UNIVERSITY OF CALIFORNIA
RIVERSIDE

Regulation of Gene Expression Patterns During Reproduction in the Female Mosquito, *Aedes aegypti*

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Cell, Molecular, and Developmental Biology

by

Lisa Kristin Johnson

December 2018

Dissertation Committee:
Dr. Alexander S. Raikhel, Chairperson
Dr. Michael E. Adams
Dr. Naoki Yamanaka

The Dissertation of Lisa Kristin Johnson is approved:

Committee Chairperson

University of California, Riverside

Acknowledgements

I would like to thank my PI Dr. Alexander Raikhel for his support, guidance and wisdom during my graduate studies at UCR. I am extremely grateful to the members of my committee: Dr. Michael Adams and Dr. Naoki Yamanaka, for their suggestions and advice, and for the time they devoted to reviewing my dissertation and serving on my defense committee. The assistance of my colleagues in the lab was invaluable. In particular, a special thank you is extended to Emre Aksoy for the countless hours and kindness with which he helped me complete this work. There are no words to adequately express the difference he made in my life. Dr. Jisu Ha became a good friend, who was always eager to assist or to listen. I'd like to thank Dr Sourav Roy and Dr. Vlastimil Smykal for their mentorship. I gained a great deal from both and realize how lucky I have been to learn from them. Keira Lucas is someone I will always count as one of my dearest friends and who added much happiness to my life. Ellie Cannel was always there for me and I am so grateful for her friendship. In, addition, many other members of the lab were essential to my success. Thank you to my family members who have helped tremendously during this journey: Nery and Belinda Beebe, Jack Thoreson, Ronnie Johnson and the Carpenter clan. Deepest thanks to Jeremy Mulford for your undying love and your constant support throughout this endeavor and for being you. You make it all worthwhile. My mom and dad are amazing and I feel like the luckiest person in the world to have them. They have always given me endless love and support. They absolutely mean the world to me and I could never thank them enough. You both believed in me and

made my success possible by teaching me to believe in myself. And finally, thank you Lola Pants for your unconditional adoration and for always being by my side.

Chapter II is a reprint, in part, of material as it appears in:

Roy, S, Saha, T T, Johnson, L, Zhao, B, Ha, J, White, K P, Girke, T, Zou, Z, Raikhel, A S (2015). Regulation of Gene Expression Patterns in Mosquito Reproduction. PLoS Genetics, 11(8), e1005450.

ABSTRACT OF THE DISSERTATION

Regulation of Gene Expression Patterns During Reproduction
in the Female Mosquito, *Aedes aegypti*

by

Lisa Kristin Johnson

Doctor of Philosophy, Graduate Program in Cell, Molecular, and Developmental Biology
University of California, Riverside, December 2018
Dr. Alexander S. Raikhel, Chairperson

Mosquitoes are the world's deadliest animals due to the link between blood feeding and disease transmission. Amino acids (AA) from a blood meal are required for initiation of egg development, a process that is central to reproductive cycles. Reproduction is governed by alternating peaks of two important insect hormones: Juvenile Hormone III (JH) and 20-hydroxyecdysone (20E). The vitellogenic, 20E-controlled phase of a reproductive cycle occurs after a blood meal and is characterized by the massive production and uptake of yolk protein precursors (YPPs). Microarray studies of the female fat body, a site of YPP synthesis, have revealed that nearly half of the genes in the *Aedes aegypti* genome are differentially regulated in discrete waves during the vitellogenic phase of a reproductive cycle. We have determined the regulatory factors responsible for each wave of up-or down-regulation by a combination of RNA interference (RNAi) and *in vitro* fat body culture (IVFBC) techniques. This study contributes to our understanding of temporal regulation of transcription during reproduction and uncovers a unique role of Ecdysone receptor (EcR) as a transcriptional

repressor. Transcriptional activation of the main YPP gene, vitellogenin, is directed by the 20E/EcR regulatory pathway and has been methodically worked out by molecular methods. However, transcriptional repression by the EcR regulatory pathway and the mechanisms by which it occurs remain a mystery. We examined the role of components of the 20E/EcR signaling cascade in transcriptional repression of a group of genes, as well as epistatic relationships of implicated pathway components using RNAi, IVFBC and cycloheximide (CHX) assays. We also addressed the involvement of co-repressor and promoter elements in 20E/EcR transcriptional repression using RNAi techniques, luciferase transfection assays, bioinformatics analysis and electrophoretic mobility shift assays (EMSA). The results of this study illuminate the complexity of gene regulation in the female mosquito during reproductive events. A detailed understanding of the nuances of gene expression during mosquito reproductive cycles is essential for development of novel strategies to control mosquito-borne disease.

Table of Contents

List of Figures

List of Tables

Chapter I

Introduction

1.1 Significance of mosquito reproduction

Vector-borne diseases are among the greatest threats to human health, causing approximately a million deaths annually. Hundreds of millions more are infected each year and left debilitated and disfigured. Roughly seventy-five percent of these deaths are caused by diseases vectored by mosquitoes, including yellow fever, dengue fever, lymphatic filariasis, malaria, West Nile virus, chikungunya and zika virus. The impact is devastating in equatorial and tropical zones but threatens subtropical and even temperate regions as well. There is a resurgence of many mosquito-borne diseases that currently lack vaccines, effective treatment or strategies for control. Even when treatment is possible, the highest incidence of infection occurs in infants and children residing in regions of extreme poverty, and therefore unable to access care (1). According to the World Health Organization (WHO), in 2015, 3.2 billion people, over half the world's population, were at risk of malaria infection, with 214 million cases and 438,000 deaths (2). In fact, in Africa, one child dies every minute from malaria alone. In recent decades, the incidence of dengue has increased dramatically. The primary vector of dengue fever, *Aedes aegypti,* is commonly known as the yellow fever mosquito, and has been found as far north as central California. Reported cases of dengue reached 2.4 million in 2015, but WHO estimates there may be 50-100 million cases of infection each year (3). The economic burden of vector-borne disease is immense, and the human suffering caused is

immeasurable. Evolution of resistance to drugs and pesticides has caused once-promising solutions to display minimized potency over time (4). Effective approaches for combating the problem are urgently needed. A detailed understanding of the molecular events underlying mosquito reproduction could help us devise novel methods to fight mosquito-borne disease in the future.

