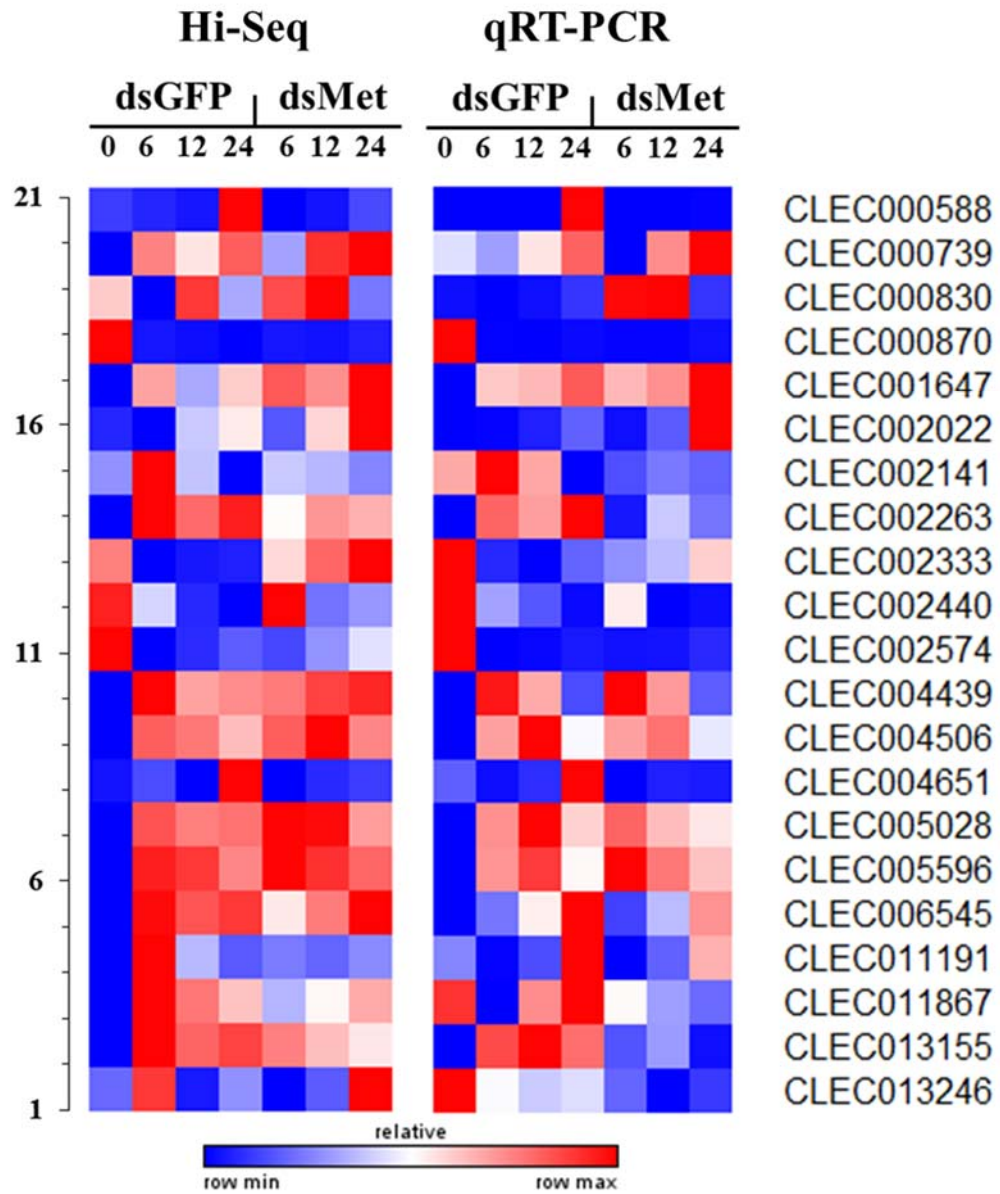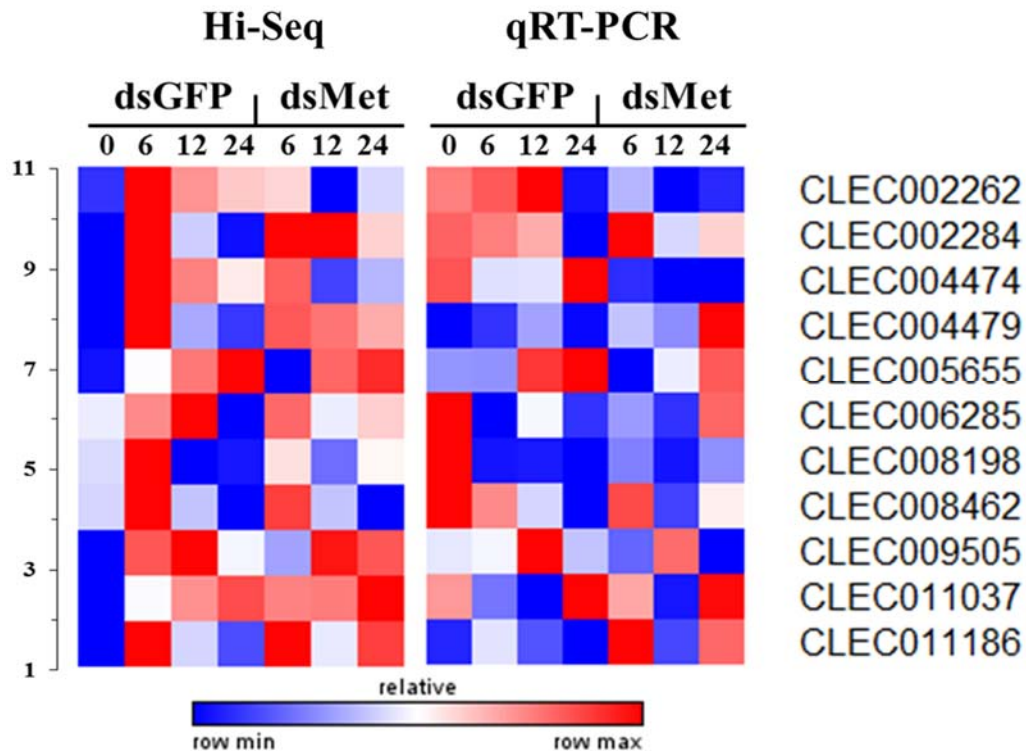
(c)

Figure 3.1. Differential expression of gene expression during first 24 h after feeding and mating in the bed bug female: (a) The heat map on the left shows changes in expression of 13953 genes from 0-24 h after feeding and mating in control insects injected with GFP dsRNA and 6-24 h after feeding and mating in insects injected with GFP dsRNA. The differential expression analysis was performed by sequencing RNA isolated from staged insects followed by alignment of sequence reads to the bed bug reference genome and cuffdiff (Trapnell et al., 2013) to calculate FPKM expression. GENE-E (broadinstitute.org) was used to create a heat map using FPKM expression values. The heat map on the right shows the differential expression of genes containing transcription factor Pfam motifs and genes containing GO terms with nucleic acid and metal binding. **(b)** 21 genes were selected based on fold increase or decrease in expression from all gene heat map shown in 1(a) and the expression of these was verified by qRT-PCR **(c)** 11 genes were selected from the hi-seq expression analysis. These genes contain transcription factor domain (Pfam), and GO terms for nucleic acid (GO: 0003676) and metal binding (GO: 0046872). The expression of these genes was verified using qRT-PCR.
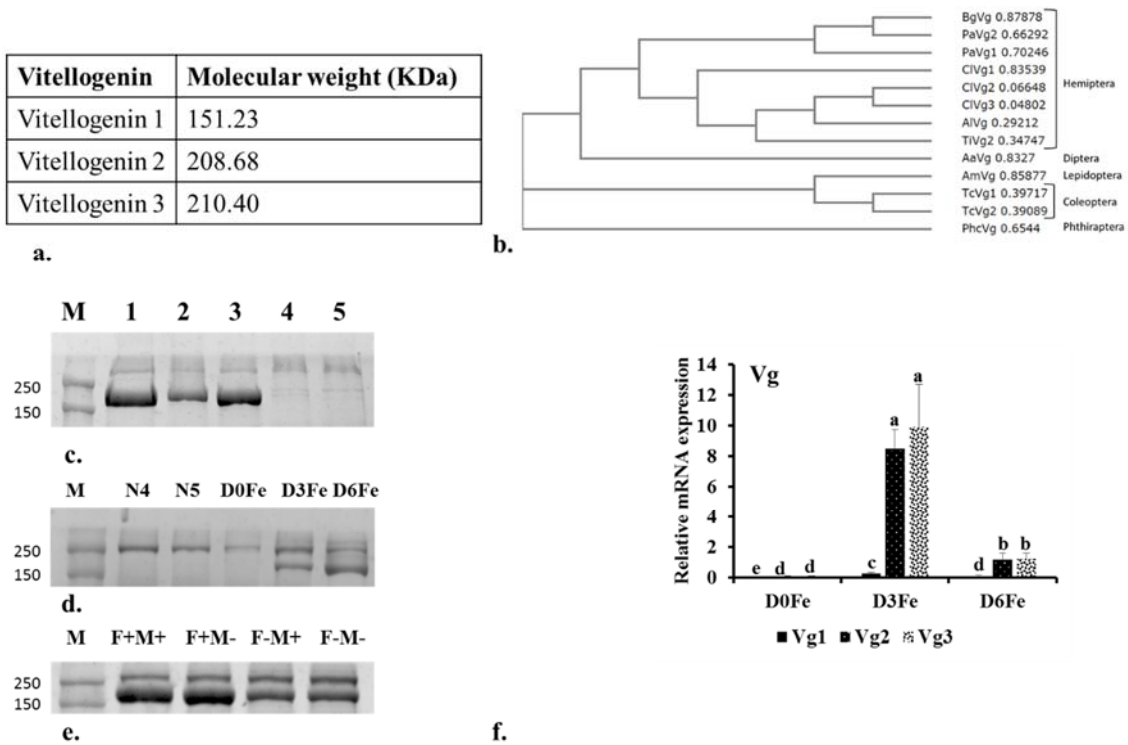
Figure 3.2. **Vitellogenin gene expression profile in bed bugs:** Expected molecular weight of vitellogenin isoforms Vg1, Vg2 and Vg3 in bed bugs as determined by ExPASy Compute pI/Mw tool (a). The phylogenetic tree shows a comparison of Vg protein sequence from different insects. Bg- *Blattella germanica*; Pa- *Periplaneta americana*; Cl- *Cimex lectularius*; Al- *Apolygus lucorum;* Ti- *Triatoma infestans;* Aa- Aedes aegypti; Am- *Apis mellifera*; Tc- *Tribolium castaneum*; Phc- *Pediculus humanus corporis*. The tree was created with clustal W with default settings (b). 10μg of total hemolymph protein from knockdown insects was loaded on the gel three days after feeding and mating of insect. Lanes shown are proteins from Marker (M), dsGFP (1), dsVg1/2 (2), dsVg1/3 (3), dsVg2/3 (4), dsVg1/2/3 (5) treated insects (c). Vg protein expression profile in the hemolymph of day two 4th instar (N4), day three 5th instar (N5), adult female at day 0 after emergence (D0Fe), adult female three days after emergence (D3Fe) and adult female 6 days after emergence (D6Fe). The single band at 200 KDa was identified as Vg protein (d). Four treatments were provided to the female bed bug F+M+ (feeding and mating), F+M- (only feeding), F-M+ (only mating), and F-M- (no feeding no mating). 10μg of total hemolymph protein was loaded on the gel three days after the treatment. After separation gel was

stained with coomassie brilliant blue stain (e). Total RNA was collected at day 0, day 3, and day 6 after emergence. qRT-PCR was performed to check expression of Vg. Expression was normalized using ribosomal protein L8 (rpl8). Each bar represents mean and SD (n=3).
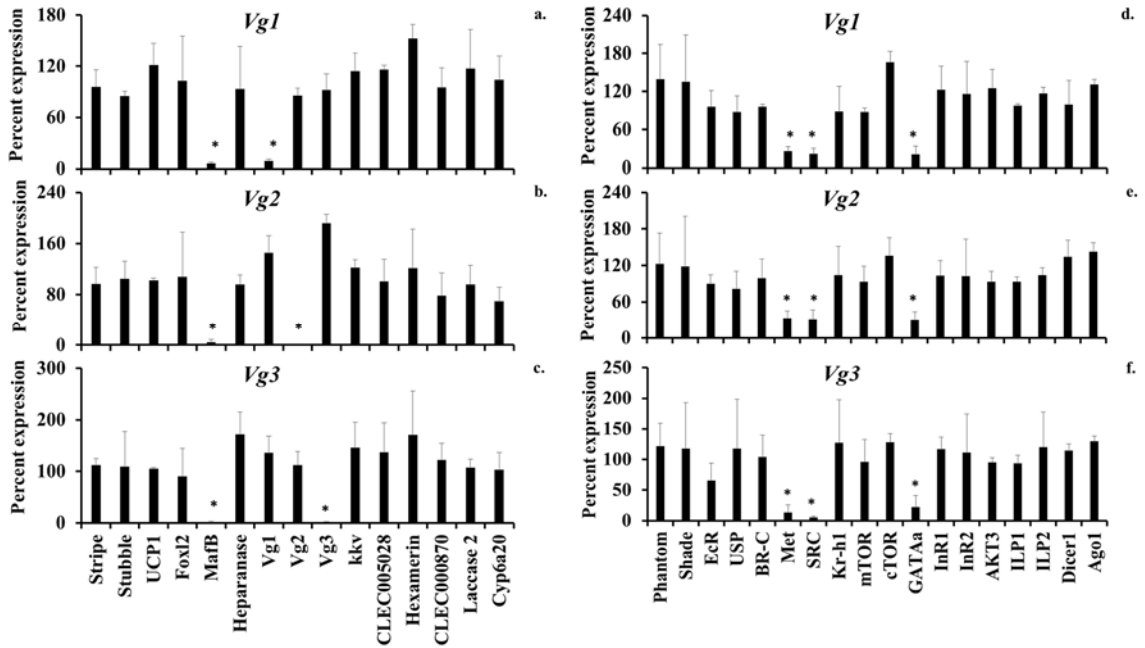
Figure 3.3. Percent vitellogenin expression in knockdown insects: Expression of all three isoforms of Vg mRNA is shown with the knockdown of genes selected from the sequencing experiment (a, b, and c): stripe, stubble, UCP1, Foxl2, MafB, HPSE, Vg1, Vg2, Vg3, kkv, CLEC005028, hexamerin, CLEC000870, laccase 2, Cyp6a20. To study the role of hormones and nutrition signaling in Vg expression knockdown of genes involved in the respected pathway were performed. Vg expression in phantom, shade, EcR, USP, Br, Met, SRC, Kr-h1, mTOR, cTOR, GATAa, InR1, InR2, AKT3, ILP1, ILP2, Dicer1 and Ago1 knockdown insects are shown (d, e, f). Knockdown was performed in virgin adult females. Insects were blood fed and allowed to mate for two days. After which total RNA was extracted and qRT-PCR was performed to check expression of Vg. mRNA expression were normalized with *ribosomal protein L8* (*rpl8*). Each bar represents the average and standard deviation of three biological replicates. Student t-test was performed (p≤0.05). All experiment were repeated twice.
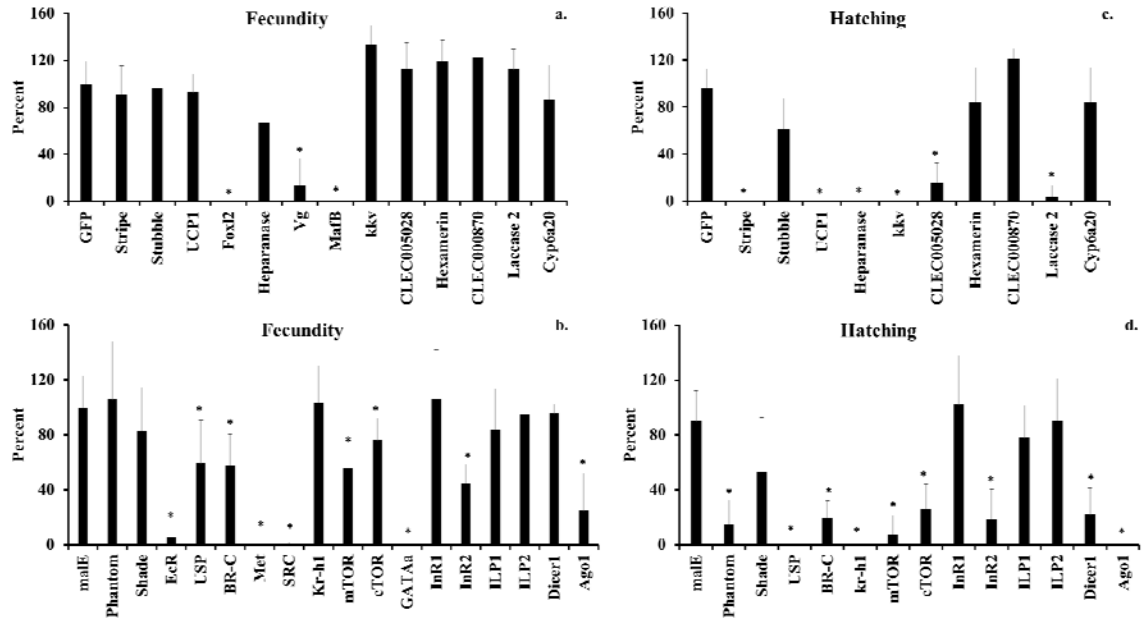
Figure 3.4. Percent fecundity and hatching in knockdown insects: Percent fecundity and hatching in Stripe, Stubble, unclassified cuticle protein 1 (UCP1), forkhead box protein L2 (Foxl2), HPSE, vitellogenin (Vg), MafB, kkv, CLEC005028, Hexamerin, CLEC000870, Laccase 2, Cyp6a20 (a and b) knockdown insects. Percent fecundity and hatching in hormonal and nutritional control of reproduction was studied with the knockdown of phantom, shade, ecdysone receptor (EcR), ultraspiracle (USP), broad complex (BR-C), methoprene-tolerant protein (Met), steroid receptor co-activator (SRC), Krüppel homologue 1 (Kr-h1), mammalian target of Rapamycin (mTOR), companion of TOR (cTOR), GATAa, insulin receptor 1 (InR1), insulin receptor 2 (InR2), insulin peptide 1 (ILP1), insulin peptide 2 (ILP2), Dicer1 and Ago1. Each bar represents percent values of average and standard deviation compared to the control. Knockdown was performed in virgin adult females. Both female and uninjected males were blood fed and allowed to mate for two days. Fecundity was recorded after 10 days and hatching after 17 days. Percent fecundity of control or (GFP/malE) insects were set at 100%. The experiment was repeated twice with n ≥ 10. Student t-test was performed (p≤0.05).
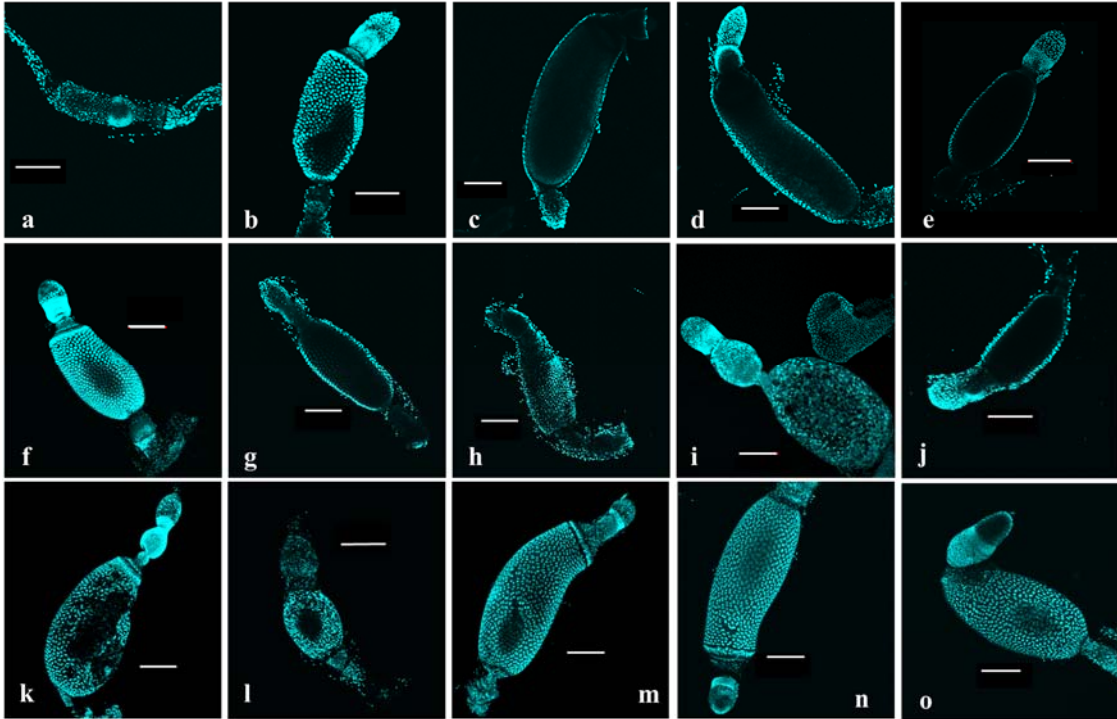
Figure 3.5. Primary oocyte development in control and knockdown insects: To study the effect of feeding and mating the insects were dissected and a representative oocyte was selected to record development. Confocal images show representative oocyte of control and treated insects fixed in 4% paraformaldehyde and stained with DAPI. Figs. a-d show the development of oocyte after feeding and mating on day 0, day1, day2, and day3. No development was observed when the insects were not mated and not fed or in the only mating group. Only mating also induced the length of the primary oocyte (Fig. e). The primary oocyte development three days after feeding and mating in insects with Vg (f), Met (g), SRC (h), Foxl2 (i), EcR (j), BR-C (k), MafB (l), GATAa (m), InR2 (n), and Ago1 (o) knockdown are shown.
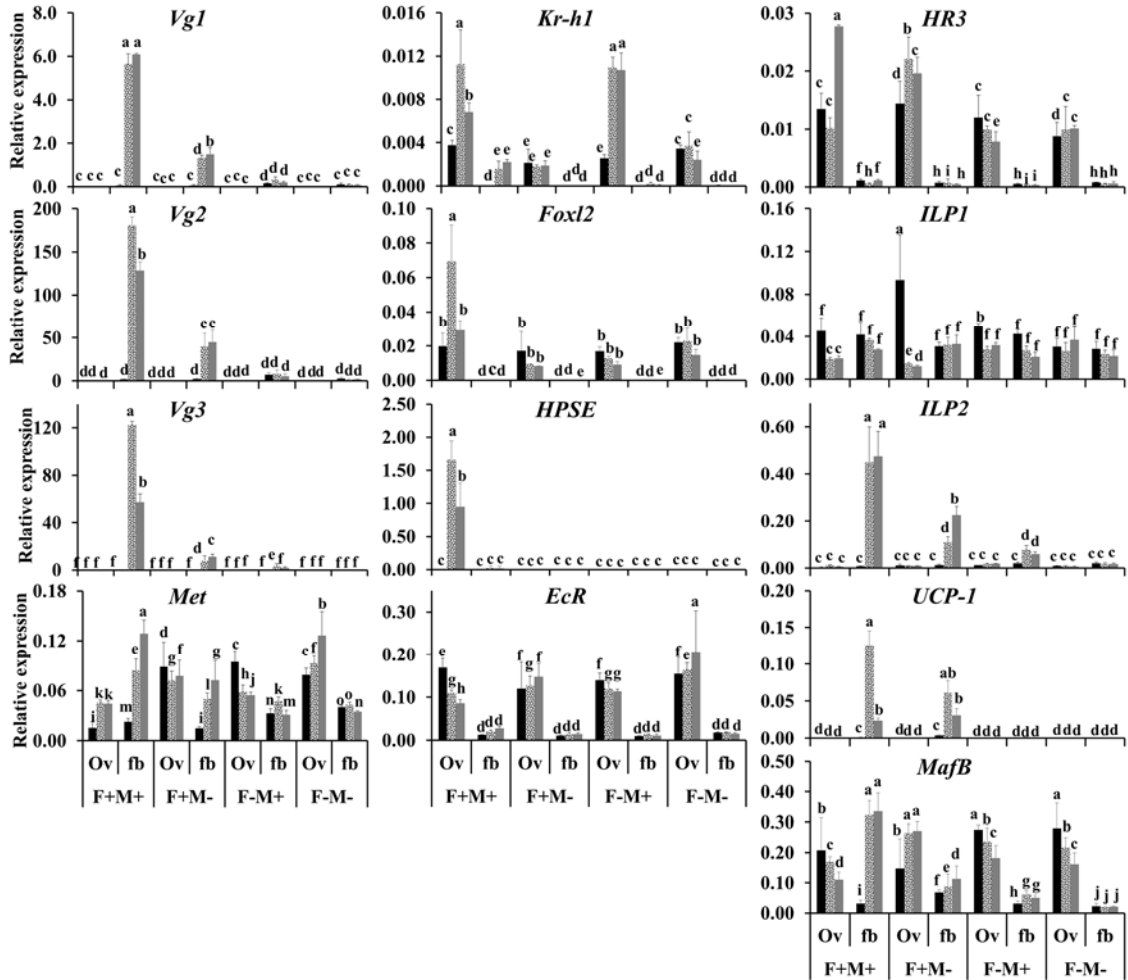
Figure 3.6. Expression analysis of genes in the ovary and fat body: Graphs shows expression profiles of genes at day 0, day 2 and day 3 in ovaries (ov) and fat body and epithelia (fb). X-axis shows treatment groups, tissue type and time day 0 (black), day 2 (pattern), day 3 (gray) and the y-axis shows the relative expression of the gene. Relative mRNA expression of *vitellogenin1 (Vg1)*, *vitellogenin2 (Vg2)*, *vitellogenin3 (Vg3)*, *methoprene-tolerant protein (Met)*, *krüppel homologue 1 (Kr-h1)*, *forkhead box protein L2 (Foxl2)*, *heparanase (HPSE)*, *ecdysone receptor (EcR)*, *hormone receptor 3 (HR3)*, *insulin peptide 1 (ILP1)*, *insulin peptide 2 (ILP2)*, *unclassified cuticle protein (UCP1)* and *MafB* was determined after normalization with *ribosomal protein 8 (rpl8)*. Virgin adult females were provided with four different treatments. F+M+ (fed and mated), F+M- (only fed), F-M+ (only mated), and F-M- (neither fed nor mated). Insects were dissected and total RNA was collected from ovaries and fat body (attached to abdominal cuticle). qRT-PCR was

performed to check expression of selected genes. Expression was normalized using expression of ribosomal protein l8 (rpl8). Data shown are mean + SD (n=3). (Alphabet represents significance at 95% CI).
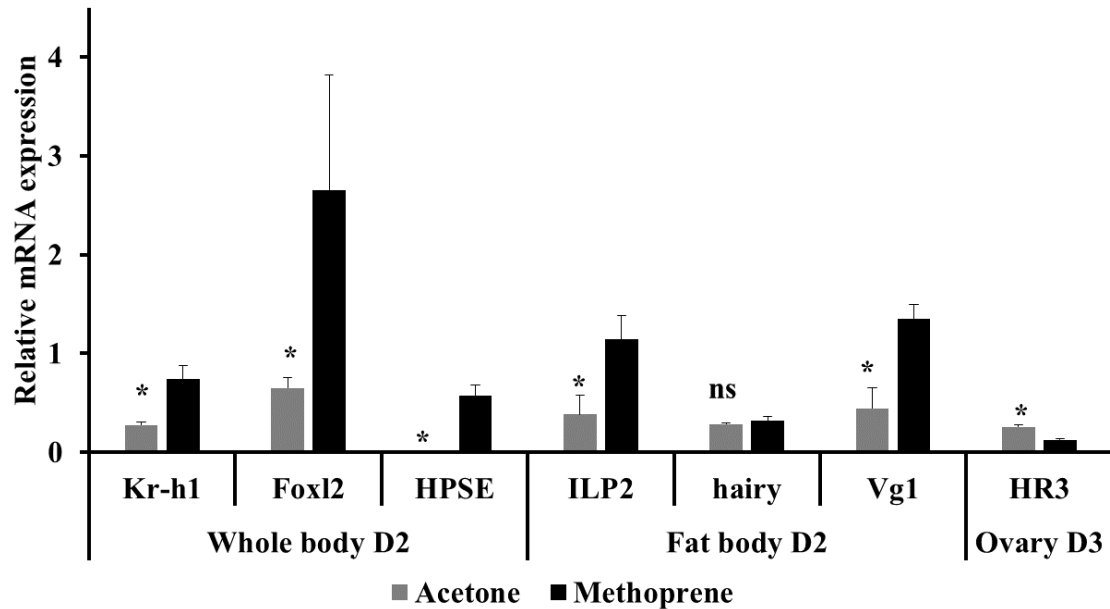
Figure 3.7 Effect of methoprene application on gene expression: Relative mRNA expression in whole body (day 2), fat body (day 2), and ovary (day 3) of krüppel homologue 1 (Kr-h1), Forkhead box protein L2 (Foxl2), heparanase (HPSE), Insulin peptide 2 (ILP2), hairy, Vitellogenin 1 (Vg1) and hormone receptor 3 (HR3) was recorded in methoprene (black) and control insects (grey). Virgin adult females were allowed to feed and 10 μg methoprene was topically applied on day 0, day 1, day 2 and day 3. qRT-PCR was performed to record an expression of genes. Expression was normalized using ribosomal protein L8 (*rl8*). Control insects were treated with the same volume of acetone. Each bar represents mean + SD; n = 3 ; p ≤ 0.05.
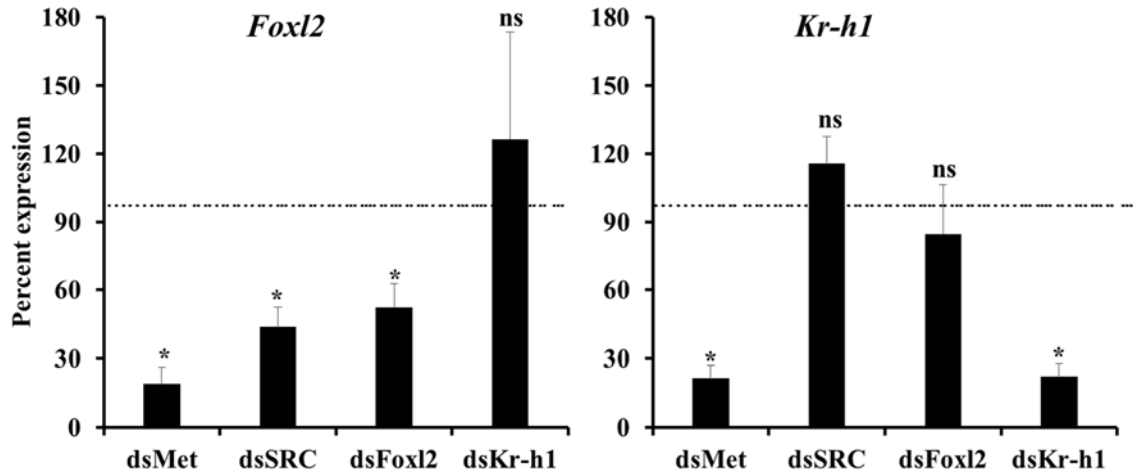
Figure 3.8. Foxl2 and Kr-h1 expression in knockdown insects: Expression of Foxl2 and Kr-h1 in Met, SRC, Foxl2 and Kr-h1 knockdown insects is shown. Knockdown was performed in virgin adult females. The insects were blood fed and allowed to mate for two days after which total RNA from whole body was collected. qRT-PCR was performed the expression was normalized with ribosomal protein l8 (*rpl8*). The experiment was repeated twice with similar results. Each bar represents average and SD of three biological replicates (p≤0.05).
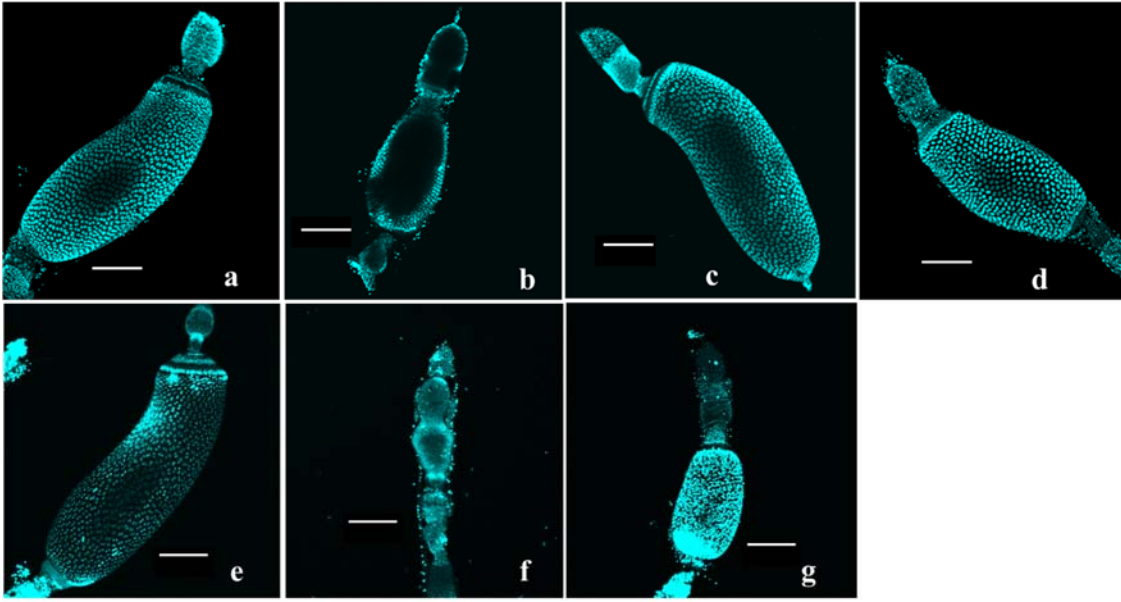
Figure 3.9. Methoprene regulates oocyte development through Foxl2: Primary oocyte development in GFP dsRNA injected insects applied with methoprene (a), insects applied with acetone (b), control insects which were blood fed and mated (c), foxl2 knockdown insects applied with methoprene (d), Kr-h1 knockdown insects applied with methoprene (e), Met knockdown insects applied with methoprene (f) and SRC knockdown insects applied with methoprene (g) are shown. Virgin adult females injected with dsRNA were blood fed and 10 µg methoprene was applied on day 0, day1 and day 2. Control insects were treated with the same volume of the acetone. Oocyte growth in blood fed and mated female bed bugs is shown for comparison.
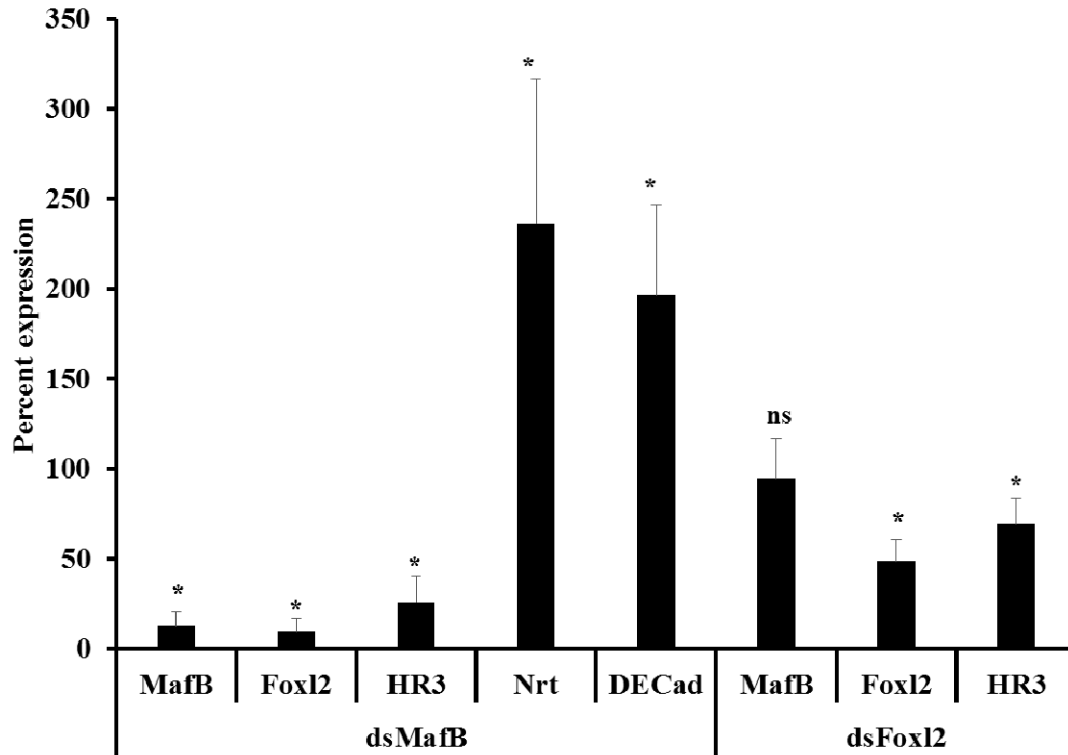
Figure 3.10. Regulation of genes downstream to MafB: Percent expression of genes in MafB and Foxl2 knockdown insects two days after feeding and mating. Percent expression of MafB, Foxl2, HR3, Nrt and DECad in MafB knockdown female adults; Percent expression of MafB, Foxl2 and HR3 in Foxl2 knockdown female adults. Total RNA was extracted from whole body samples. The experiment was repeated twice with similar results. Each bar represents average and SD of three biological replicates. ($p \leq 0.05$).
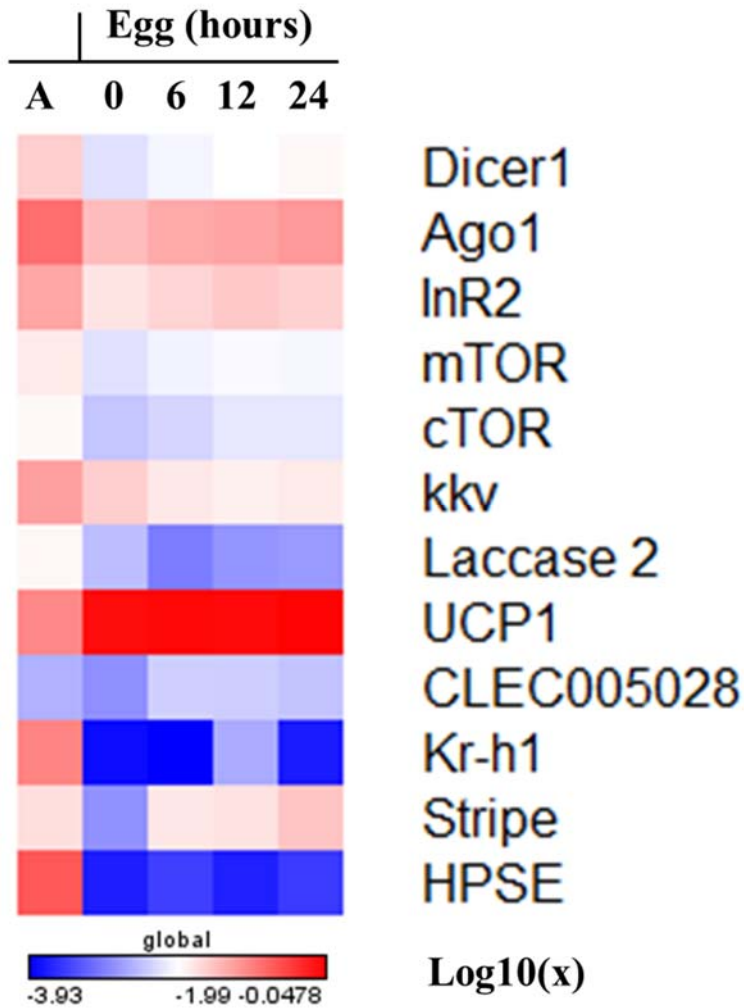
Figure 3.11. Expression profile of genes in embryo and adult female bed bug: Heat map showing relative expression of genes in adult females two days after feeding and mating, and at 0, 6, 12 and 24h after egg laying was calculated. Expression of Dicer1, Ago1, InR2, mTOR, cTOR, kkv, Laccase 2, UCP1, CLEC005028, Kr-h1, Stripe and HPSE was recorded. GENE-E was used to make heat map. The heat map shows log to the base 10 value of the relative expression.
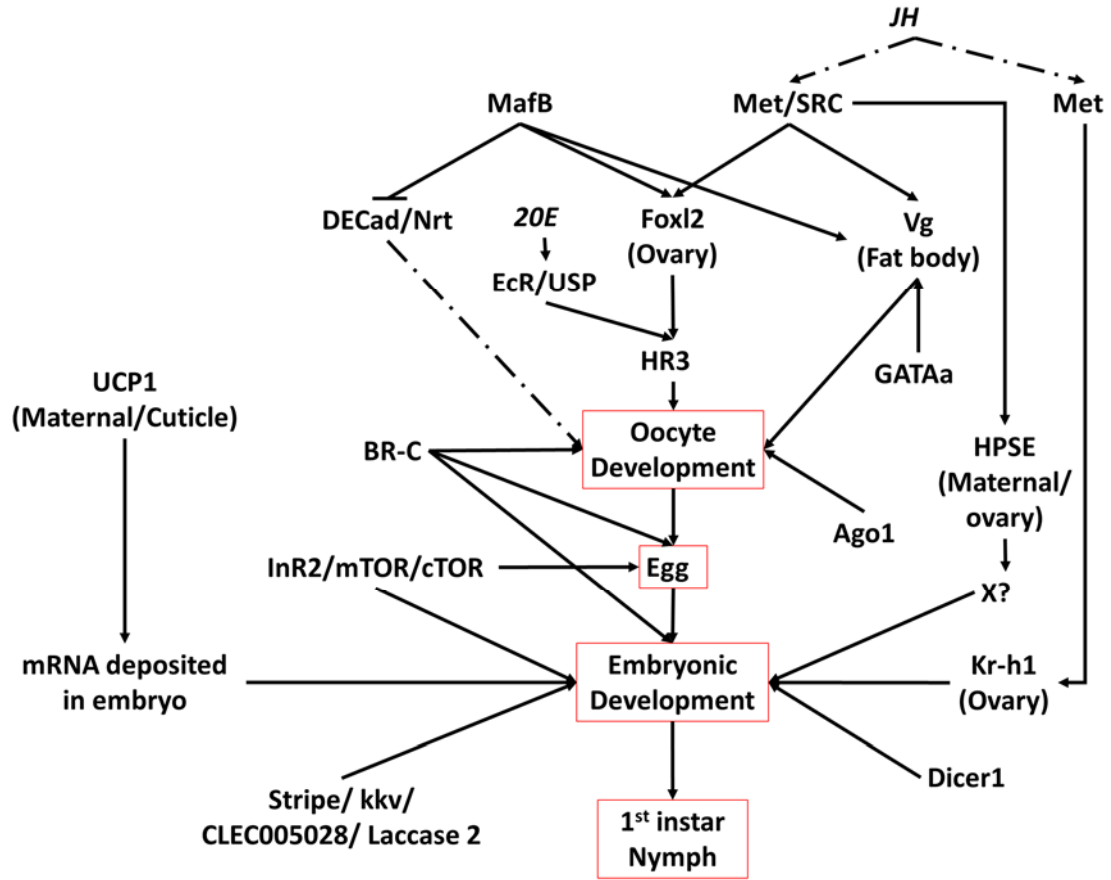
Figure 3.12. Summary of genes regulating reproduction in bed bugs: Schematic diagram of the interplay of various genes regulating ovarian as well as embryonic development in the bed bug. The pathways in bold lines represent the results from this study. Whereas the dotted lines represent the extrapolation of results from the literature. (Solid lines represent results from this experiment; dotted lines shows extrapolation from literature).