1.2 Introduction to mosquito reproductive biology

Anautogenous female mosquitoes require a blood meal to produce eggs during a gonadotrophic cycle, intimately linking reproduction to disease transmission. Amino acids (AA) and other factors in blood initiate the synthesis of hormones that drive rapid physiological changes in the principal reproductive organs: the ovaries and the fat body (FB) (5). The FB, composed of sheets of tissue lining the abdomen walls, is analogous to the vertebrate liver and adipose cells combined (6). It is a versatile and dynamic organ, with functions in energy storage, lipid metabolism, nutrient sensing, and immunity (7–10). During reproduction, the FB responds to hormonal signals by secreting massive amounts of yolk protein precursors (YPP) essential for oocyte maturation (11, 12). Vitellogenin (Vg) is the primary and most abundant YPP, and vitellogenesis is the central event of reproduction (13).

A reproductive cycle consists of two phases: previtellogenesis and vitellogenesis, and the transition between them is triggered by intake of blood (5, 14). Previtellogenesis begins upon eclosion and lasts about 72 hours. Also known as the post-eclosion (PE) stage, it is a period of growth and preparation for both the demands of reproduction and

the ability to respond to various signals (15). Behavioral changes also occur, such as receptivity to mating and host-seeking (16). At the end of the PE stage, a period of arrest continues until a suitable host is found (17). Vitellogenesis, or the post-blood meal (PBM) stage, begins upon acquisition of a blood meal, which initiates massive changes including YPP synthesis and uptake. These changes result in egg maturation and deposition, and ultimately the completion of a reproductive cycle in about 72 hours. Termination of one cycle occurs concomitantly with preparation for the next.

The reproductive cycle is controlled by alternating peaks of two important insect hormones: juvenile hormone (JH) and 20-hydroxyecdysone (20E) (18, 19). These hormones and related components of their signaling pathways play an important role in developmental transitions during immature stages and again later in the mature insect, as the main regulators of reproductive events. Interestingly, JH controls reproduction in most insects, and only dipteran insects exclusively use ecdysteroids as the primary player (12). In the mosquito, JH titer is high during the preparatory PE period, dropping sharply immediately following a blood meal (18). The titer of 20E, however, shows an opposite trend, rising after a blood meal to its maximum at about 16 hours PBM and then declining (19). At the termination of vitellogenesis, 20E levels have dropped to baseline, and JH levels surge again, setting the stage for another cycle.

JH is a uniquely structured hormone that is classified as a sesquiterpenoid. It was named for the observation that in development, it prevented larvae from proceeding through metamorphosis and maintained the juvenile state. During the previtellogenic phase of reproduction, JH acts through its receptor, Methoprene-tolerant (Met) to

upregulate genes involved in preparation and competency for vitellogenesis (15). The resulting physiological changes, in combination with nutritional cues upon acquisition of a blood meal, lead to a rapid response.

In the mosquito midgut, the availability of amino acids sends signals to the brain. The brain responds by releasing ovarian ecdysteroidogenic hormone (OEH), synthesized in medial neurosecretory cells (NSCs) during previtellogenesis and stored in the corpora cardiaca (20). Release of OEH as well as insulin-like proteins (ILPs) stimulate the follicle cells of the ovary to secrete ecdysone (E) into the hemolymph, where it is taken up by peripheral tissues and modified to the active hormone 20-hydroxyecdysone (20E) (21). The response to 20E is the massive production of vitellogenin and the moderate production of other YPPs, including lipophorin (Lp), vitellogenic carboxypeptidase (LCP) and vitellogenic cathepsin B (10, 22, 23). These YPPs are secreted into the hemolymph and subsequently internalized by the ovaries where their accumulation is vital for supporting the remaining events of reproduction. Internalization of YPPs, an essential process in nutrient uptake, occurs via receptor-mediated endocytosis (11).

Although 20E is the master regulator of vitellogenesis, there are other factors that play an important role during the PBM period. The insulin signaling pathway and the amino acid (AA) target of rapamycin (TOR) signaling pathway are activated by a blood meal and act as nutrient sensing systems (24). Insulin-like peptides (ILPs) are structurally and functionally analogous to vertebrate insulin. In drosophila, ILPs have been shown to have roles in growth, fecundity, diapause and longevity (25–29). In the mosquito, *Aedes aegypti,* there are eight ILPs and one insulin receptor (InR) which mediates their action

and is expressed in the ovaries during the PBM phase (5). They are released from the brain in response to a blood meal and signaling occurs through the PI3K pathway, resulting in phosphorylation of downstream targets (30, 31).

AAs from a blood meal are monitored by the TOR signaling pathway (5) In the fat body, AAs are taken up by FB cells, trophocytes, through the action of transporters (32). The SLC7 family of transporters are essential to this process, as shown by RNAi experiments that resulted in reduced TOR-signaling, YPP expression and egg production (33, 34). Another upstream component of the nutrient-sensing pathway is the GTPase Rheb, shown to be indispensable for AA-mediated TOR activation (35). TOR's direct action on downstream components of the nutrient-sensing pathway play a significant role in the level of YPP gene expression. TOR's direct phosphorylation of the S6 kinase results in translation of a GATA transcription factor that binds directly to the vg promoter, replacing a negative GATA factor and resulting in a high level of expression (5, 36–38). Another downstream target that acts as a translational repressor is 4E-BP, which becomes hyper-phosphorylated after a blood meal. However, blocking this action by TOR is not sufficient to block hyper-phosphorylation (39, 40).