Chapter 4: The role of ABC transporters in development and resistance in the bed bug, *Cimex lectularius*

Introduction

ATP-binding cassette (ABC) transporters superfamily constitutes one of the largest ubiquitous families of protein membrane translocators in all the living species (Jones and George, 2004). They are involved in active transport of various substrates, either inside or outside, the cell membrane. Structurally, ABC transporter consists of the transmembrane domain (TMD) embedded in the lipid bilayer of the cell membrane and the nucleotide binding domain (NBD) in the cytoplasm connected through intracellular domain (ICD). ICD joins the membrane-spanning helices of ABC domain with that of NBD thereby providing communication between two (Rees et al., 2009). A functional ABC transporter consisting of two TMDs and two NBDs. All these four domains if present in one protein form a full transporter. In case, these are spread over many proteins; these are called half-transporter. In the case of latter, each domain of TMD and NBD form a homo-or heterodimer to be fully functional. Once the substrate binds on to TMD, it confers conformation changes in NBD that facilitate binding of two ATP within its dimer. This in turn leads to changes in TMD to allow expulsion of substrate outside. Later, ATP is hydrolyzed to provide energy for opening NBD dimer and returning of the TMD to its initial state (Andersen and Leevers, 2007; Linton, 2007). ABC transporters are involved in various physiological functions like ion and substrate transport, signal transduction and translation. These are also reportedly involved in genetic diseases (Dean et al., 2001; Rees et al., 2009). In prokaryotes, both kinds of transporters, importers as well as exporters are found. In eukaryotes such as insects, however, only the exporters transporting lipids and many other metabolites are found. These are of special importance as they are involved in insecticide transport and resistance (Dermauw and Van Leeuwen, 2014). At present, more than 400 arthropod ABC transporters are known. Similar to those found in other living organisms, ABC transporter family is divided into eight subfamilies from ABCA to ABCH, on the basis of high sequence similarity in NBDs (Dermauw and Van Leeuwen, 2014).

In insects, ABC transporters are involved in the transport of eye pigment. The white, brown and scarlet genes of *Drosophila* transport guanine or tryptophan precursors

of red (Kim et al., 2013) (drosopterins) and brown (ommochromes) eye pigments. White and brown gene product form a guanine specific transporter, while white and scarlet, a tryptophan-specific transporter (Mackenzie et al., 1999). ABC transporters are also involved in the transport of cuticular lipids or neutral lipids like triglycerides in *Tribolium* (Broehan et al., 2013), 20-hydroxyecdysone (Hock et al., 2000; Retnakaran et al., 2001) in *Drosophila* and spruce budworm larvae, lipid-modified Drosophila germ cell peptide chemoattractant (Ricardo and Lehmann, 2009), allelochemicals like nicotine in *Manduca sexta* (Gaertner et al., 1998; Murray et al., 1994) and colchicine in *D. melanogaster* (Tapadia and Lakhotia, 2005). ABC transporter Sulfonylurea receptor allows Ca^{+2} necessary for the proper cuticle synthesis by the vesicles in the integument of *Periplaneta americana* (Abo-Elghar et al., 2004). They are also associated with insecticide transport and resistance in many insect species (Dermauw and Van Leeuwen, 2014). Resistance to *Bacillus thuringiensis* protein toxin like Cry1Ac was correlated to a mutation in ABCC2 transporter in *Heliothis virescens* (Gahan et al., 2010) and Cry1Ab and Cry1Ac in *Helicoverpa armigera* (Xiao et al., 2014). Single amino acid mutation in ABC transporter in *Bombyx mori* imparted resistance to Cry1Ab (Atsumi et al., 2012) and ABCA2 mutation in *H. armigera* and *H. punctigera* imparted resistance to Cry2Ab (Tay et al., 2015).

In the bed bug, *C. lectularius* 51 ABC transporters are identified through blast analysis (Benoit et al., 2016). ABC transporters were named based on their similarity to *D. melanogaster*. These were classified into eight families from ABCA-H. Of these ABC transporters, 23 are in ABCG family; two in a closely related family ABCH and four MDR transporters from ABCC family. These transporters are involved in the transport of pigment precursors, lipids, steroid hormones, and xenobiotics (Labbe et al., 2011). ABCG transporters have been previously implicated in deltamethrin resistance in the bed bug (Zhu et al., 2013). However, more than one ABC transporters may also be involved in resistance mechanism. In this chapter we identified ABC transporters through RNAi-mediated knockdown studies that are involved in development and reproduction. We have also identified potential candidate transporters that might be involved in resistance in bed bugs. This study lays the foundation for further research on basic biology of molting, metamorphosis and reproduction in the bed bug and identifies specific ABC transporters

as targets of insecticide action. These transporters can also be used in the control of bed bugs through RNAi based insecticides.

Materials and methods

Insects: Bed bugs were originally collected from the infested apartment in Plainview, New York in April 2007. This bed bug population named *C. lectularius* NY-1 was maintained at 26.7 °C, 65 ± 5% RH and a photoperiod of 14: 10 h (L: D). The bugs were fed defibrinated rabbit blood, supplied by the Quad Five Company, Montana, USA. as per the method proposed by Montes *et al.* (2002) (Montes et al., 2002). Insects were fed once in a week. The 4th and 5th instar nymphs, 1-2 day old, were used to study molting and metamorphosis. Fifth instar nymphs that were collected in a 96-well plate after feeding metamorphosed into adults which were then used as virgin adults, 1-2 day old to study reproduction.

RNA isolation, cDNA synthesis, and Quantitative real-time PCR: Total RNA was isolated from three insects per biological replicate using TRI reagent (Molecular Research Center Inc., Cincinnati, OH). Three replicates were used. Total RNA was treated with DNase I (Ambion Inc., Austin, TX). cDNA synthesis (Promega) and qRT-PCR was performed using Applied Biosystems Step One Plus TM (Life Technologies TM Real-Time PCR System, Carlsbad, CA). Primers are shown in the **Table S1**. The mRNA levels were normalized using the internal control, ribosomal protein L8 (RPL8).

Double-stranded RNA synthesis, injection and mating experiments: The fragments of about 300-400 bp coding regions of genes were PCR amplified using the primers reported in **Table 1S.** These DNA fragments were used to prepare dsRNA, as described by MEGA script RNAi Kit (Ambion Inc., Austin, TX). The 4th and 5th instar insects, 1-2 day old were used for the experiment. The insects were anesthetized with ethyl ether vapor for two minutes and lined on a glass slide covered with double-sided tape. One µg of dsRNA in 0.1 µl was injected in to the abdomen of the 4th and 5th instar insects using NANOJECT II (Drummond Scientific Company). The glass needles were prepared by using needle puller (Idaho Technology, Salt Lake City, Utah). The dsRNA of GFP gene was injected into insects belonging to the 4th and 5th instar to serve as a control. The insects were incubated for five days after which they were allowed to blood feed. As a consequence, these insects molted or metamorphosed depending up on their instar. Virgin

adult female insects, 1-2 day old were used to study reproduction. One µg of dsRNA for the specific ABC transporter gene in 0.2 µl was injected into the ectospermalege of each insect. Insects were incubated for five days before blood feeding. The uninjected virgin adult males were blood fed. The engorged male and female adults were paired in a 48-well plate and allowed to mate for two days. The males were then removed and disposed of safely.

Dissection and Confocal Microscopy: Knockdown and control adult females were dissected in 0.01 M phosphate buffer saline (PBS) (pH 7.2) three days after feeding and mating to study the development of primary oocyte. Some representative ovariole were fixed in 4% paraformaldehyde (in 1x PBS at pH 7.2) overnight. The tissue was rinsed with PBS before staining with DAPI. Observations were recorded with confocal microscopy under illumination with light at 405 nm wavelength.

Protein extraction and SDS-PAGE: Total protein from hemolymph and ovaries of insect was extracted three days after treatment. Holes were made in the abdomen of the insect using a needle. The insect was kept in 20 µl PBS containing proteinase inhibitor and centrifuged at 1000 g for 1 minute at 4 °C. The supernatant obtained was quantified for its total protein content using Bradford method(Bradford, 1976). After denaturing 10 µg of the sample with protein dye containing β-mercaptoethanol and SDS, protein samples were loaded on 6% SDS-PAGE along with marker lane and run at 60V, 25mA until the tracking dye reached the end of the gel. The gel was stained using 0.01% Coomassie brilliant blue dye and recorded in white light using a gel documentation system. The protein band at 200 KDa was identified as vitellogenin 2 and 3 (unpublished data).

Bed bug populations used for resistance experiment: Six bed bug populations were collected and maintained in the laboratory as per conditions described earlier. These are LA-1 (Los Angeles, collected in 2007), CIN-1 (Cincinnati, OH, 2005), NY-1(Plainview, NY, 2007), CIN-10 (Cincinnati, OH, 2012), LEX-8 (Lexington, KY, 2012) and CIN-11 (Cincinnati, OH, 2012).

Statistical analysis: Statistical analysis was performed using Statistix 10.0, Analytical Software. Tallahassee, FL. Student t-test (unpaired t-test) was performed for comparing significance in a knockdown. One way ANOVA and Tukey HSD test was

performed for checking the significance of expression profiles of genes. The level of significance was set at P≤0.05.

Results

Identification of Genes: ABC transporters were identified and annotated from bed bug genome (Benoit et al., 2016). The following ABC transporters: - ABCA-UE, ABCB7, MDR1, ABCE1, ABCF1, ABCG20-2, ABCG22, ABCG23-2, ABCG4-3, White2 and Scarlet were identified through tblastn search as homologs of ABC genes from *Tribolium* showing phenotypic effects which include defects in molting and metamorphosis. RNAi-mediated knockdown of mRNA in immature and adult stage of bed bug was performed to functionally characterize these transporters.

RNAi-mediated knockdown of ABC genes in the fourth instar nymphs: Out of 11 ABC transporter genes knocked down four showed the effect on molting (Fig. 4.1 and Table 4.1). Knockdown efficiency in fourth instar nymphs varied from 50 to 99% (Fig. S4.1).

Knockdown of selected genes from the ABCA family i.e. ABCA-UE and ClABCA1 did not show any phenotypic defects. Similarly knockdown of ABCB7 from ABCB family and MDR1 and ABCC5K from ABCC family individually or together did not show any phenotypic defects. The knockdown insects molted or metamorphosed into their next stage similar to the control insects. ABCE1 knockdown showed 29% mortality during ecdysis in fourth instar nymphs while remaining 71% metamorphosed into normal fifth instar nymphs (Fig. 4.1b and Table 4.1). ABCF1 knockdown in the fourth and fifth instar nymph showed a phenotype similar as control dsGFP injected insects. The insects molted into the next stage i.e. fifth instar nymphs. Knockdown of ABCG20-2 mRNA in fourth instar nymphs produced 100% mortality as compared to the control (Fig. 4.1c and Table 4.1). ABCG20-2 fourth instar knockdown insects did not grow or molt to the next instar (Fig. 4.1c). ABCG22 mRNA knockdown in the fourth instar nymph did not show any effect on molting (Table 4.1). ABCG23-2 mRNA knockdown in the fourth instar nymphs showed 100% molting to the last instar stage. However, 18% nymphs showed mortality after molting to the fifth instar stage (Fig. 4.1d). Knockdown of ABCG4-3 mRNA in the fourth instar nymph also affected molting (Fig. 4.1e) and caused 100% mortality. The old cuticle appeared to be attached to the new cuticle. The insects were not

able to emerge. White 2 and Scarlet mRNA knockdown did not show any effect on molting. Eye color observed in the next instar stage was same as in control insects.

RNAi-mediated knockdown of ABC genes in the fifth instar nymphs: Knockdown of selected ABC genes in the fifth instar stage showed 40 to 95 % RNAi efficiency (Fig. S4.2). Knockdown of selected genes from ABCA, B and C family was performed. No phenotypic defects were observed with the knockdown of ABCA-UE, ABCB7, MDR1 or ABCC5K. All the nymphs metamorphosed to the adult stage. Control nymphs were injected with dsRNA derived from GFP gene. These insects also metamorphosed to adults. ABCE1 knockdown in the fifth instar nymph affected them adversely at pharate stage leading to 100% mortality (Fig. 4.2b and Table 4.1). Control insects injected with dsGFP metamorphosed into normal adults. ABCF1 knockdown did not show any phenotypic effects. Knockdown of ABCG20-2 mRNA in the fifth instar nymphs produced 100% mortality as compared to the control (Table 4.1). No development of fifth instar nymphs was observed with the knockdown of ABCG20-2 mRNA (Fig. 4.2c). ABCG22 mRNA knockdown in the fifth instar nymphs did not show any effect. The knockdown insects molted into the fifth instar stage. Effect of ABCG23-2 knockdown was studied on metamorphosis in fifth instar nymphs (Fig. 4.2d and Table 4.1). All these nymphs metamorphosed into normal adults. However, 45.5% mortality was observed in the adult stage. Knockdown of ABCG4-3 mRNA in the fifth instar nymphs did not metamorphose into adults to the extent of 60%. Wing pads appear to show development (Fig. 4.2e). White 2 and scarlet mRNA knockdown was not observed.

RNAi-mediated knockdown of ABC genes in the adults: All ABC transporter knockdown in adults showed 50 - 95 % RNAi efficiency (Fig. S4.3). Knockdown of ABCA-UE in the adults did not show any detectable phenotypic changes as compared to the control. Knockdown of ABCB7 in female adults did not show any effect on fecundity.

We were not able to confirm the effect on hatching. Knockdown of ABCC family transporter MDR1 significantly reduced the fecundity 58.3±47.5% (Fig. 4.3a). Egg hatching was also significantly reduced to 50±50.2% (Fig. 4.3b). Fecundity of control insects injected with dsGFP was adjusted to 100 %. ABCE1 knockdown in the female adults did not show any fecundity. Fecundity was also severely suppressed to 20% ±10 with the knockdown of ABCF1 (Fig. 4.3a). No hatching was observed in these insects.

Knockdown of ABCG20-2 mRNA in the female adults did not show any effect on fecundity as compared to the control. However no egg hatching was observed in the ABCG20-2 females (Fig. 4.3a and b). Fecundity and hatching observed in ABCG22 knockdown insects were not significantly different from that of control. ABCG23-2 mRNA knockdown in the female adults neither affected fecundity nor egg hatching. However, 100% mortality was observed of the first instar nymphs in the next generation as compared to the control (Fig. 4.4c and d). ABCG4-3 mRNA knockdown in female adults resulted in 100% loss of fecundity as compared to the control (Fig. 4.3a and b). Knockdown of White2 mRNA in adults did not affect fecundity or hatching. However, the eye color in first instar nymph progeny was orange in case of the White2 knockdown parents (Fig. 4.4a) and the dark brown in the case of control or dsGFP treated parents (Fig. 4.4b). Knockdown of scarlet mRNA did not show any effect on reproduction in the bed bug. The eye color observed in the treatment was similar as in control (Table 4.1).

Total protein expression profile in case of ABCE1 and ABCF1 knockdown female adults from day three hemolymph and ovaries showed reduced Vg protein as compared to the control (Fig. 4.5).

Oocytes from female adults were dissected and recorded after staining with DAPI. A representative oocyte from control insects injected with GFP dsRNA (Fig. 4.6a) showed maximal growth at day 3. No difference in oocyte length was observed with MDR1 and ABCG20-2 knockdown (Fig. 4.6b and f). Knockdown of ABCE1, ABCF1, and ABCG4-3 showed reduced length of theee primary oocyte (Fig. 4.6c-e).

Role of ABC transporters in resistance

Relative expression analysis of selected ABC transporters was performed on different populations of the bed bug. Fold change in expression was calculated by dividing expression of the respective population with LA1 and the results were shown on a heat map (Fig. 4.7). *C. lectularius* CIN11 showed four-fold higher expression of ABCG23-6 and an eight-fold higher expression of ABCG5-1 as compared to LA-1. Other populations tested were CIN10, LEX8, NY1 and CIN1.

Discussion

Bed bug ABC transporters were found to be involved in molting, metamorphosis, and reproduction. They might also have a role in resistance to pyrethroid insecticide. There

are 51 ABC transporters in bed bugs (Benoit et al., 2016). ABC transporters were selected, and knockdown was performed to find their function.

In mammals, the ABCA1, ABCA3, ABCB4, ABCG1, and ABCG5/8 are implicated in lipid transport the deficiency of these transporters causes diseases in humans (Quazi and Molday, 2011). They do not have any know function in insects except in *Tribolium*. Knockdown of TcABCA-9A/9B showed stage-specific mortality in *Tribolium* adults and defects in wing development, elytra shortening and blisters (Broehan et al., 2013). Closest homolog to TcABCA-9A/9B was identified in *Cimex* to be ClABCA-UE. Knockdown of ABCA-UE did not give any phenotype, suggesting that the transporter might lack function in bed bugs. Lipid transport in bed bug might be primarily done by ABCG family of transporters. About half of the transporters were classified in ABCG family. In humans ABCB transporter MDR1 have been reported to be involved in multiple drug resistance in human cancer (Ueda et al., 1987). In insects i.e. *Helicoverpa armigera* they are involved in insecticide resistance (Srinivas et al., 2005). In *Tribolium* knockdown of ABCB5A is required for female fertility. Severe molting defects in pupal to adult molt were also observed (Broehan et al., 2013). ABCB gene Dmmdr49 is involved in the transport of chemoattractant for the germ cell migration in *Drosophila* embryonic development (Ricardo and Lehmann, 2009). ABCB7 knockdown in fourth and fifth instar nymphs did not show any phenotypic defects. Hence, this transporter may not be involved during molting or metamorphosis. The role of ABCB7 in embryonic development was not confirmed as we were not able to replicate the experiment. MDR1 was found to be the closest homolog of TcABCC9A and DmSUR. DmSUR is the target of diflubenzuron. It is involved in chitin synthesis in *D. melanogaster* and *B. germanica* (Abo-Elghar et al., 2004). MDR1 knockdown did not show any phenotypic defects during molting or metamorphosis. However, fecundity and hatching were reduced in female adults. Knockdown of chitin synthase gene in bed bug was performed to confirm these results. Knockdown of kkv (CS-1) in fourth instar nymphs produced molting defects. Knockdown of kkv mRNA in female adult bed bug did not affect fecundity, but hatching was reduced to zero (unpublished data). This suggests a different role of MDR1 in development in bed bugs. Knockdown of ABCC5K second closest homolog to TcABCC9A did not show any phenotypic defects. Co-knockdown of ClMDR1 and ClABCC5K had no effect on molting

or metamorphosis on bed bug suggesting the role of Ca^{+2} signaling is regulated by other ABC transporters. ABCE and ABCF family of transporters lack TMDs. They have a role in ribosomal assembly, translational initiation (Andersen and Leevers, 2007) and mRNA transport. The function of ABCE1 homolog of TcABCE-3A and Dmpixie was studied through knockdown. Knockdown of ABCE1 mRNA increased mortality during molting and metamorphosis. No fecundity was observed in adults. Mortality in nymphs might be due to a defect in protein synthesis of housekeeping genes. Both instars where stuck at pharate stage showing the importance of protein synthesis at this stage. Reduction in fecundity was attributed to decreased production of vitellogenin protein in hemolymph and oocytes (Fig. 4.4). Knockdown of ABCF1 did not show any phenotypic defects during molting or metamorphosis showing that this gene may not have any function during this stage. However, ABCF1 knockdown reduced fecundity and stopped hatching in female adults. Vg protein was also decreased with ABCF1 knockdown (Fig. 4.4). ABCF1 is homologue of ABCF-2A of *Tribolium*. Knockdown of TcABCE-3A and TcABCF-2A showed a phenotype similar to ABCE1 knockdown in *Cimex*. However in *Tribolium* phenotype were much sever showing 100% mortality in insects during quiescent and pharate adult stage. ABCF1 may not have any function during molting or metamorphosis in *Cimex*. ABCG transporters are often associated with transport of steroid or lipids. Subfamily G proteins are half-transporters and form homo- or heterodimers (Tarr et al., 2009). They have NBD at the N-terminal and TMD in the C-terminal the reverse orientation of all the other ABC transporters. ABCG in have been shown to be involved in the transport of lipids, sterols, drugs and chemotherapeutic treatments of tumors. Their deficiency causes pulmonary inflammation (Baldan et al., 2008). In *Tribolium* ABCG-4C and ABCH-9C have been implicated in lipid transport to the cuticle. Knockdown of these genes in *Tribolium* causes desiccation phenotype and decreased lipid in the cuticle. ABCG20-2 and ABCG23-2 are homologs of ABCGH-9C in bed bugs. Knockdown of ABCG20-2 mRNA caused mortality in nymph possibly due to desiccation caused by decreased cuticular lipids. No hatching was observed in these insects possibly due to a decrease in transport of lipids to the embryo. Knockdown of ABCG23-2 mRNA showed mortality in the next stage of the insect. This pattern of mortality was consistent in all the stages of *Cimex*. Showing that the knockdown of this transporter has a delayed effect.

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Knockdown of ABCG4-3 mRNA homolog of TcABCG-4C in *Cimex* produced sever phenotype. This transporter has also been implicated in the transport of cuticular lipids. Insects looked desiccated. Mortality observed could due to lack of cuticular lipids. ABCG4-3 might have a role in primary oocyte development. The length of primary oocyte was decreased with the knockdown of ABCG4-3. ABCG22 is the homolog of *Drosophila* DmE23 and *Tribolium* TcABCG8A. DmE23 is implicated in ecdysone transport (Hock et al., 2000). Ecdysone induces DmE23 in *Drosophila* regulates ecdysone-mediated gene activation to regulate metamorphosis (Hock et al., 2000). Knockdown of ABCG22 in *Cimex* did not give the expected phenotype. No phenotypic defects were observed during molting, metamorphosis or reproduction in insects. The second closest gene to DmE23 was found to be ClWhite2. Knockdown of White2 in bed bugs has been shown to cause red eye phenotype in the progeny. Hence, it is possible that the role of Ecdysone transport in *Cimex* has been substituted with a gene carrying a low homology with DmE23. White2 was found to be homologs to DmWhite and TcABCG9B. TcABCG9B is also known as TcWhite. Knockdown of TcWhite and TcScarlet in *Tribolium* resulted in white-eye phenotype. Knockdown of White2 and Scarlet in *Cimex* fourth instar nymph did not show any phenotypic change in the fifth instar. The results were similar to that observed in Tribolium. Knockdown in fifth instar nymph also did not show any phenotypic changes. However, knockdown of White2 but not Scarlet in adults showed red eye phenotype in first instar nymphs. White2 is similar to DmWhite. DmWhite is involved in the transport of guanine and tryptophan molecules that serve as precursors of red (drosopterins) and brown (ommochromes) pigment of the eye, respectively (Kim et al., 2013). Mutations in White gene causes a major decrease in the red eye pigment in *Drosophila* (Mackenzie et al., 1999). Knockdown of White2 did not decrease red eye pigment in first instar bed bugs. However, the brown pigment was reduced. This shows White2 in *Cimex* is a tryptophan transporter required for the synthesis of brown pigment.

Besides the role of ABCG transporter in development, they may also be involved in insecticide resistance. As was shown by the correlation of high expression of ABCG5-1 and ABCG23-6 in the pyrethroid resistant CIN11 bed bug population. LA-1 population showed a LC$_{50}$ of 0.003mg/cm^2 as obtained by disk residual bioassay (Zhu et al., 2013). CIN11 population was found to be highly resistant to deltamethrin with an LC50 of 0.2%

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as determined by topical application of deltamethrin (Fig. S4.4). It is common for multiple mechanism of resistance to occur in insects. Sequence analysis of sodium channels have also revealed the existence of two knockdown mutations in their sequence (Fig. S4.5). Other genes showing high expression in CIN11 population w.r.t. LA-1 include Cyp397A1 (5 fold) and cuticular gene C2 (22 fold) (Fig. S4.6). A Large number of transporters in the ABCG family in *Cimex* shows that these insects might have evolved a complex and more specific system of transport of lipid and steroid molecules. The selection of ABC transporters was made based on their similarity to *Tribolium* ABC transporters identified through tblastn. This method of selection might not be appropriate as bed bug transporters may not be closely related to the *Tribolium*. Hence, further knockdown studies need to be conducted to find the role of all ABC transporters in bed bugs. Further study needs to be conducted to find the function of ABCG family of transporters in detail.

Table 4.1. Summary of phenotype obtained with knockdown of ABC transporters in *Cimex lectularius*.

Summary			% Mortality		Phenotype		
S.No.	Gene Family	Gene	N4 injections	N5 injections	N4 injections	N5 injections	Adult injections
1	ABCA	ClABCA-UE	0	0	5th instar	Adult	Same as control
3	ABCB	ClABCB7	0	0	5th instar	Adult	NA
4		ClMDR1	0	0	5th instar	Adult	decreased fecundity and hatching
5	ABCC	ClABCC5K	0	0	5th instar	Adult	Same as control
6	ABCE	ClABCE1	28.6	100	28.6% insects stuck at pharate stage showed mortality	stuck at pharate stage	No Fecundity; decreased Vg expression; reduced length of oocyte
7	ABCF	ClABCF1	0	0	5th instar	Adult	Decreased fecundity; no hatching; reduced Vg expression; reduced length of oocyte
8		ClABCG20-2	100	100	No development; desiccated	No development	No hatching
9		ClABCG22	0	0	5th instar	Adult	Same as control
10		ClABCG23-2	18.2	45.5	Mortality observed after molting	Mortality observed after metamorphosis	Mortality in first instar nymphs
11		ClABCG4-3	100	60	did not molt; dessicated; defects in molting	No development; did not metamorphose	No Fecundity; reduced length of oocyte
12		ClWhite2	0	0	NA	NA	Red eye in first instars
13	ABCG	ClScarlet	0	0	5th instar	NA	Same as control
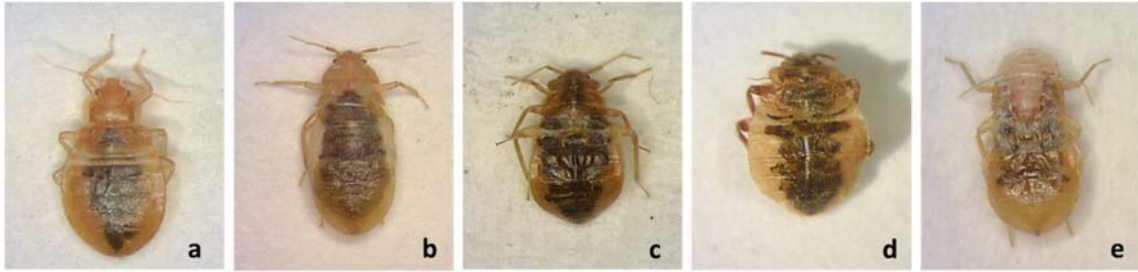
Figure 4.1. Effect of knockdown of ABC transporters on fourth instar nymphs: dsRNA targeting ABC transporter mRNA sequence were injected into the fourth instar nymphs to study its effect on molting. After injection insects had been incubated for five days, they were then allowed to feed. The phenotype was recorded after five days. The fourth instar nymphs injected with dsGFP molted into fifth instar nymph (a). Knockdown of ABCE1 injection in fourth instar nymph showed defects in molting. 29% insects are showing defects also showed mortality (b). ABCG20-2 knockdown insects showed 100% mortality; no development was observed in these insects (c). Knockdown of ABCG23-2 in fourth instar nymphs showed 18% mortality in the next stage of the insects. All insects were able to molt (d). 100% mortality was observed in insects when ABCG4-3 knockdown was carried out. The insects showed defects in development (e).

Figure 4.2. Effect of knockdown of ABC transporters in fifth instar nymphs: ABC transporters were knockdown through RNAi-mediated gene silencing through the introduction of dsRNA in the insect. 1ug of dsRNA was injected into the insects they were incubated for five days after which they were allowed to feed and molt. dsRNA derived from GFP mRNA was used as a control. The control insects metamorphosed into adults (a). Knockdown of ABCE1 resulted in 100% mortality. The insects were stuck at pharate stage (b). Knockdown of ABCG20-2 resulted in 100% mortality at 5th instar stage the insects did not show any sign of development (c). All the insects metamorphosed to adults with knockdown of ABCG23-2. However, 45.5% mortality was observed in the adult stage (d). Knockdown of ABCG4-3 showed 60% mortality. The insects did not show any sign of development (e).

(a)
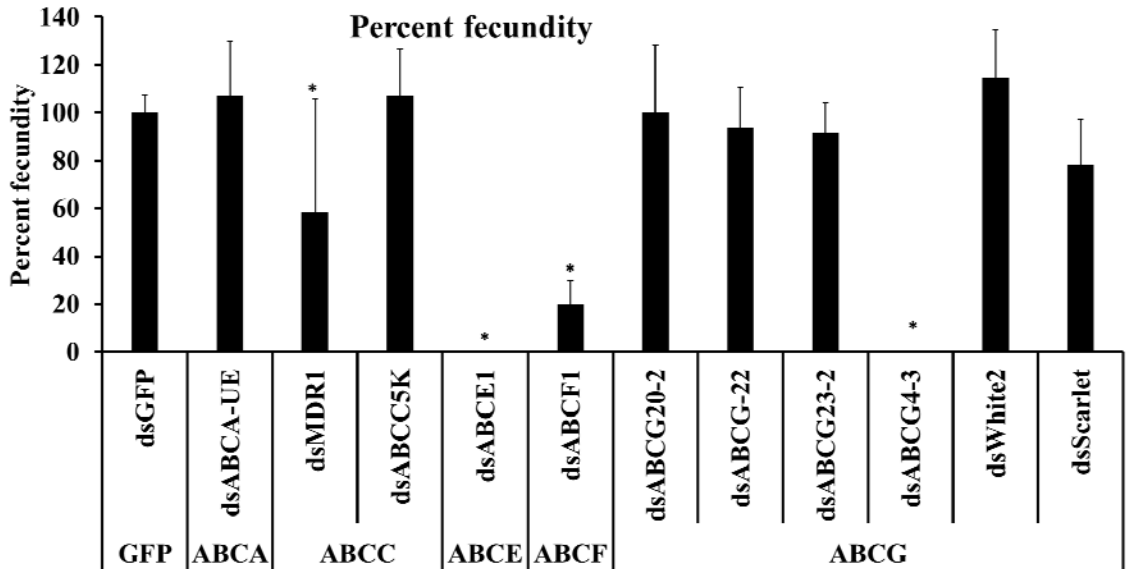
(b)
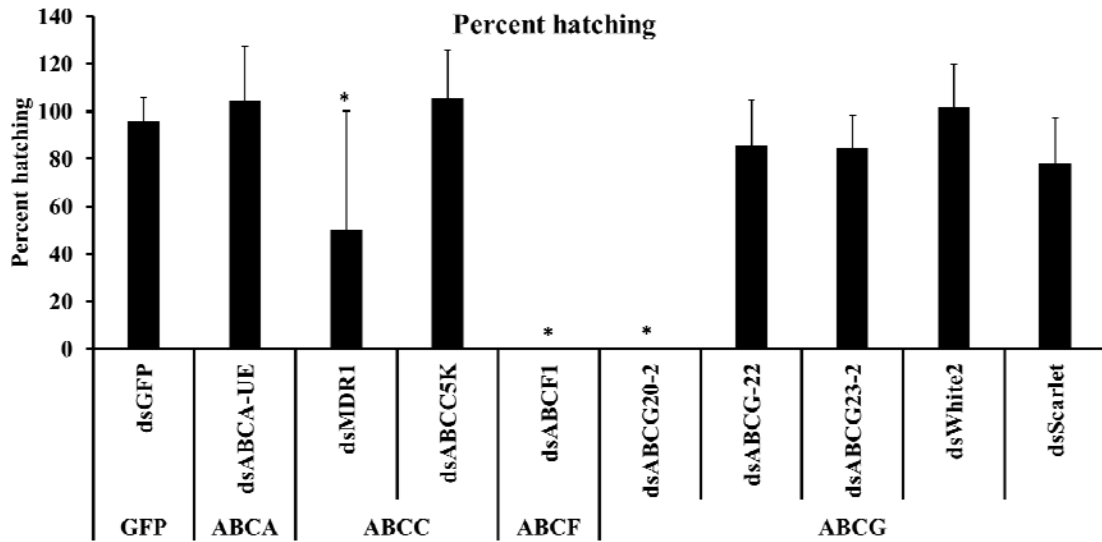
Figure 4.3. Fecundity and hatching in knockdown insects.