1.3 Molecular biology of mosquito vitellogenesis

Much of what we know about how the 20E regulatory pathway operates has been garnered from several decades of research in drosophila (41). Ashburner, in a landmark study in 1974, observed chromosomal puffing patterns in the polytene chromosomes of drosophila in response to a pulse of 20E (42). This visualization of the sequential

activation of regional gene transcription led him to propose that 20E activates temporally distinct cascades of gene up-regulation, in which the products of early genes in the hierarchy act as transcription factors (TFs) that regulate the expression of later genes. This idea became what is known as the Ashburner model, which was experimentally confirmed and expanded through subsequent studies over the next several decades.

Another breakthrough came in 1991 with the identification of EcR as the functional receptor for the 20E signal (43). EcR is a nuclear receptor (NR) that, upon activation by its ligand, physically interacts with gene regulatory regions of DNA to induce a transcriptional response. Identification and characterization of other components of the 20E pathway followed, and while this accumulated knowledge has provided a solid foundation, 20E-controlled reproduction in anautogenous mosquitoes is unique in the requirement of a blood meal for initiation of these events. Historically lacking the advanced molecular toolkit available for drosophila research, gonadotrophic processes in the mosquito were deciphered primarily through reverse genetic techniques, and the activation of vg expression by the 20E pathway served as an excellent model for these studies.

EcR, which is orthologous to the vertebrate farnesoid-X receptor, heterodimerizes with another NR ultraspiracle (USP), ortholog of the vertebrate retinoid-X receptor, to form a functional receptor complex (44). A hydrophobic pocket in the ligand-binding domain (LBD) of EcR binds 20E with high affinity (45, 46). Binding of 20E by EcR results in a conformational change that reveals a short region of the DNA-binding domain (DBD). This region recognizes and binds to a RE, made up of a complementary sequence

of nucleotides within DNA, allowing direct interaction between the hormone/receptor complex and the regulatory region of a target gene. Several EcR/USP REs are found in the vg promoter, contributing to the high level of activation that is observed (47). Like many NRs, both EcR and USP are alternately spliced resulting in their existence as isoforms that have distinct spatial and temporal expression profiles and have diverged in function. In *Aedes aegypti*, there are two isoforms for both EcR, designated EcR-A, EcR-B, and USP, designated USP-A and USP-B (48, 49).

The early genes in the 20E regulatory hierarchy encode products that enhance or restrict the transcription of downstream genes. Some of these TFs act by direct interaction with DNA or the 20E/EcR complex, but they can also act by recruiting other cofactors. One of these early genes codes for E74, belonging to the ETS family of TFs, which was shown in drosophila to be essential for stage- and tissue-specific expression of 20E during metamorphosis (50). There are two transcripts in *Aedes aegypti*: E74A, expressed during the termination stage of vitellogenesis, and E74B, expressed during the peak of 20E titer (51). E74A has no effect on the expression of vg, but E74B acts synergistically with EcR to activate vg expression by direct binding both to the EcR complex as well as to the E74 REs found in the vg promoter (52, 53). This interaction results in high levels of vg transcription.

E75 is an ecdysone-inducible early gene that codes for a NR that is homologous to the vertebrate Rev-erb NR. During *D melanogaster* development, E75 is required for ecdysteroidogenesis (54) It has been demonstrated that E75 is a receptor for nitric oxide (NO) (55, 56), and therefore may be involved in the sensing of oxygen supply. It was also

reported that E75 acts as a repressor by competing with EcR/USP during development by exclusion from gene promoters due to overlapping REs (57). The E75 group of transcripts produce three isoforms in *Aedes aegypti* designated E75A, E75B and E75C (58). RNAi experiments have demonstrated that they are also differentially involved in the specificity of the 20E response: E75A was shown to elevate vg expression while E75C has the opposite effect (59). E75 reversibly binds heme as its ligand, reinforcing the blood meal activation of 20E (56). E75A also heterodimerizes with the NR HR3, blocking its activating effect (60). E75B lacks the ability to bind DNA. In *Aedes aegypti,* all three isoforms display similar expression profiles during early vitellogenesis (59).

The Broad (Br) family of transcription factors are C2H2-type zinc finger molecules that act by binding DNA. There are four apparent isoforms in *Aedes aegypti:* Z1, Z2, Z3 and Z4. With the exception of Z3, all are expressed early in response to a blood meal and enhance the transcription of the other early genes (61–63). The vg promoter contains response elements for broad isoforms, with Z1 and Z4 repressing vg expression and Z2 displaying an activating effect (61).

Another NR that plays an important role in 20E-directed regulation is hormone receptor 3 (HR3). It has been shown that HR3 directs the timing of transitions during immature stages in drosophila and plays a similar role during mosquito vitellogenesis (64). It drives the expression of the NR βFTZ-F1 which determines competence for a 20E response (65). βFTZ-F1 recruits the histone acetyltransferase p160/SRC (FISC) to the activated EcR complex and the acetylation of local chromatin drives a high level of vg transcription (66). HR3 is highly expressed between 24-36h PBM and acts to attenuate *vg*

expression before the termination of vitellogenesis (64). Again, the sequential expression pattern of HR3 and βFTZ-F1 is observed, with βFTZ-F1 controlling the timing of entry into a subsequent reproductive cycle (66).