RNAi-mediated knockdown was carried of selected ABC transporters. (a) The top graph shows percent fecundity on y-axis and gene name on the x-axis. Fecundity of control (dsGFP) insects was considered as 100%. (b) The bottom graph shows percent hatching in dsRNA injected insects. (n≥10; p≤0.05)
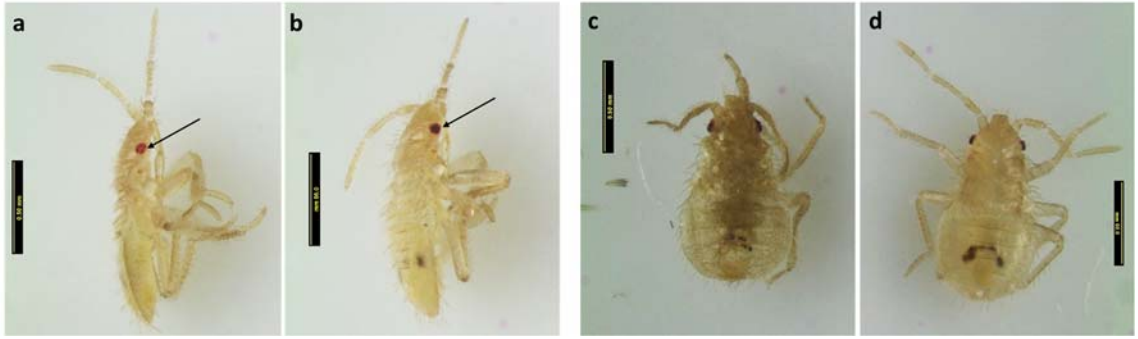
Figure 4.4 Phenotypic effect of knockdown on progeny: ABC transporters knocked downed in virgin adult female 1-2 day old. The insects were fed and mated after a five days incubation. The phenotype of progeny was recorded. Knockdown of White2 mRNA in adults produced red eye in progeny (a). The control insects were injected with dsRNA derived from GFP. Progeny from control insects had a dark brown colored eye (b). Knockdown of ABCG23-2 in adults showed mortality in the progeny (c). The control insects were alive (d).
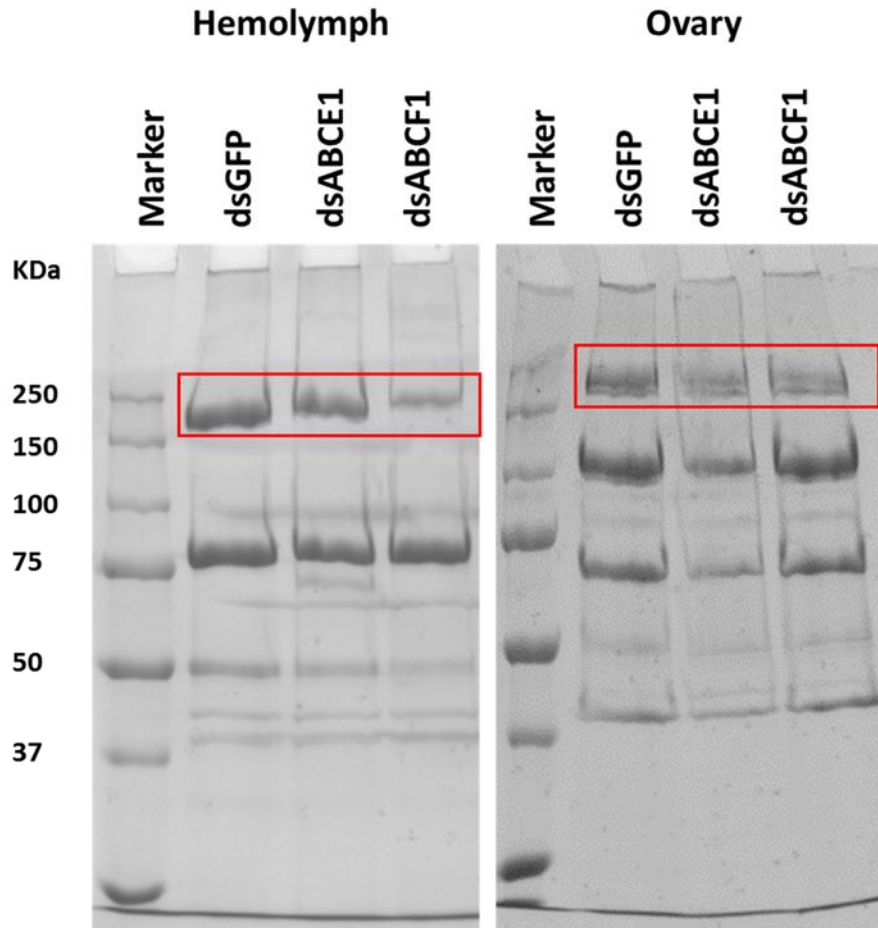
Figure 4.5. Total protein profile from ABCE1 and ABCF1 knockdown insects.
RNAi-mediated knockdown of ABCE1 and ABCF1 mRNA was performed. Same amount
of dsRNA derived from GFP was injected as a control. The insects were incubated for five
days after which they were allowed to feed and mate. Total protein was extracted three
days after feeding and mating. 10ug of total protein from hemolymph (a) and 5ug of total
protein from ovaries (b) was loaded on the gel. Vitellogenin bands are shown in the red
box. Vg protein bands had decreased intensities in insects showing knockdown of ABCE1
and ABCF1 mRNA.
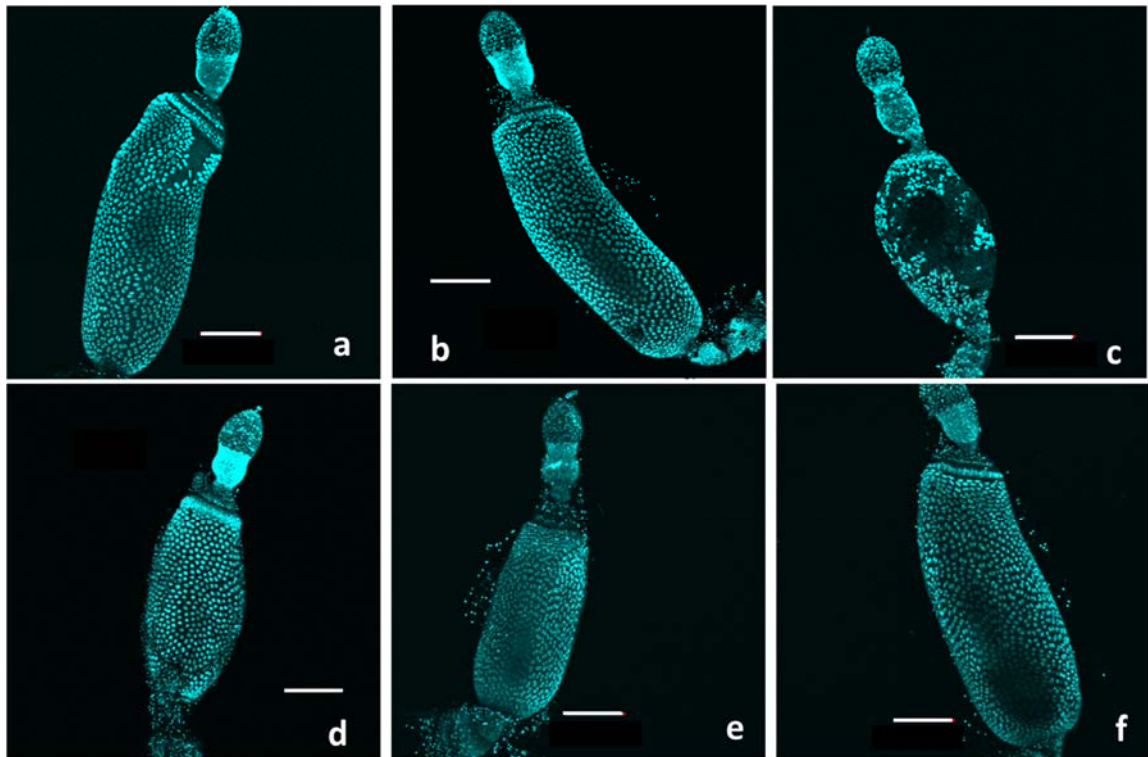
Figure 4.6. Primary oocyte development in knockdown insects. RNAi-mediated knockdown using specific dsRNA derived from selected ABC transporters was performed. The insects were allowed to feed and mate after five days of incubation. Insects were dissected after three days to observe the development of oocyte. The ovaries were fixed in 4% paraformaldehyde overnight. Oocytes were washed with phosphate saline and stained with DAPI. dsGFP injected insects were used as a control. GFP knockdown insects showed the maximum development of oocytes at day three (a). Decrease in oocyte development was observed in ABCE (b), ABCF (c) and ABCG4-3 (e) knockdown insects. MDR1 and ABCG20-2 knockdown insects did not show any difference in oocyte development (d and f). Bar represents 200um.

Figure 4.7. Heat Map showing fold change in gene expression of selected ABCG and MDR transporters in CIN10, LEX8, CIN11, LA1, NY1 and CIN1 populations of bed bugs. Fold change was calculated by dividing relative expression of the gene in a population divided by relative expression in LA1.

Chapter 5: Conclusion and future directions:

Bed bugs and the problems they cause in terms of nuisance as well as public health hazards are on the rise in many countries, especially the developed countries. They cause allergic reactions, are potential vectors of disease causing micro-organism (Lowe and Romney, 2011; Salazar et al., 2015), they are most importantly considered as a social stigma. In recent times, insecticide resistance in the bed bug has developed to the extent that some of the pyrethroid are no longer effective for their control (Zhu et al., 2013). In view of the economic importance of the bed bug and lack of knowledge of the molecular basis of its growth and development, we initiated studies to elucidate genes associated with molting, metamorphosis, and reproduction. The long-term goal of these studies is to understand the basic biology and to identify novel targets for insect control.

qRT-PCR analysis at different time interval have revealed higher expression of Kr-h1 during penultimate stage. Kr-h1 expression drops in the last instar stage possibly in the absence of JH, allowing expression of E93 gene. Kr-h1 knockdown in penultimate stage of the insect resulted in the development of adult characters like wing pads, darker sclerotization, and absence of unsclerotized integument (characteristic of last instar nymph). Dissections of precocious adults have shown for the first time the development of male and female genitalia; development of seminal vesical, male accessory glands, and male accessory gland reservoir in the male and, the formation of common and lateral oviducts and ovaries in the female. This showed that Kr-h1 acted as a repressor of adult characteristics. Expression of ecdysone early genes HR3 and HR4 were found to increase before every molt in the bed bug. Knockdown of E93 in fifth instar nymph caused the development of supernumerary nymphs. Knockdown and JH application studies also showed that E93 and Kr-h1 negatively regulate each other. The mechanism of their regulation remains unknown and is a topic for future studies. Kr-h1 expression is often correlated with high JH titers. Kr-h1 is known to be directly induced by JH in the presence of its receptor Methoprene-tolerant protein (Met) and steroid receptor coactivator (SRC) (Kayukawa et al., 2012). Recording titers of JH and ecdysteroid would also be essential to make this study on molting and metamorphosis complete. Knowledge of type of JH isoform still lacks in the bed bugs. This would potentially be helpful in studying the interaction between JH and its receptor Met, which would be helpful in designing or

selecting inhibitors to block the JH action pathway. Through this study we also observed that a blood meal triggers a change in gene expression which results in the growth of the immature insect by molting to the adult stage.

Besides the growth of an insect JH and ecdysone also have a role in reproduction. In bed bugs Vg mRNA expression and oocyte development was induced with only a blood meal, however mating further enhanced these effects for having an optimal effect for normal reproduction to take place. The expression pattern in fat body and ovary revealed the expression of Vg in fat body only. Various genes like Kr-h1, ILP2, and MafB were also induced in fat body by feeding and mating. Whereas Foxl2, HPSE, HR3, and Kr-h1 are induced in ovaries of fed and mated insects. Knockdown of mTOR or Insulin signaling pathway genes did not show any effect on Vg expression or oocyte length.

In bed bugs, JH affected reproduction by regulating vitellogenesis, oogenesis, and embryogenesis. Juvenile hormone regulates Vg expression through Met/SRC in the fat body, oocyte maturation through Foxl2 in the ovary; embryogenesis through the maternal expression of HPSE in the ovary and Kr-h1 expression in the embryo. In the bed bug, Foxl2 expression was predominantly observed in both female and male reproductive organs. Knockdown studies to elucidate the juvenile hormone pathway have shown that Foxl2 acts downstream to Met/SRC but independent to Kr-h1, an early gene in the pathway. The kr-h1 expression is found to be independent of SRC as opposed by what has been shown in other organisms (Zhang et al., 2011). Role of Foxl2 in insects has not been previously recorded. H3 an ecdysone inducible gene was also found to be regulated by a JH-inducible gene Foxl2. Hence, Foxl2 provides a link between JH and ecdysone action pathways during regulation of ovaries. In the bed bug, embryogenesis takes a long time of about 4-5 days. Kr-h1 knockdown shows embryonic mortality or egg mortality. This could be due to deposition of processed siRNA or dsRNA into the developing oocyte. Tracking of P^{32} labeled radioactive dsGFP was not able to detect siRNA in adults or egg possibly due to sensitivity issues. Maternal expression of heparanase (HPSE) mRNA is found to be essential for embryonic development in the bed bugs. In the bed bugs, HPSE possibly regulates embryonic development through hydrolyzing heparin sulfate or heparin sulfate proteoglycan which could have effected concentration of factors, cell signaling and cell-cell interactions. Zinc finger containing gene Stripe and CLEC005028 showed embryonic

lethality. Zinc finger proteins regulate a wide variety of DNA, RNA and protein- protein interactions. The embryonic lethality due to stripe is possibly through defects in muscle development might be due to a knockdown in the embryo. Stripe is expressed early in the bed bug embryo. Knockdown of Stubble in the bed bug during its adult stage did not decrease fecundity or hatching as compared to the control. Vg expression was also shown to be regulated by MafB. MafB also regulated Foxl2 in the oocyte. MafB regulates oocyte development by suppressing the expression of various cell adhesion molecules like Nrt and DEcad which enables proper interaction of follicle cells with the developing oocyte. Since Vg knockdown also affects oocyte development, MafB acts through more than one pathway to regulate oocyte development in the bed bug. The role of MafB in regulating Vg expression in insects or arthropods has not yet been documented. Insect cuticle synthesizing genes like kkv encoding a chitin synthase enzyme (CS-1), unclassified cuticle protein 1 (UCP1), and Laccase2 were also identified which affected embryonic development. Further work need to be done to find out the stage in embryonic development affected, and the mechanism of action of how transcription factors regulate reproduction.

ABC transporters are the important group of transmembrane proteins associated with many physiological functions. Selected ABC transporters tested from family A, B, C and F did not show any effect on molting or metamorphosis. This could be because many transporters might have overlapping substrate specificity. Knockdown of ABCE1 mRNA increased mortality during molting and metamorphosis. It also completely suppressed fecundity. Mortality in nymphs might be due defect in protein synthesis of housekeeping genes. Both instars were stuck at pharate stage showing the importance of protein synthesis at this stage. Knockdown of ABCG20-2 and ABCG4-3 mRNA caused nymphal mortality in the fourth instar stage. ABCG23-2 knockdown also showed mortality but in the next stage of insect growth. Knockdown of ClWhite2 in the bed bug has been shown to cause red eye phenotype in the progeny. This might be due to incomplete knockdown of the gene. ABCG4-3 might have a role in primary oocyte development. The length of primary oocyte decreased with the knockdown of ABCG4-3. ABCG20-2 mRNA knockdown did not affect fecundity, but no hatching was observed possibly due to a decrease in transport of lipids to the embryo. Knockdown of ABCG23-2 mRNA showed mortality in the first instar next stage of the insect. This pattern of mortality was consistent in all the stages of *C.*

lectularius, showing delayed effect of the knockdown of this transporter. The role of ABCG transporters in the transport of lipids can be confirmed by lipid staining experiments.

Besides, the ABCG transporter in development may also be involved in insecticide resistance, as shown by the correlation of high expression of ABCG5-1 and ABCG23-6 in the resistant CIN11 bed bug population. Thus, ABCG subfamily of transporters remains one of the most important in regulating lipid and xenobiotic metabolism in *C. lectularius*. Knockdown experiments need to be conducted to verify their role in resistance.

The research reported here showed that JH and 20E regulate molting and metamorphosis and reproduction in *C. lectularius*. Based on our data and data reported from other holometabolous and hemimetabolous insects, we conclude that higher levels of JH during early nymphal stages induce Kr-h1 expression and suppress E93 expression and prevent metamorphosis. The decrease in JH levels in the last instar nymph results in a decrease in Krh1 expression allowing expression of E93, which promotes adult characteristics. JH and transcription factor MafB has also emerged as major players in reproduction in bed bugs. They regulate reproduction at every stage from vitellogenesis, oogenesis to embryogenesis. More research needs to be conducted to find their mechanism of actions. ABC transporters were also found to play a major role in molting, metamorphosis, and reproduction. Considering the economic importance of bed bug as a parasite the role of ABC transporters in resistance was also studied. High expression of ABCG23-6 and ABCG5-1 were found to correlate with deltamethrin resistance in bed bugs.

Through this study Foxl2 and ABCG20-2 have emerged as a major candidate that could be used in RNAi-based insecticide. Foxl2 is a JH inducible transcription factor found to be regulated by MafB and Met/SRC complex. Knockdown of Foxl2 completely suppressed fecundity. Knockdown of ABCG20-2 also completely suppressed fecundity and was also found to cause 100% mortality. These features make Foxl2 and ABCG20-2 and excellent candidate genes for RNAi based insecticides. An important limitation for the use of RNAi based insecticide is the delivery of dsRNA into the bed bug. In theory, dsRNA can be conjugated to nanoparticles which would then deliver the dsRNA into the bed bug. Nanoparticles would also increase the stability of dsRNA in the field. Bed bugs feed on

human blood hence it is difficult to deliver the dsRNA through feeding. The deliver method would require the penetration of nanoparticle conjugated dsRNA to penetrate through the cuticle or epidermis of the nymphs where sclerotization is absent. The sequence of DNA selected for the preparation of dsRNA should not have close homology with the sequence of other organisms including, beneficial insects and humans. Nanoparticle conjugation with dsRNA has been shown possible for the control of mosquito (Das et al., 2015). Another approach would be do genetically engineer the endosymbiotic bacteria like *Wolbachia* and *Arsenophonus* to produce dsRNA which will then kill the bed bug. The endosymbiont expressing the dsRNA will have to be laterally transmitted to other bed bugs hence eliminating all the population. *Wolbachia* is known to be vertically transmitted and it is an obligate endosymbiont in many insects and arthropods, hence it may not be a good candidate. Another approach is to design chemical inhibitors of these gene products. Which could be developed for controling these insects.. These novel approaches will add to the existing arsenal for the control and eradication of bed bugs.

Appendix:

Table 1S: Primers used in this study and reported in manuscript.

S.No.	dsRNA primers	Forward primer	Reverse primer
1	Stripe	GAAACCGAGAAAGTACCCAAAC	ATCTTTCTTCCCTCGGGATTTA
2	Stubble	TAAATTTCCCGTCTCGTCTTC	GGGTGTATGACAATCCTTTCT
3	UCP1	ATGGCCGTGGCTATCGT	TGCACGTGCTGAACAACAG
4	Foxl2	CTCAACGACTGTTTCGTCAAAG	CCGTTCATAGGTATCTGCATAGT
5	MafB	GGACACCAATCTGAGGATTAC	GTGTTGGTGGTGGTATTCTAA
6	Heparanase (HPSE)	CTTCCACTTCTTGCTCCTAC	GGCACTTTGCACTTGATTAC
7	Vg1	CTACGGACAAGACGAAGAATTT	CTTACTGGTGTACAGGTTGTTT
8	Vg2	GTTCTCGGAAACGTCCTTAACA	CGCATTTCTTGGGTACTTGAAC
9	Vg3	CCATGAAAGAGAAAGCTTACAA	CTGGGAGAAGTCTGGTTTATTT
10	kkv	CAATACGATCAAGGAGAGGATCG	GCTCCCACCAACATAAGGAATA
11	CLEC005028	AACAACATCGTGACGGAGATAG	TCCAGATGGGACAGAAACATATC
12	Hexamerin	CAAGGAAGGCGAGACTTTCTAC	GAAAGTCAGGTTGTGGCTATCT

13	CLEC000870	CAAGGGAAATCTGAACGACAAC	CTTACAGCCACTCCAGTCATAG
14	Laccase2	GACGGTATTTATGGAAGCATTGTC	CAGTGAGTTGATCATACGGAATCT
15	Cyp6a20	CACCCTTCACTTTGCTCTTTATG	CGAATCTTTCCGGATCGAATTTAC
16	Phantom	ACATCACACTGACTGAACTGGCG	GAAACGTCGCTGCTCCTTCCATTT
17	Shade	CCTTGGAAAGCTCAACTCGTCCT	TGTGCTTTGTCATACTTGGCTGGC
18	EcR	TATGTAGACAACAGGGCAAGGCC	GGAAACGTCTTCTGGCACGGATT
19	USP	TGAGGAGTTCATTCCAACCCGCAT	TCTATGGGCATGAAACGTGAGGCT
20	BR-C	TGAGGCAAAGGTCTCTTTCGTCGT	TTCATCGGCATTGCATTGTCCTGG
21	Met	CCAAGCTCGACCAGAGAGATAAGA	AGCTGCTCCGTGCTTGTAT
22	SRC	AGCAAAGAGGAGGTCCTAGGGAAA	CATATTTGCACACTCGCTGCTGCT
23	Kr-h1	TTCGGATAACAACCTCCGCTTGGA	GGTCCACAATCGGAGTCACACA
24	mTOR	ATGGCAGGAGAAAGTTACCAGCGA	AAGAGGCGTACTTAAGCCAGGTGT
25	InR1	GTGTTCCCGAACTTGGCCGTTATT	AAACACAATTGGACGGACCACTGC
26	InR2	GCTCCTTATATACCATCTGAACC	CTTGGAATCCTTCTCCCATAAC
27	ILP1	TTCTGACCGCTCACAACCGATGA	TCCCTCGCTGCAGTACGATTCAA

28	ILP2	CCTATAATTTCGAGGATG ATCGTATGAATG	CATCCTTGACCATCAGC ACAATAGG
29	Akt3	GGGTGATTTGTATTTCGG AGAACCCG	ACAACCCAGTTCAAGA TCGAGGCT
30	fkh1	CGGCACTTTGACGAAAC AGTCGTT	ATTCTGGGATTCAGGCT ATGGGCA
31	cTOR	CAACTGAAGAAGACACT CCTAATA	GGATCTACTCTTTCTAC CACTTTC
32	GATA	AGCAGTCGGTTACAGCA TCAGGAA	TGTACATCGCGGTTGTG TAGTCGT
33	TSC2	TGAGCACGGTCTTCACCT ATCACA	TATGCAGTCGTTGTGGT CGAACCT
34	Dicer1	GAGGACGAGAAGAGATT CATTAG	GATTTATCCCGATTTAA CCTTTCC
35	Ago1	TGCCGCATAACCACATCT AA	AGACAAATGCCCGAGG AAG
36	ABCA-UE	TATCTGCCGTATCCATTT ATAGAG	AAAGGGAACCAGTAGG ATTTAG
37	MDR1	CAATGCTCTTCCTCCCAT AAT	GCCAGGATTCATTGTCT CTC
38	ABCC-5K	CATCGTCGATCCCAGTA AAG	GTGGAGTAAGTGGCGA ATAG
39	ABCF1	GGAACCACAGACTGAGA ATAG	CCGTTTCCCTAATAAGT CTCTC
40	ABCG23-2	CTACTCGGACTGCCATTA GTA	GACATATCCATCCAGA CGTTAAG
41	ABCB7	CTACTCAGCAGACGGTA TTATTT	CTACTCAGCAGACGGT ATTATTT
42	Clst	CCGGTCTAGACAGCTAT ACA	CAGTGATCCCATACGC TAAC