The preceding examples illustrate the extensive work that has been applied to understanding the coordinated actions of EcR and the early gene TFs in the 20E hierarchy. These factors drive successful reproduction by orchestrating each successive stage: from initiation of vitellogenesis when conditions are right, to the timely termination of vitellogenenic events. This elegant system of signaling is used reiteratively during the most vital processes of both immature and adult life stages in response to different hormonal, nutritional and environmental cues. The control of gene expression is central to life. However, in teasing out the mechanisms of gene regulation, the complexity becomes apparent. The use of a limited set of tools to generate many responses to the integration of various conditions and signals requires a dynamic system of regulation.

1.4 Regulation of Gene Expression

As we have seen with the well-studied example of vitellogenesis during female mosquito reproduction, organisms possess the intrinsic ability to alter molecular and genetic activity when it is necessary to produce protein. Gene expression changes occur in response to environmental stimuli such as the lack or availability of nutrients, in order to maintain homeostasis, and to execute large-scale biological transitions such as

embryonic development or reproduction, all requiring complex programs of sequential gene up- or down-regulation. Alterations in normal gene expression patterns can result in abnormalities that lead to disease, abnormal development or death of the organism. How are the vast number of signals that are received transduced and integrated by a limited number of factors into accurate transcriptional responses? Although genetic output can be regulated at many levels, including chromatin domain, transcription, RNA transport, mRNA degradation, translation and post-translational modifications, the vast majority of gene regulatory events occurs at the level of transcription, and at the heart of transcriptional regulation is the activity of receptors. The activating or deactivating of receptors initiates cascades of modulations driven by the receptor directly, or through intermediaries that are directly affected by the receptor. There are multiple receptor types, but here we will focus on NRs.

NRs, like EcR and USP, form a superfamily of highly conserved proteins that share common structural and functional features and are activated by the binding of non-polar regulatory molecules that can cross the plasma membrane, including hormones, retinoic acid, fatty acids, phospholipids vitamins, and even small gas molecules and heme. (44, 55–57, 67). The NR model of genetic regulation began to form during the 1960s, primarily based on Ashburner's observations and the work of Wigglesworth in the field of insect physiology (68). These early studies sparked several decades of research that supported and expanded the NR gene regulatory model. The field was energized by the cloning of the glucocorticoid (GR) and estrogen receptors (ER) in the mid-1980s, which created a flurry of NR discovery and characterization. The high degree of

structural homology, underlying a conservation of sequence and modularity was indicative of a protein with vital biological importance, and indeed, NRs today make up 13% of pharmaceutical drug targets (69). Ultimately, 48 NRs in the human genome were cloned as well as homologous counterparts in other organisms. They have been found to exist in all metazoans and although highly conserved, have branched out in some species due to isoform formation (the worm *C. elegans* has 284 NRs compared to human's 48). NRs are composed of several distinct, modular domains, with the highest degree of conservation located within the DBD and the LBD (70). A linking region between the DBD and the LBD and the amino terminal region display much greater variability in sequence (44). The ligand-receptor relationship is highly specific and is based on tertiary structure resulting from nucleotide sequence of segments of the LBD. Folding of the receptor results in the formation of a ligand-binding pocket with specificity for a particular agonist, however, there are a number of NRs known as orphan receptors for which a ligand has not been identified. The DBD contains regions of sequence complementarity to REs within target gene promoters. These facilitate a physical connection between the receptor and specific genes, linking the activity of the receptor to gene expression. There are two additional features in NRs known as activation function 1 (AF1) and activation function 2 (AF2). AF1 is ligand-independent and contributes to relatively weak transactivation. AF2 is involved in ligand-dependent transactivation and has additional roles in dimerization and co-regulator interaction (69). NRs frequently homo- or hetero- dimerize with other NRs to form a functional receptor complex and are divided into classes based on differences in dimerization as well as cellular localization.

REs, generally located within the 5' promoter region of genes, act as allosteric modulators of gene expression. A cluster of genes containing a particular RE can therefore be activated by a common signal, a necessity during biological transitions. These palindromic sequences occur as direct or inverse repeats and display varying degrees of degeneracy. EcR/USP binding is fairly promiscuous compared to other receptors, and binding to a number of imperfect versions of its RE has been reported. This variability probably contributes to fine-tuning of the transcriptional response. NRs also modulate their activity through the recruitment of co-regulators. These molecules alter the stability of complexes at response elements by creating a stable platform for additional co-factor docking and by affecting the interaction between receptors, response elements and general transcription factors (71). Co-regulators often possess chromatin-modifying activity, and the addition or transfer of methyl or acetyl groups to local histones alters the environment to favor or restrict gene transcription.

The classic paradigm of NR signaling states that the binding of ligand is the switch that turns the receptor on. Normally, in the off position, the receptor complex, with an empty ligand-binding pocket, is associated with inhibitory elements such as co-repressors. Ligand-binding turns the switch on, by affecting a conformational change in the receptor. This conformational change allows the release of inhibitory elements and their replacement by recruited activating elements. The entire activating complex interacting with DNA upregulates the transcription of target genes. For much of observed NR activity, this model provides a good basic explanation. In recent years, however, one exception after another has emerged in which the binding of a liganded receptor complex

to its RE results in the repression of target genes rather than activation (72). Once exceptions, recent genome-wide studies have concluded that nearly half the genes regulated by NR ligands are downregulated (73). Aligning these differences with the accepted model has proved to be a difficult task. However, some progress has been made in understanding negative regulation by NRs.

1.5 Negative regulation

One of the earliest examples of negative gene regulation was the down-regulation of specific target genes by the ligand-bound glucocorticoid receptor (GR) (73, 74). Then, repression of thyroid-stimulating hormone (TSH) by the thyroid receptor (TR) was found to occur in the presence of its ligand T3 (75, 76). Other cases followed, where genes appeared to be repressed in the presence of ligand-bound NRs and in some cases, a ligand-bound receptor appeared to be responsible for the up- and down-regulation of two different sets of genes simultaneously (67, 77, 78).

NRs often have several isoforms that display different expression profiles or are expressed in different tissues, and have adopted different, sometimes opposing functions in regulating gene expression. Thyroid hormone receptor β-2 (TR β-2) is restricted to the central nervous system and was found to play a unique role in negative regulation of target genes by thyroid hormone (T3). Different isoforms of TR were found to have different affinities for co-regulatory elements, resulting in differential transcriptional potential (79–82). In human breast cancer cells, target genes were uniquely regulated by

two isoforms of the progesterone receptor (PR) (83). Components of signaling pathways such as co-regulators also may have different isoforms with distinct functions. For example, the ecdysone signaling pathway is mediated by transcription factor isoforms that have differing roles in regulation. In the expression of vg, E75A acts as an activator while its isoform E75C acts as a repressor (84). The number of interacting isoforms combined with the sequential nature of signal transduction pathways suggests that differences in regulation of members of the signaling pathway may play a role in variation of NR activity. In addition to isoform variation, the epistatic relationship between factors in a signaling pathway can influence the outcome, with autoregulatory and feedback loops complicating the matter even more.

REs are an additional variable element found to direct up- vs. down- regulation. REs that differ slightly in nucleotide sequence may preferentially attract positive or negative regulatory complexes (85, 86). It was reported that the liganded glucocorticoid receptor induced repression directly by binding to negative response elements (nREs) unrelated to GR positive REs. The GR nRE was found in over 1000 mouse/human ortholog genes that are repressed *in vivo* (78). Other studies involving repression of thyroid stimulating hormone (TSH) gene identified a nTRE in the proximal promoter, between the TATA box and the transcriptional start site. Here it was proposed that repression was the result of steric interference between the hormone/receptor complex and components of the transcriptional machinery (87). Binding sites for TR and the strongly activating transcription factor Sp1 overlap in the beta-amyloid precursor protein (APP) promoter, suggesting that nREs function through both promoter interference and

competition mechanisms (88). T3 binding facilitated the formation of TR-RXR heterodimers, which bind the TRE, thereby displacing SP1 from the overlapping sequences. The proximity of response elements to the transcription start site can also lead to steric hindrance and preferential binding. However, the difference in sequence alone between n- and p- REs can profoundly influence transcriptional outcome. Different GR binding sites require or exploit different activation domains within the receptor, and even a single base-pair change influences GR conformation (89). Many studies have now demonstrated that the organization and number of response elements within promoters is a significant determinant of regulatory outcome (73, 79, 90–96).

The recruitment of co-factors and their effect on chromatin structure can influence the choice between activation and repression. Recruitment of cofactors occurs in specific sequences with even a slight deviation in order resulting in a different effect (97). In addition, the distinction between co-activators and co-regulators has become blurred in recent years. RIP140, a co-regulator that directs development, metabolism and other key processes, has properties of both a co-repressor and a co-activator (98). SRC1, long classified as a co-activator, plays a repressive role in TR-mediated gene regulation. Conversely, the classical co-repressor NCor/SMRT has been shown to facilitate the activation of target genes (71, 73, 99, 100). Cofactors that act as chromatin modifiers, such as histone acetyltransferases and methyltransferases, further influence gene expression by altering the degree of accessibility of target genes (101, 102).

There are numerous ways in which regulation of gene expression by hormone NRs can be modified and structuring a complete model that accounts for these

differences will be challenging. It is essential to understand the mechanistics of all aspects of gene regulation, even those that seem to be exceptions, to gain a complete view of biological complexity and the elegant methods that biological systems employ to adapt to that complexity.

1.6 Dissertation objectives and aims

Regulation of gene expression patterns during each reproductive cycle of the female mosquito, *Aedes aegypti*, is directed by rising and falling levels of 20E and JH, in addition to nutritional cues. Clusters of specific genes are sequentially expressed or inhibited at precise timepoints to ensure that reproduction is successfully carried out. Similarly-expressed gene clusters may be similarly-regulated; either directly by hormone/receptor interaction, or indirectly through hormone/receptor interaction with a molecular middleman. The factors responsible for each cluster of temporally-regulated genes during a reproductive cycle were previously unknown and are the focus of Chapter Two. A cluster of genes that peak in expression at 36h PBM exhibit an expression profile that is inversely correlated to the 20E titer profile. This suggests that 20E, acting through its receptor, EcR, negatively regulates these genes. If and how negative regulation is exerted, however, whether directly or indirectly, and which transcription factor isoforms are involved are unresolved issues that will be discussed in Chapter Three. This prospective ability of 20E and EcR to play both activating and repressive roles would allow a limited number of factors to control a wide array of responses. How the switch is flipped from up- to down-regulation when needed is unknown, and this complexity of

regulatory conservation and the mechanism by which it occurs are questions approached in Chapter Four. Negative regulation is a subject that remains unclear and merits further examination. The information gleaned from these studies could contribute to our understanding of NR action and potentially be used to develop novel methods of controlling reproduction in the world's primary disease vector. A more thorough understanding of how gene expression is directed during all phases of reproduction is a promising path to reducing disease transmission by mosquitoes and significantly reducing human suffering.