S.No.	qRT-PCR primers	Forward primer	Reverse primer
43	ClE93	GACGTCATCACTAACAC GATAA	GAACACTCATCTCTCCT CTTTG
S.No.	qRT-PCR primers	Forward primer	Reverse primer
1	Stripe	TACAACGCACAGATTGT AGTC	CTCGTCGAAGGGTTGT ATTC
2	Stubble	TGGACCACACAGTAATG ATATTG	GATGTCACTTTCAGGA AGACAG
3	UCP1	CCGTTGCCGTATCTTACA AC	GGTAGTCGAGTGAACA GCTA
4	Foxl2	GACTACTGCCACATTCAC TAC	GTACGAGTATGGAGGC TTTG
5	MafB	GATGAAAGGGAGTGATG GTAG	CGACTTCTTCTCTGGGA AAG
6	Heparanase (HPSE)	TCATCAGTTCACATACTT CCTATC	CGACTGGTTTGTTCTCA TACA
7	Vg1	CGTGTTGAGCTTTACGCG AGCAAT	ACCTGTCGGTCGTCATG AAATCGT
8	Vg2	TATGTTCATGAAGACAC CGTTT	GCCTCGACTGAGATGT TGACG
9	Vg3	CTCATGTTATTGAAGATT TTATTACCCCT	GAGAATGGATGGAGAC ATTAGCA
10	kkv	TGTCGATCCCGTGTATGT	CCTCTCTTCCTCCAATT CTTTC
11	CLEC005028	CAGCTACGGCAGTGATA TTT	GACGGTGAGCGTGTAT ATTG
12	Hexamerin	CGAACTTCCTCCGTTCTA TG	CGGTGTTGTTGTATTCG TATTT

13	CLEC000870	GTACGAGGAACTTCAAT CTCTG	GGTATAATCCTTGGGTC AACATA
14	Laccase2	CCAAGATCTGCTACTACC ATTT	TTATCGTGAGCATTCCT CTTTC
15	Cyp6a20	CTATAAAGTGACGCTCA ACGA	CTGCGTTTGGTGATGTT AATC
16	Phantom	TAATCGGCATTGTAGGG CCTGTCA	CTTTCAGGTAGCGCAA CAAACGGT
17	Shade	TCCAAATCGCTTCTTCCC TCACGA	GCGAACAACAAAGCCC TTGACGAT
18	EcR	ATCCTCTTCGCCAATAAC CAGCCT	TGCCTGCAGAACCTAA GTAGACCT
19	USP	TGTGTCGACAGAAACGT CACCGAT	TGTTAAGGTTGCCCTCG CTTAGGT
20	BR-C	ATCGCCAAGGGAAGACA TGGAAGA	TGGAGTCTTCCCGCTCA TTGTGAA
21	Met	TCGTCCCTGGTCACAGA AACGAAA	ACTGCGGTTGATCGCTC TTCGATA
22	SRC	ATGAGCTCACTTTCCGTC AAGCCT	GGTCGCAGATGCTTGC TGTTCTTT
23	Kr-h1	ACGCTTTGGCGTACTGA ATAACGG	TTTCGGGATCGCCGATT TCTGTCT
24	mTOR	AAGGATGGCTCCCGATT ATGAGCA	CCAAATCATCGCCTTGC GTGTGTT
25	InR1	AGACGGCAGATGAGCTT GCTAAGT	AATGGAGGCTGTTCAA GGGTCTCA
26	InR2	GACTGAACTGATAGCTG AAGTAA	CCTCAATTGAATAGAC AACATATCC
27	ILP1	AACGTGGCTACAACATG GCGTTTC	ATCCCTCGCTGCAGTAC GATTCAA

28	ILP2	GTGGCCGGACTTTAACG AAGATGT	ATCCATTGCAAATGAC TCGGAGCG
29	Akt3	GGGCTGTGGAATCAATT CAGCCAT	GCAGCCATTTCATCGG ACGTGTTA
30	fkh1	GCGGCTTCTTTCGTTCTC GTCTTT	GGGCAAAGGATCATTC TGGACGTT
31	TOR	GGACAATGGCCTCAAGT GTATGAAGC	ACCAATCGACCGACAA AGGTACGA
32	cTOR	CCACCTATCACCTATCAA CATATC	ATCTAGAAGCTGGGTG TAAATTC
33	GATAa	ACTAGAGAGCAAAGTCT GGCAGCA	CCAATGCGTCGGCCAT AGTGAAA
34	TSC2	AGTAGCGTTCAGATCAG TTGGCGA	TTGCAGACAGACGGGA AAGATGGA
35	VgR	ATGCTTCCTGCAGCCATT TGTGTC	ATCCGTAGGGCAAGCA CAAGTGTA
36	JHAMT1	TTACGTGTCCAAGATCCC ACACGA	AACGATCAAACCCGTG TAGTCCGT
37	JHAMT3	GCTCTTCGTTTGGGATTC T	CTTCTCAGAGGTGAAA GTCTAC
38	HR3	TTGACCCTGTCAACGAG ACAAGC	ATCACATGCGAGGGTT GCAAGG
39	HR4	AAGAAACCTCGATGATC TCTTAG	GAGTTCAAGGTAGAAG GGTAATC
40	hairy	AGCATCCAACCAGTGCC AGTTT	TGGTGACCATGACGTT GATCGGTA
41	BR-CZ3	AACTCACAAATCGCTTC AGCACCG	AATTTCTTGCATGCCTG ACCCACC
42	BR-CZ2	GCTGGCCTTAGAGCACA ACACTTT	AGACAGCTTGGCAGGA ACATCACA

44	Ago1	CATCGTTATTTGGAAGTGGTTC	ACCTCCTGATTTACCAATGTTC
45	Dicer1	CCATCATTTGCATTCGACATAG	GATTATGAAGTCGAGAGCTACAG
46	CLEC002262	GCGAGAAGGAAGACAAGTTC	TGGCCCAGATGAGACATAA
47	CLEC002284	GAAGGTTTGGAAGAGGACAG	TGTCGTCCGAGAGGATTT
48	CLEC004474	TAGAAGCGGCCTGTCAT	CAGTGGTTGAACTGGAAGAG
49	CLEC004479	AAGCAAGAGATGGAAGGAATAA	GGACTTGAGGTATCGGATTTC
50	CLEC006285	CCTTTCCCTTGCTCTCATAAT	CGTCCTTGAACGACCTAAAG
51	CLEC008198	GGCTCACACTTAACGGTATTTA	GCACTTTCACGAAGCATTTG
52	CLEC008462	TTGAAACGTCACATCAGGAA	CGAGGCGTTCATCGTATATC
53	CLEC009505	CCTGAGGACGTTAACAACAA	GGCATACTTGGAAGGAGTATC
54	CLEC011037	GAAACGAAGGGAAACATAGGA	GTTCGATGATTGTGGAGGAG
55	CLEC011186	GAACACTTGGCCTCCATAATC	CATCCATGTGCATTCCCTTT
56	CLEC000739	AGCAAAGGTCGCTCATTAC	GTCAGCGTTAAGGTGAACA
57	CLEC000830	GTAATGTTAAACCGCCTTCTTAC	ACTGTCTTTGGATCAGGATATG
58	CLEC002141	GAAATCTCTTCCAACCTACCTG	GTACTGTGAACTGAAGAGATCG

59	CLEC002333	GAGGTTATGGATCAGGAGGATA	CCTACGAGTACTTTCGAACTTG
60	CLEC002440	GCTTGTCACCGATGTTGAT	ATGATCTTGGCCGACTCTT
61	CLEC004439	TCTCATGCTGTTCCTTTGTC	GCGTAGGTGTTAGAGTAAGAAG
62	CLEC011191	ACCTTATCTGCTAGACAAGAATAC	CGCAGATAGTAGGACCAATTTA
63	CLEC011867	GCAGAATAAACCTAGAACCAGA	TTCACAGATCCTCGATGTAAAG
64	CLEC013155	CATACACAGCCCATTCTAGTT	CGAGTTCCTCAGCTTATTCTC
65	CLEC013246	CGATCCAAGAATTTCACCTCTA	GTGTCGACCTTGGTTCTATTC
66	Nrt	CATCAATGGCTCGTAGAAGATG	CTGAGTGGAGAGTAGTACCAATTA
67	DECad	CACAACAGGAGAGCCAAATA	CATCCTCTGGAATAGCTTCAG
68	ABCA-UE	CAAAGACCGTTCCAGGATTTA	GTATGATCCCGGGTGTATCT
69	MDR1	TACCCAGGATACTAGCAAGAG	GGAGACCAATAGAAGCGTAAATA
70	ABCC-5K	Abc5 (Zhu et al., 2013)	Abc5 (Zhu et al., 2013)
71	ABCF1	TGCCTTGGATGAGAACTTTAC	CTTTGCCTTTAGCTGCAATAC
72	ABCG23-2	Abc7 (Zhu et al., 2013)	Abc7 (Zhu et al., 2013)
73	ABCB7	GAACTTTCTCGGGTCTGTTTAT	GTAGTTATCGCAAGTGGAAGAG
74	E93	AAGCGTTGGTGAGTAAAGAG	GTTGGAAGGAGGATAGGAAATAG

Table 2S: Phenotype observed with Met mRNA knockdown in fourth instar nymphs.

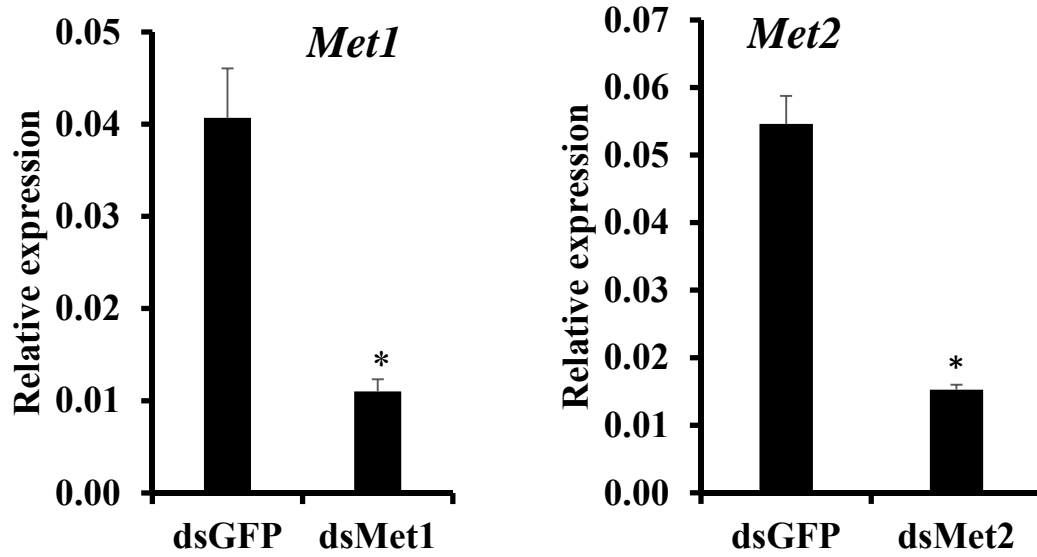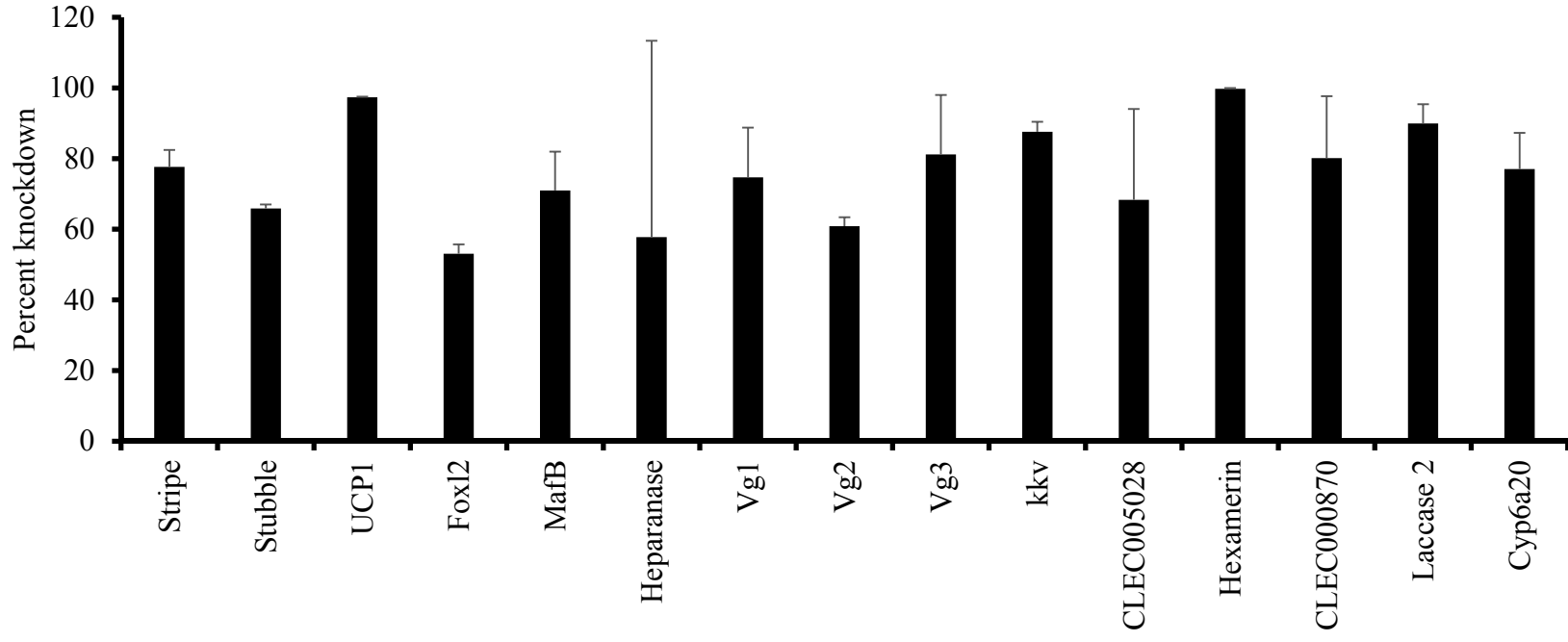| Percent	injection in 4th instar nymph		
Gene	Mortality	N5	Phenotype
malE	0	100	N5
Met	0	100	N5

Fig. S1.1: Knockdown of Methoprene tolerant protein was performed on newly molted fourth instar nymphs. The insects were incubated for five days before feeding. Total RNA was collected two days after feeding and knockdown was observed. Two different fragments of Met gene, Met1 and Met2 were used to perform knockdown separately. Each bar represents Mean + SD of three biological replicates (Star represents level of significance with Student t-test at p≤0.05).

(a)

(b)

Fig. S2.1: Identification of vitellogenin protein by MALDI TOF/TOF. Peptides showed identity with CLEC001323 (Vg2) and CLEC001325 (Vg3) proteins.

(a)