1.7 References

1. Bhatt S, et al. (2013) The global distribution and burden of dengue. *Nature* 496(7446):504–507.
2. WHO (2017) Global Vector Control Response - Background document to inform deliberations during the 70th session of the World Health Assembly. *Who* 2030:47.
3. World Health Organization, Dengue (2017) *SEARO*.
4. Matthews KR (2011) Controlling and Coordinating Development in Vector-Transmitted Parasites. *Science (80-)* 331(6021):1149–1153.
5. Hansen IA, Attardo GM, Rodriguez SD, Drake LL (2014) Four-way regulation of mosquito yolk protein precursor genes by juvenile hormone-, ecdysone-, nutrient-, and insulin-like peptide signaling pathways. *Front Physiol* 5 MAR(March):1–8.
6. Raikhel A (2004) Vitellogenesis of disease vectors, from physiology to genes. *Biology of Disease Vectors*, ed WC M (Elsevier - Academic Press), pp 329–346.
7. Zhang Y, Xi Y (2014) Fat Body Development and its Function in Energy Storage and Nutrient Sensing in Drosophila melanogaster. *Tissue Sci Eng* 6(1):1–8.
8. Klowden MJ, Davis EE, Bowen MF (1987) Role of the fat body in the regulation of host-seeking behaviour in the mosquito, Aedes aegypti. *J Insect Physiol* 33(9):643–646.
9. Klowden MJ (1997) Endocrine aspects of mosquito reproduction. *Arch Insect Biochem Physiol* 35(4):491–512.
10. Hays AR, Raikhel AS (1990) A novel protein produced by the vitellogenic fat body and accumulated in mosquito oocytes. *Roux's Arch Dev Biol* 199(2):114–121.
11. Raikhel AS, Dhadialla TS (1992) Accumulation of yolk proteins in insect oocytes. *Annu Rev Entomol* 37(1):217–251.
12. Raikhel AS, Brown MR, Belles X (2005) Hormonal control of reproductive processes. *Compr Mol insect Sci* 3(1):433–491.
13. Raikhel AS, Lea AO (1983) Previtellogenic development and vitellogenin synthesis in the fat body of a mosquito: An ultrastructural and immunocytochemical study. *Tissue Cell* 15(2):281–299.
14. Attardo GM, Hansen IA, Raikhel AS (2005) Nutritional regulation of vitellogenesis in mosquitoes: Implications for anautogeny. *Insect Biochem Mol Biol* 35(7):661–675.
15. Zou Z, et al. (2013) Juvenile hormone and its receptor, methoprene-tolerant,

control the dynamics of mosquito gene expression. *Proc Natl Acad Sci* 110(24):E2173–E2181.

16. Clements A (1992) *The biology of mosquitoes: Development, Nutrition and Reproduction.* doi:10.1016/0169-4758(93)90183-G.

17. Sappington TW, Raikhel AS (1998) Molecular characteristics of insect vitellogenins and vitellogenin receptors. *Insect Biochem Mol Biol* 28(5–6):277–300.

18. Shapiro AB, et al. (1986) Juvenile hormone and juvenile hormone esterase in adult females of the mosquito Aedes aegypti. *J Insect Physiol* 32(10):867–877.

19. Hagedorn HH, et al. (1975) The ovary as a source of alpha-ecdysone in an adult mosquito. *Proc Natl Acad Sci U S A* 72(8):3255–9.

20. Lea AO (1972) Regulation of egg maturation in the mosquito by the neurosecretory system: the role of the corpus cardiacum. *Gen Comp Endocrinol* 3:602–608.

21. Raikhel AS, Roy S, Saha TT, Zou Z (2017) Regulatory Pathways Controlling Insect Reproduction. *Annu Rev Entomol* 63(1).

22. Cheon HM, Seo SJ, Sun J, Sappington TW, Raikhel AS (2001) Molecular characterization of the VLDL receptor homolog mediating binding of lipophorin in oocyte of the mosquito Aedes aegypti. *Insect Biochem Mol Biol* 31(8):753–760.

23. Cho WL, et al. (1999) Mosquito cathepsin B-like protease involved in embryonic degradation of vitellin is produced as a latent extraovarian precursor. *J Biol Chem* 274(19):13311–21.

24. Colombani J, et al. (2003) A nutrient sensor mechanism controls Drosophila growth. *Cell* 114(6):739–49.

25. Okamoto N, Yamanaka N (2015) Nutrition-dependent control of insect development by insulin-like peptides. *Curr Opin Insect Sci* 11:21–30.

26. Okamoto N, et al. (2009) A Fat Body-Derived IGF-like Peptide Regulates Postfeeding Growth in Drosophila. *Dev Cell* 17(6):885–891.

27. Smykal V, Raikhel AS (2015) Nutritional control of insect reproduction. *Curr Opin insect Sci* 11:31–38.

28. Sim C, Denlinger DL (2013) Insulin signaling and the regulation of insect diapause. *Front Physiol* 4:189.

29. Wu Q, Brown MR (2006) SIGNALING AND FUNCTION OF INSULIN-LIKE PEPTIDES IN INSECTS. *Annu Rev Entomol* 51(1):1–24.

30. Riehle MA, Brown MR (1999) Insulin stimulates ecdysteroid production through a

conserved signaling cascade in the mosquito Aedes aegypti. *Insect Biochem Mol Biol* 29(10):855–60.

31. Hansen IA, Attardo GM, Roy SG, Raikhel AS (2005) Target of rapamycin-dependent activation of S6 kinase is a central step in the transduction of nutritional signals during egg development in a mosquito. *J Biol Chem* 280(21):20565–20572.

32. Attardo GM, Hansen IA, Shiao S-H, Raikhel AS (2006) Identification of two cationic amino acid transporters required for nutritional signaling during mosquito reproduction. *J Exp Biol* 209(Pt 16):3071–3078.

33. Hansen IA, et al. (2011) AaCAT1 of the yellow fever mosquito, Aedes aegypti: a novel histidine-specific amino acid transporter from the SLC7 family. *J Biol Chem* 286(12):10803–10813.

34. Carpenter VK, et al. (2012) SLC7 amino acid transporters of the yellow fever mosquito Aedes aegypti and their role in fat body TOR signaling and reproduction. *J Insect Physiol* 58(4):513–22.

35. Roy SG, Raikhel AS (2011) The small GTPase Rheb is a key component linking amino acid signaling and TOR in the nutritional pathway that controls mosquito egg development. *Insect Biochem Mol Biol* 41(1):62–69.

36. Martín D, Piulachs MD, Raikhel a S (2001) A novel GATA factor transcriptionally represses yolk protein precursor genes in the mosquito Aedes aegypti via interaction with the CtBP corepressor. *Mol Cell Biol* 21(1):164–174.

37. Attardo GM, Higgs S, Klingler KA, Vanlandingham DL, Raikhel AS (2003) RNA interference-mediated knockdown of a GATA factor reveals a link to anautogeny in the mosquito Aedes aegypti. *Proc Natl Acad Sci U S A* 100(23):13374–13379.

38. Park J-H, Attardo GM, Hansen IA, Raikhel AS (2006) GATA factor translation is the final downstream step in the amino acid/target-of-rapamycin-mediated vitellogenin gene expression in the anautogenous mosquito Aedes aegypti. *J Biol Chem* 281(16):11167–11176.

39. Hay N, Sonenberg N (2004) Upstream and downstream of mTOR. *Genes Dev* 18(16):1926–1945.

40. Roy SG, Raikhel AS (2012) Nutritional and hormonal regulation of the TOR effector 4E-binding protein (4E-BP) in the mosquito Aedes aegypti. *FASEB J Off Publ Fed Am Soc Exp Biol* 26(3):1334–1342.

41. Yamanaka N, Rewitz KF, O'Connor MB (2013) Ecdysone Control of Developmental Transitions: Lessons from *Drosophila* Research. *Annu Rev Entomol* 58(1):497–516.

42. Ashburner M (1974) Sequential gene activation by ecdysone in polytene chromosomes of Drosophila melanogaster. II. The effects of inhibitors of protein

synthesis. *Dev Biol* 39(1):141–157.

43. Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P. and Hogness D. (1991) The drosophila EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* 67(1):59–77.

44. Cruz J, Sieglaff DH, Arensburger P, Atkinson PW, Raikhel AS (2009) Nuclear receptors in the mosquito Aedes aegypti: Annotation, hormonal regulation and expression profiling. *FEBS J* 276(5):1233–1254.

45. Wang S-FF, et al. (2000) Differential Expression and Regulation by 20-Hydroxyecdysone of Mosquito Ultraspiracle Isoforms. *Dev Biol* 218(1):99–113.

46. Wang SF, Ayer S, Segraves WA, Williams DR, Raikhel AS (2000) Molecular determinants of differential ligand sensitivities of insect ecdysteroid receptors. *Mol Cell Biol* 20(11):3870–3879.

47. Kokoza VA, Raikhel AS (2011) Targeted gene expression in the transgenic Aedes aegypti using the binary Gal4-UAS system. *Insect Biochem Mol Biol* 41(8):637–644.

48. Cronauer M V., Braun S, Tremmel C, Kröncke KD, Spindler-Barth M (2007) Nuclear localization and DNA binding of Ecdysone receptor and ultraspiracle. *Archives of Insect Biochemistry and Physiology* doi:10.1002/arch.20184.

49. Kapitskaya M, Wang S, Cress DE, Dhadialla TS, Raikhel AS (1996) The mosquito ultraspiracle homologue, a partner of ecdysteroid receptor heterodimer: Cloning and characterization of isoforms expressed during vitellogenesis. *Mol Cell Endocrinol* 121(2):119–132.

50. Fletcher JC, Burtis KC, Hogness DS, Thummel CS (1995) The Drosophila E74 gene is required for metamorphosis and plays a role in the polytene chromosome puffing response to ecdysone. *Development* 121(5):1455–1465.

51. Sun G, Zhu J, Li C, Tu Z, Raikhel AS (2002) Two isoforms of the early E74 gene, an Ets transcription factor homologue, are implicated in the ecdysteroid hierarchy governing vitellogenesis of the mosquito, Aedes aegypti. *Mol Cell Endocrinol* 190(1–2):147–157.

52. Sun G, Zhu J, Chen L, Raikhel AS (2005) Synergistic action of E74B and ecdysteroid receptor in activating a 20-hydroxyecdysone effector gene. *Proc Natl Acad Sci* 102(43):15506–15511.

53. Sun G, Zhu J, Raikhel AS (2004) The early gene E74B isoform is a transcriptional activator of the ecdysteroid regulatory hierarchy in mosquito vitellogenesis. *Mol Cell Endocrinol* 218(1–2):95–105.

54. Bialecki M, Shilton A, Fichtenberg C, Segraves WA, Thummel CS (2002) Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from

metamorphosis in Drosophila. *Dev Cell* 3(2):209–220.

55. Caceres L, et al. (2011) Nitric oxide coordinates metabolism, growth, and development via the nuclear receptor E75. *Genes Dev* 25(14):1476–1485.

56. Reinking J, et al. (2005) The Drosophila nuclear receptor e75 contains heme and is gas responsive. *Cell* 122(2):195–207.

57. Johnston DM, et al. (2011) Ecdysone- and NO-Mediated Gene Regulation by Competing EcR/Usp and E75A Nuclear Receptors during Drosophila Development. *Mol Cell*. doi:10.1016/j.molcel.2011.07.033.