(b)
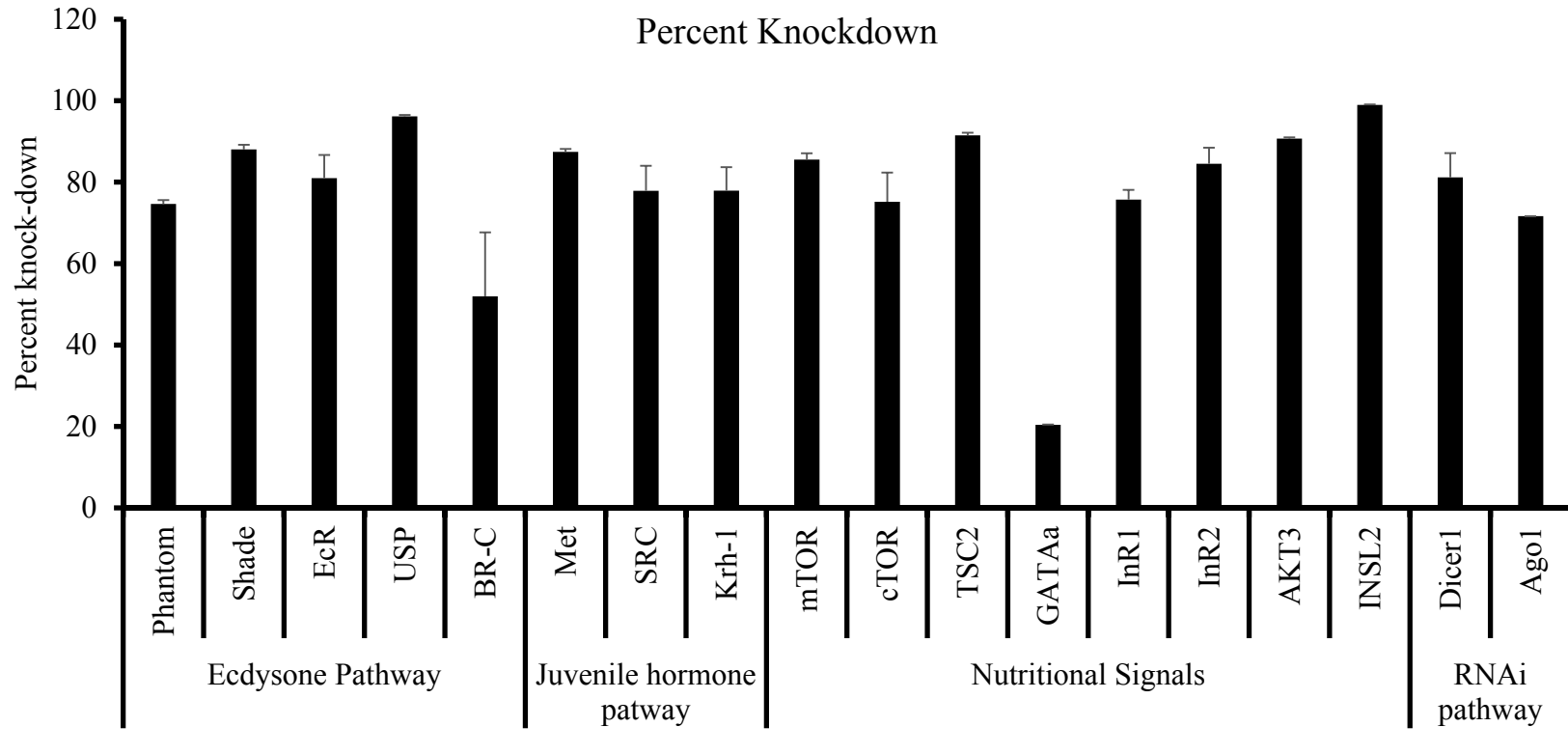
Fig. S2.2: Knockdown percentage of genes used in chapter 3.
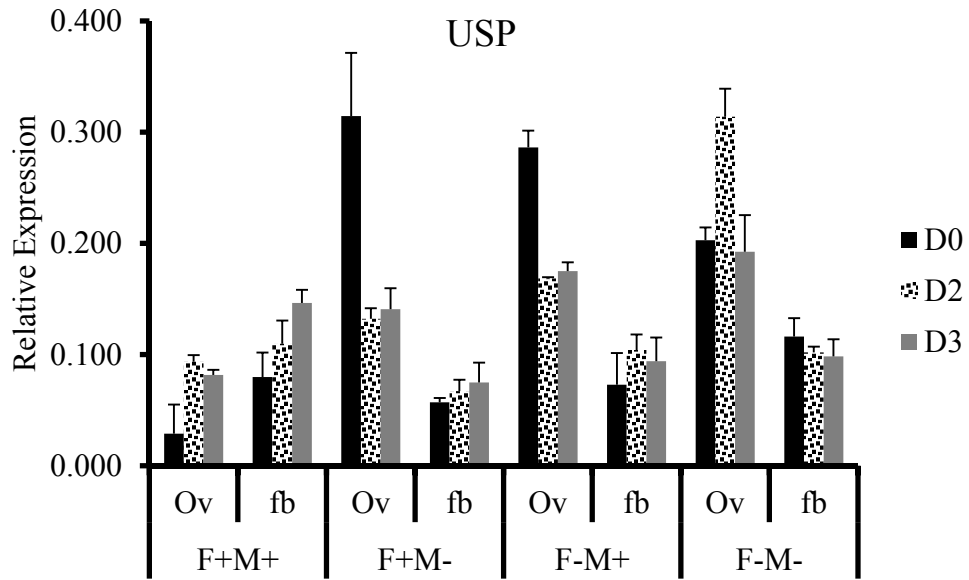
Fig. S2.3: Expression profile of genes in ovary (Ov) and fat body/ epidermis (fb) of *Cimex lectularius* after treatment at different time intervals. Female bed bugs were provided four treatments, F+M+ (blood fed and mated), F+M- (only blood fed), F-M+ (only mated), and F-M- (not blood fed and not mated). Expression profile of genes were recorded using qRT-PCR at day 0 (D0), day 2 (D2) and day 3 (D3) shown on x-axis. Expression values are normalized to ribosomal protein L8. Each bar represents mean + SD of three biological replicates.
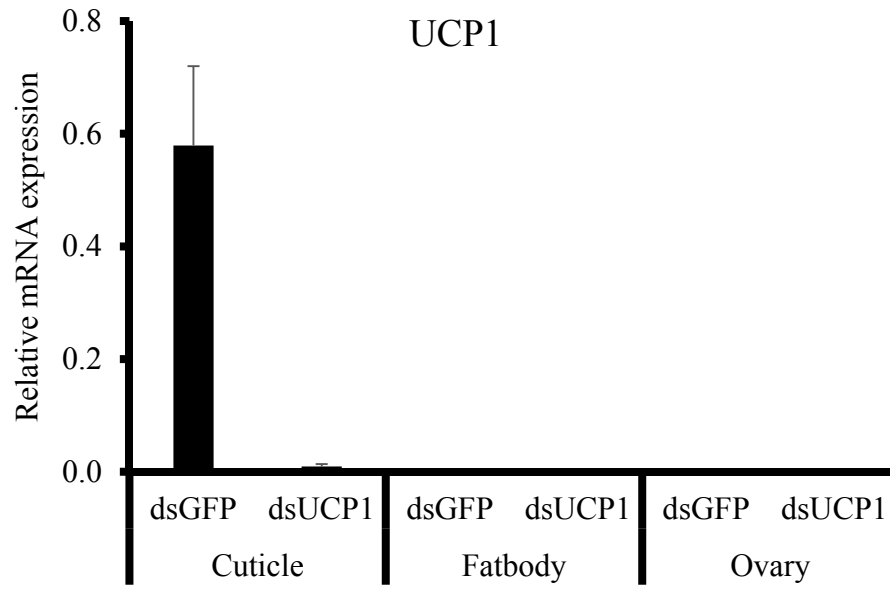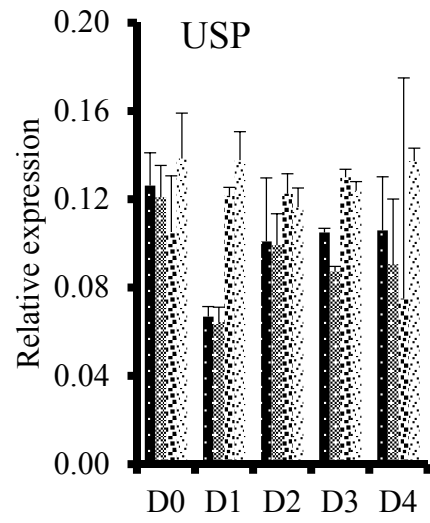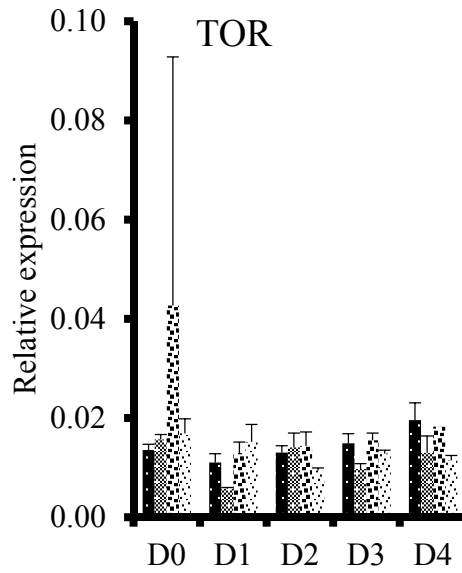
Fig. S2.4: UCP1 mRNA expression and knockdown in cuticle, fat body and ovaries in knockdown (dsUCP1) and control (dsGFP) adult female two days after feeding and mating. mRNA levels were normalized to *ribosomal protein L8* (*rpl8*). Each bar represents mean + SD of three biological replicates.
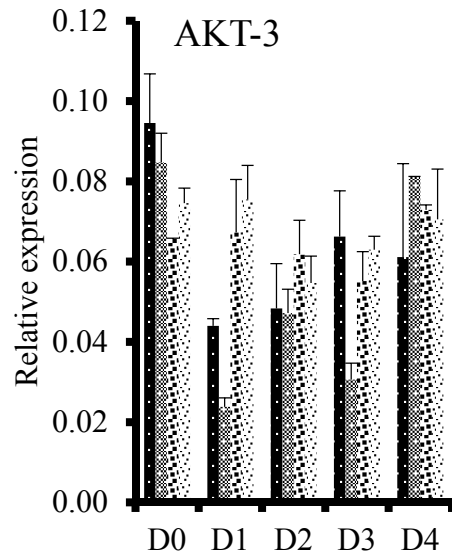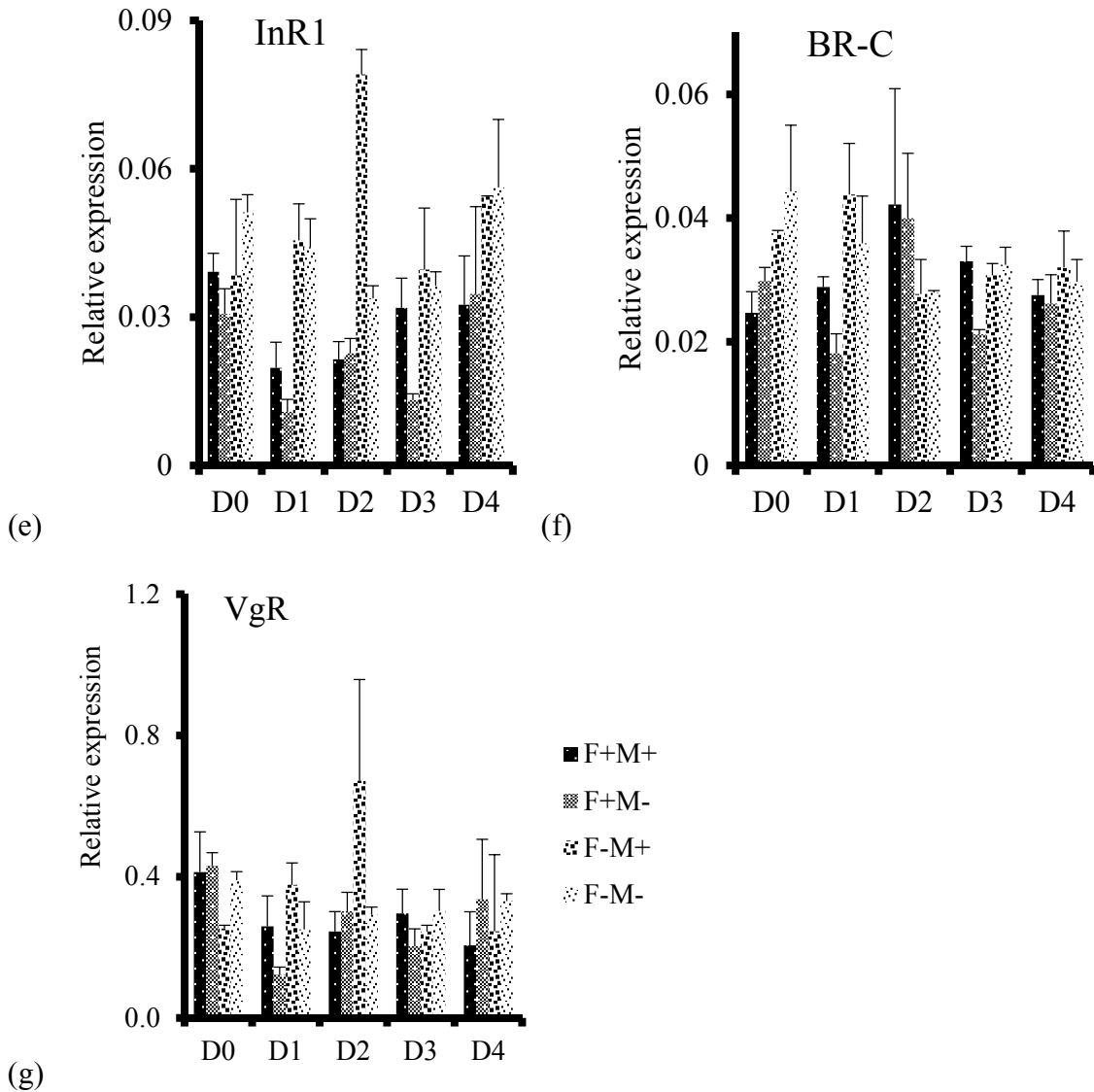
(a)

(b)

(c)

(d)

(e)

(f)

(g)
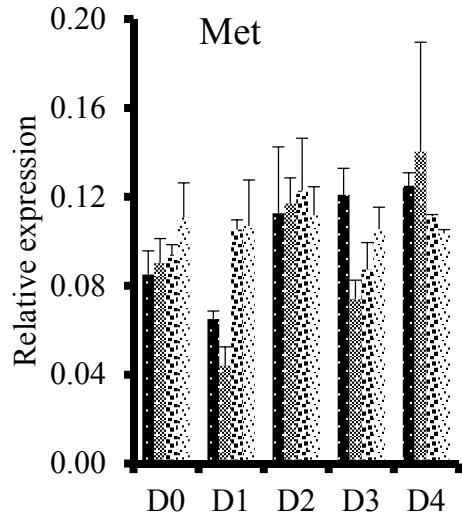
Fig. S2.5: Expression profile of genes in whole body of *Cimex lectularius* after treatment at different time intervals. Female bed bugs were provided four treatments, F+M+ (blood fed and mated), F+M- (only blood fed), F-M+ (only mated), and F-M- (not blood fed and not mated). Expression profile of genes were recorded using qRT-PCR at 24 h time interval starting from day 0 (D0) to, day 4 (D4) on x-axis. Expression values are normalized to ribosomal protein L8. Each bar represents mean + SD of three biological replicates.
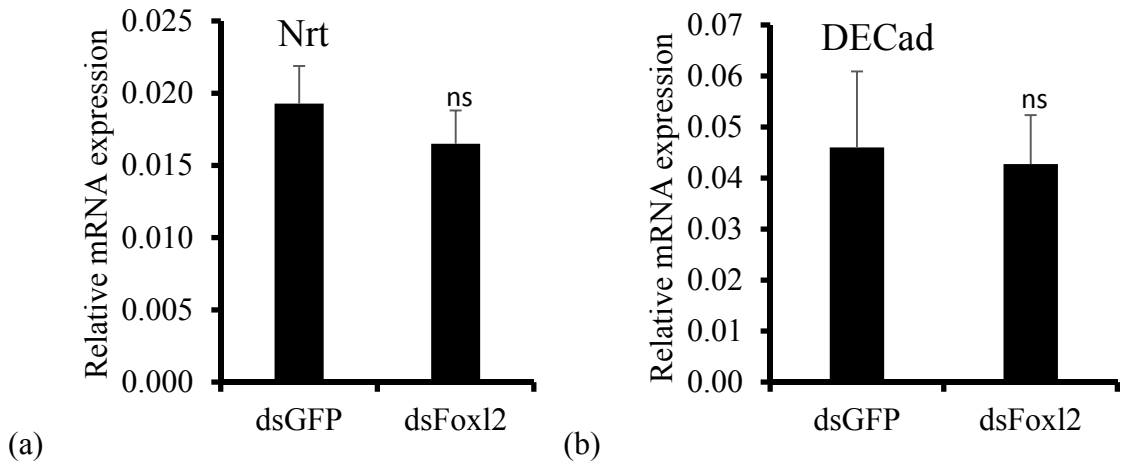
Fig. S2.6: Relative expression of Nrt and DECad mRNA in control (dsGFP) and Foxl2 knockdown insects. mRNA expression is normalized to ribosomal protein L8 (rpl8). Each bar represents mean + SD of three biological replicates. (p≤0.05).
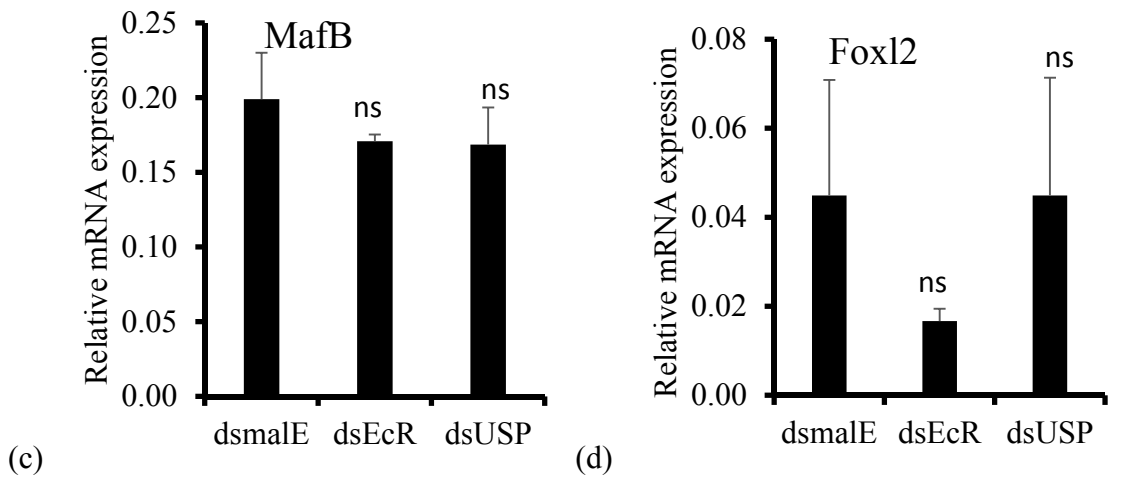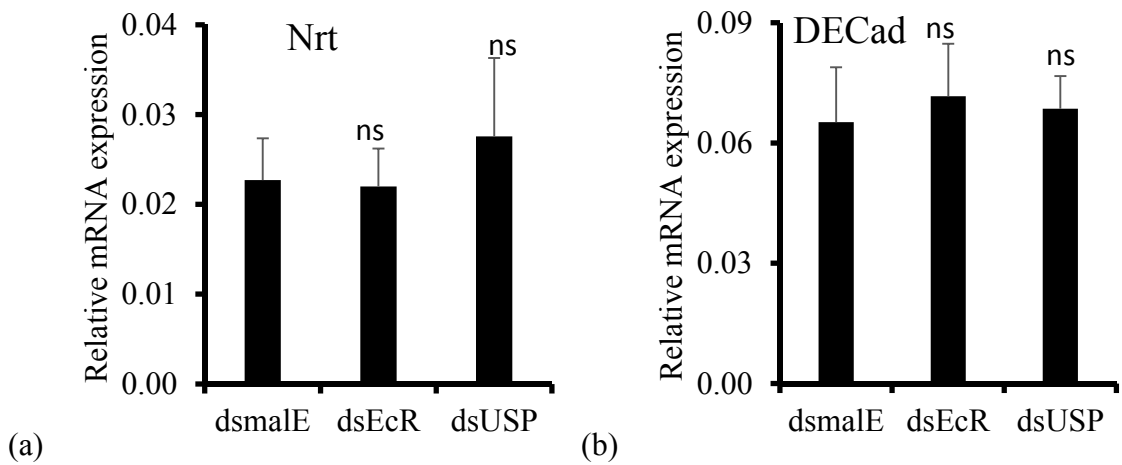
Fig. S2.7: Relative expression of Nrt, DECad, MafB, and Foxl2 mRNA in control (dsmalE) and EcR and USP knockdown insects. mRNA expression is normalized to ribosomal protein L8 (rpl8). Each bar represents mean + SD of three biological replicates. (p≤0.05).

(a) (b)
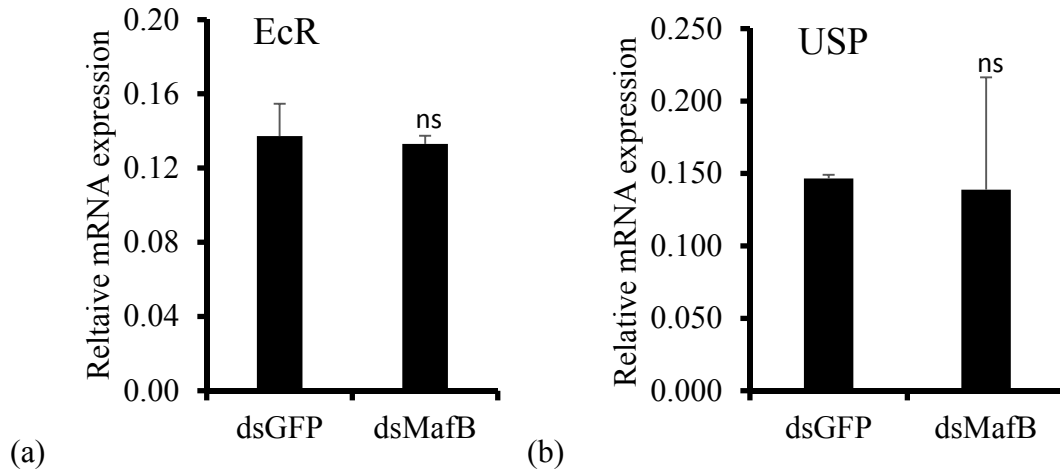
Fig. S2.8: Relative expression of EcR and USP mRNA in control (dsGFP) and MafB knockdown insects. mRNA expression is normalized to ribosomal protein L8 (rpl8). Each bar represents mean + SD of three biological replicates. (p≤0.05).

Fig. S2.9: *Foxl2* mRNA expression in male (Male_R) and female (Female_R) reproductive tissue as compared to rest of the body in male (Male_R') and female (Female_R'). mRNA levels were normalized to *ribosomal protein L8* (*rpl8*). Each bar represents mean + SD of three biological replicates.
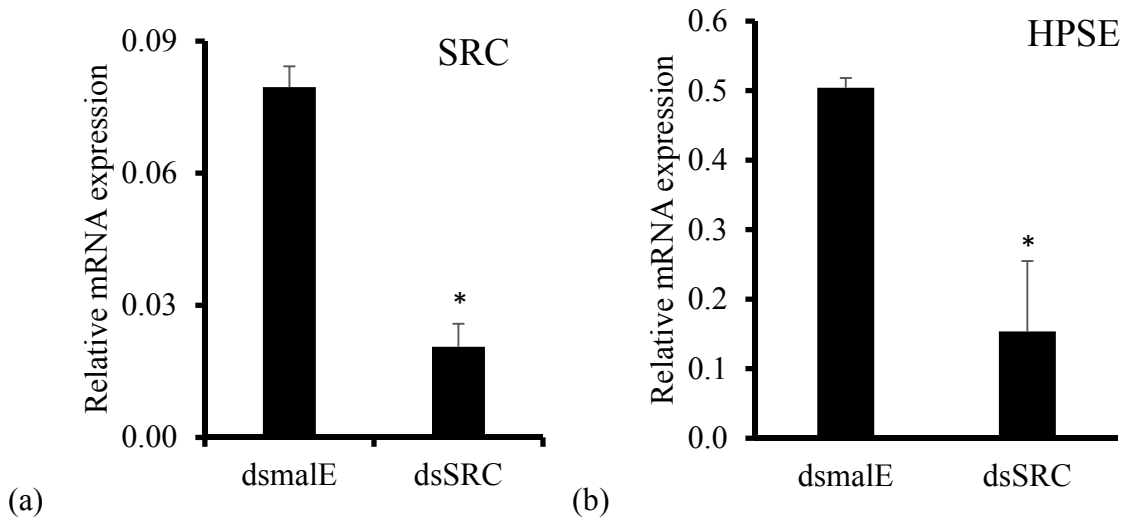
Fig. S2.10: HPSE expression in SRC knockdown female bed bugs: qRT-PCR was performed on female bed bugs after SRC knockdown. mRNA expression is normalized to ribosomal protein L8 (rpl8). Each bar represents mean + SD of three biological replicates. (p≤0.05).
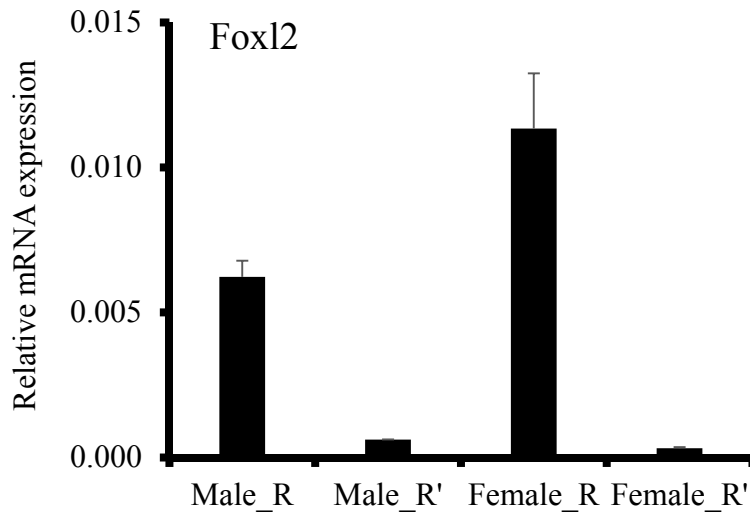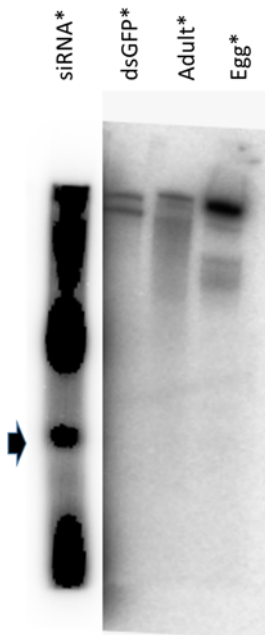

Fig. S2.11: dsRNA processing in bed bugs: P32 labelled dsGFP was injected in bed bugs. Total RNA was separated on gel to identify siRNA processing.
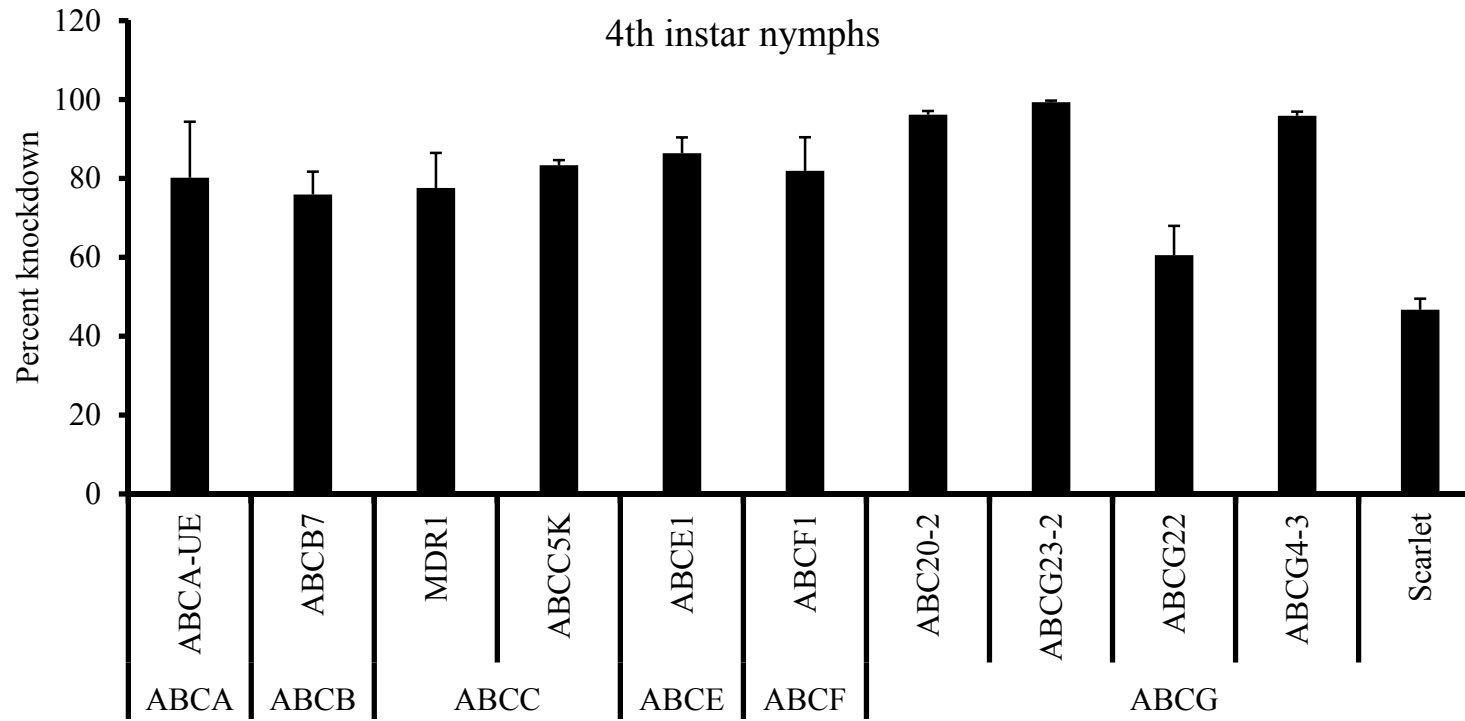
Fig. S4.1: Knockdown of ABC transporters in fourth instar nymphs.
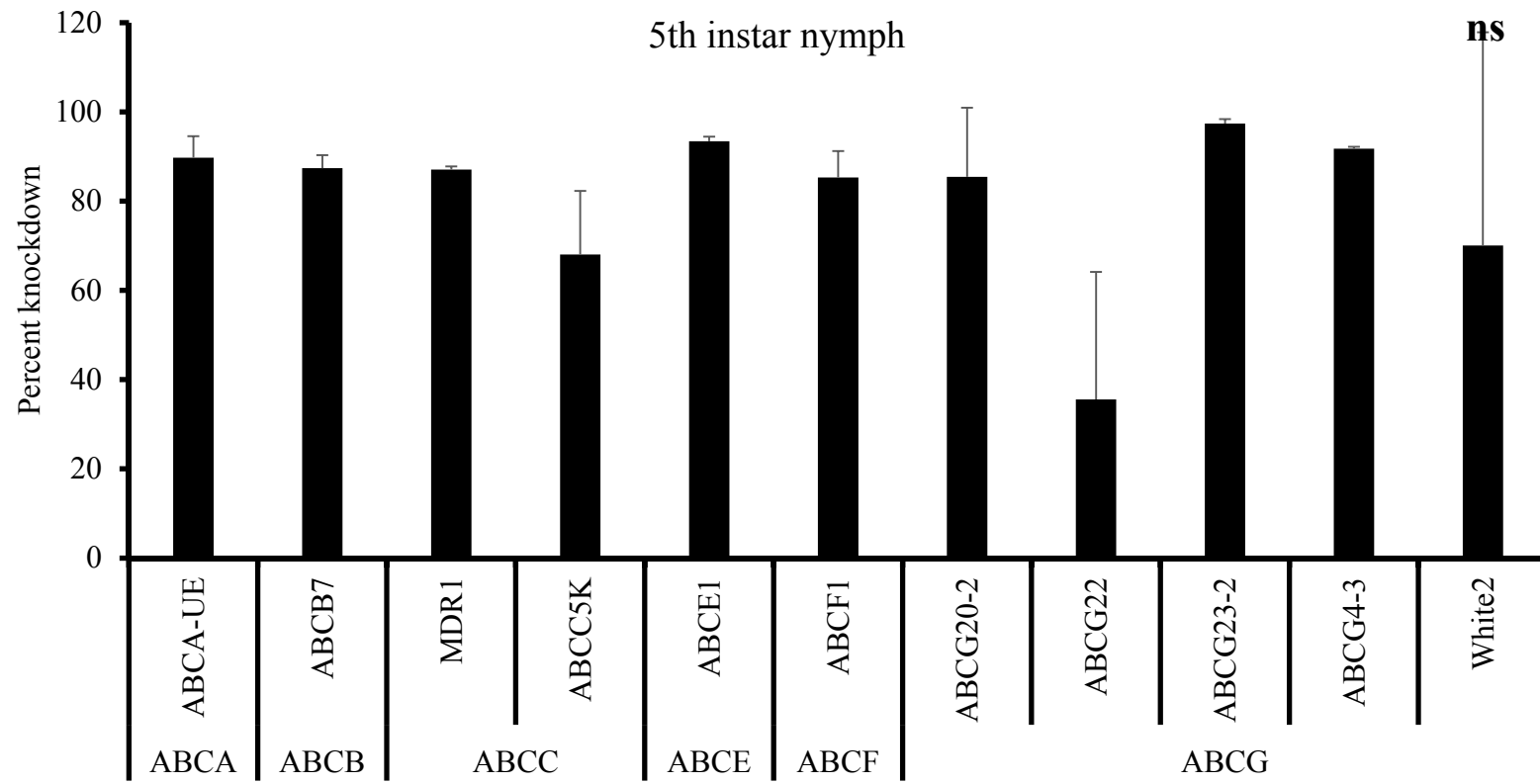
Fig. S4.2: Knockdown of ABC transporters in fifth instar nymphs.
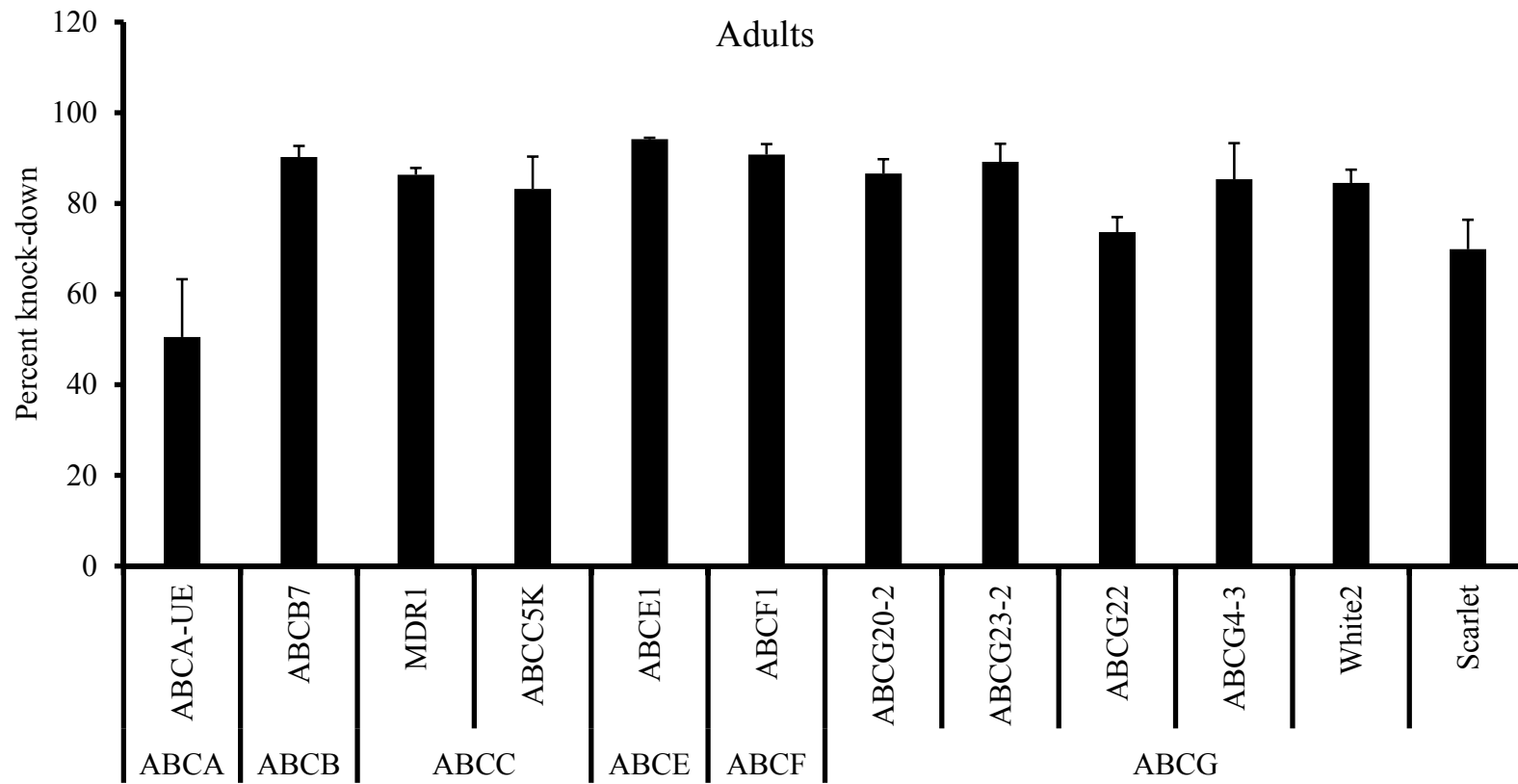
Fig. S4.3: Knockdown of ABC transporters in adults.
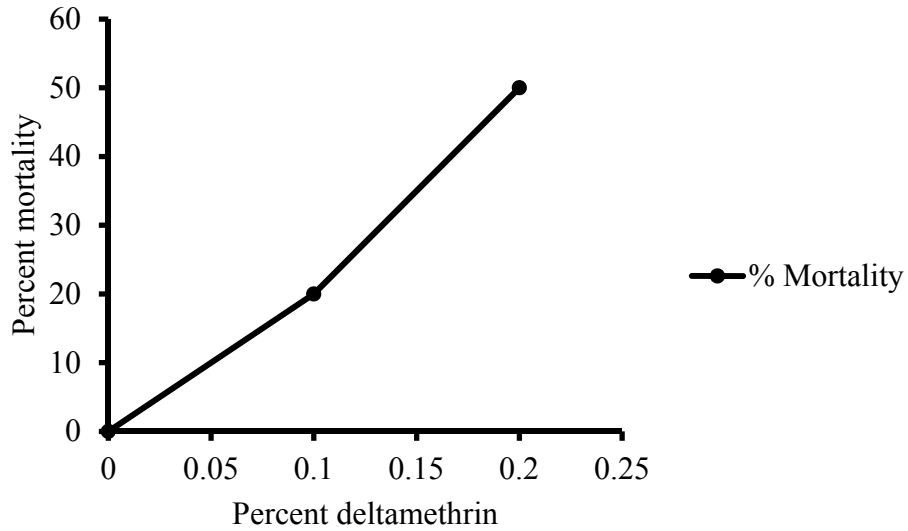
Fig. S4.4: Percent mortality vs. percent dose of technical grade deltamethrin. 50% percent mortality was achieved at 0.2% deltamethrin concentration.

Fig. S4.5: Genomic DNA was sequenced from the bed bug sodium channel to determine valine to leucine mutation (V419L) and leucine to isoleucine (L925I) mutation in the *Cimex lectularius* CIN11 population. These mutations have also been previously reported to be involved in pyrethroid resistance in bed bugs (Zhu et al., 2010).
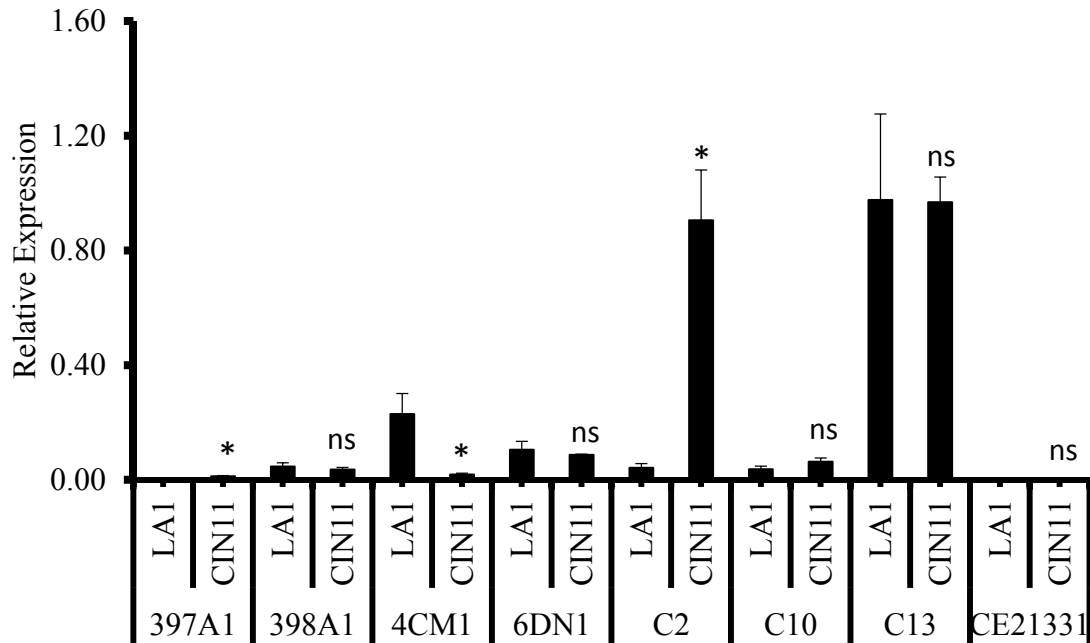
Fig. S4.6: Relative expression of genes associated with resistance in bed bugs. Relative expression of cytochrome P450 - Cyp397A1, Cyp398A1, Cyp4CM1, Cyp 6DN1, cuticular genes - C2, C10, C13 and carboxyl esterase CE21331. Expression values are normalized using ribosomal protein L8 (rpl8). (n≥3 ; p≤0.05).

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VITA

EDUCATION

1. **MSc Biochemistry** (Aug 2007 – Dec 2009). Punjab Agricultural University, Ludhiana, Punjab, India (6.94/10).

2. **BSc (Hons.) Microbiology** (June 2004 – June 2007). University of Delhi, New Delhi, India (62.07%).

PROFESSIONAL POSITIONS

1. **Research Assistant:** (2011-present 2016): University of Kentucky, Lexington, KY.

2. **Teaching Assistant**: (2014) spring semester. Department of Entomology, University of Kentucky, Lexington, KY

SCHOLASTIC AND PROFESSIONAL HONORS

1. Present's prize in Entomology: Second prize in student competition, 63rd Entomological Society of America Annual Meeting. November 2015 – Minneapolis, MN.

2. Graduate student travel award: November 2015, University of Kentucky, Lexington, KY. US$400.

PUBLICATIONS

1. Joshua B. Benoit, Zach N. Adelman, Klaus Reinhardt, Amanda Dolan, Monica Poelchau, Emily C. Jennings, Elise M. Szuter, Richard W. Hagan, **Gujar H**. *et al* (**2016**). Unique features of a global human ectoparasite identified through sequencing of the bed bug genome. *Nature Communications* 7, Article number: 10165 doi:10.1038/ncomms10165.

2. Zhu, F., **Gujar H.**, J.R. Gordon, K.F. Haynes, M.F. Potter, and S.R. Palli. (**2013**). Bed bugs evolved unique adaptive strategy to resist pyrethroid insecticides. Scientific Report 3:1456; DOI:10.1038/srep01456 (From Publishers of Nature) Note: this work gained national media attention with articles in Nature Publishing Group, National Geographic News and UK Ag News.

3. **Gujar H.** and Sohal B.S. (**2010**) Susceptibility status of diamondback moth, *Plutella xylostella* (L) to some insecticides and role of general esterases and acetylcholinesterases

in imparting resistance. *Pesticide Research Journal.* 21 (1): 50-54.

PROFESSIONAL PRESENTATOINS

1. **Gujar, H.** (2016). Hormonal and nutritional regulation of molting, metamorphosis, and reproduction in bed bugs, *Cimex lectularius*. Exit seminar presented to the Department of Entomology at the University of Kentucky, Lexington, KY.

2. **Gujar, H.** and Palli, S.R. (2015) Feeding and mating initiate juvenile hormone regulation of reproduction in bed bugs. Oral presentation. 63rd Entomological Association of America Annual Meeting, Minneapolis, MN.

3. **Gujar, H.** and Palli, S.R. (2015) Molecular analysis of juvenile hormone regulation of reproduction in the bed bug, *Cimex lectularius.* Oral presentation. "The 2015 Ohio Valley Entomological Association Annual Forum and Student Paper Competition". Lexington, KY.

4. **Gujar, H.** and Palli, S.R. (2012) Hormonal regulation of reproduction in bed bug, *Cimex lectularius.* Oral presentation. Entomological society of America Annual Meeting, Knoxville, TN.

5. **Gujar, H.** and Sohal, B.S. (2009) Esterase as a tool for monitoring insecticide resistance in *Plutella xylostella* (DBM). Poster presentation. 13th Punjab Science Congress, Punjab University, Chandigarh, Punjab, India.