58. Pierceall WE, et al. (1999) E75 expression in mosquito ovary and fat body suggests reiterative use of ecdysone-regulated hierarchies in development and reproduction. *Mol Cell Endocrinol* 150(1–2):73–89.

59. Cruz J, Mane-Padros D, Zou Z, Raikhel AS (2012) Distinct roles of isoforms of the heme-liganded nuclear receptor E75, an insect ortholog of the vertebrate Rev-erb, in mosquito reproduction. *Mol Cell Endocrinol* 349(2):262–271.

60. Hiruma K, Riddiford LM (2004) Differential control of MHR3 promoter activity by isoforms of the ecdysone receptor and inhibitory effects of E75A and MHR3. *Dev Biol* 272(2):510–521.

61. Zhu J, Chen L, Raikhel AS (2007) Distinct roles of Broad isoforms in regulation of the 20-hydroxyecdysone effector gene, Vitellogenin, in the mosquito Aedes aegypti. *Mol Cell Endocrinol* 267(1–2):97–105.

62. Chen L, Zhu J, Sun G, Raikhel AS (2004) The early gene broad is involved in the ecdysteroid hierarchy governing vitellogenesis of the mosquito Aedes aegypti. *J Mol Endocrinol* 33(3):743–761.

63. Devaiah BN, Gegonne A, Singer DS (2016) Bromodomain 4: a cellular Swiss army knife. *J Leukoc Biol* 100(4):679–686.

64. Li C, et al. (2000) Conserved Molecular Mechanism for the Stage Specificity of the Mosquito Vitellogenic Response to Ecdysone. *Dev Biol* 224(1):96–110.

65. Mane-Padros D, Cruz J, Cheng A, Raikhel AS (2012) A Critical Role of the Nuclear Receptor HR3 in Regulation of Gonadotrophic Cycles of the Mosquito Aedes aegypti. *PLoS One* 7(9):e45019.

66. Zhu J, Chen L, Sun G, Raikhel AS (2006) The competence factor beta Ftz-F1 potentiates ecdysone receptor activity via recruiting a p160/SRC coactivator. *Mol Cell Biol* 26(24):9402–12.

67. Darwish HM, DeLuca HF (1996) Analysis of binding of the 1,25-dihydroxyvitamin D3 receptor to positive and negative vitamin D response elements. *Arch Biochem Biophys* 334(2):223–234.

68. Wigglesworth V (1955) The Physiology of Insect Metamorphosis. *AIBS Bull* 5(1):12–12.

69. Kapitskaya MZ, Li C, Miura K, Segraves W, Raikhel AS (2000) Expression of the early-late gene encoding the nuclear receptor HR3 suggests its involvement in regulating the vitellogenic response to ecdysone in the adult mosquito. *Mol Cell Endocrinol* 160(1–2):25–37.

70. Huang P, Chandra V, Rastinejad F (2010) Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics. *Annu Rev Physiol* 72:247–72.

71. Szapary D, Huang Y, Simons SS (1999) Opposing Effects of Corepressor and Coactivators in Determining the Dose-Response Curve of Agonists, and Residual Agonist Activity of Antagonists, for Glucocorticoid Receptor-Regulated Gene Expression. *Mol Endocrinol* 13(12):2108–2121.

72. Santos GM, Fairall L, Schwabe JWR (2011) Negative regulation by nuclear receptors: a plethora of mechanisms. *Trends Endocrinol Metab* 22(3):87–93.

73. Surjit M, et al. (2011) Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. *Cell* 145(2):224–241.

74. Vander Kooi BT, et al. (2005) The Glucose-6-Phosphatase Catalytic Subunit Gene Promoter Contains Both Positive and Negative Glucocorticoid Response Elements. *Mol Endocrinol* 19(12):3001–3022.

75. Wondisford FE, et al. (1989) Thyroid hormone inhibition of human thyrotropin beta-subunit gene expression is mediated by a cis-acting element located in the first exon. *J Biol Chem* 264(25):14601–4.

76. Chatterjee VK, Lee JK, Rentoumis A, Jameson JL (1989) Negative regulation of the thyroid-stimulating hormone alpha gene by thyroid hormone: receptor interaction adjacent to the TATA box. *Proc Natl Acad Sci U S A* 86(23):9114–8.

77. Swami S, Krishnan A V., Peng L, Lundqvist J, Feldman D (2013) Transrepression of the estrogen receptor promoter by calcitriol in human breast cancer cells via two negative vitamin D response elements. *Endocr Relat Cancer* 20(4):565–577.

78. Dostert A, Heinzel T (2004) Negative glucocorticoid receptor response elements and their role in glucocorticoid action. *Curr Pharm Des* 10(23):2807–16.

79. Takeda T, Nagasawa T, Miyamoto T, Hashizume K, DeGroot LJ (1997) The function of retinoid X receptors on negative thyroid hormone response elements. *Mol Cell Endocrinol* 128(1–2):85–96.

80. Schroeder A, Jimenez R, Young B, Privalsky ML (2014) The ability of thyroid hormone receptors to sense t4 as an agonist depends on receptor isoform and on cellular cofactors. *Mol Endocrinol* 28(5):745–57.

81. Abel ED, et al. (1999) Divergent roles for thyroid hormone receptor β isoforms in the endocrine axis and auditory system. *J Clin Invest* 104(3):291–300.

82. Bleicher L, et al. (2008) Structural basis of GC-1 selectivity for thyroid hormone receptor isoforms. *BMC Struct Biol* 8(1):8.

83. Sar P, Peter R, Rath B, Das Mohapatra A, Mishra SK (2011) 3, 3′5 triiodo l thyronine induces apoptosis in human breast cancer MCF-7cells, repressing SMP30 expression through negative thyroid response elements. *PLoS One* 6(6). doi:10.1371/journal.pone.0020861.

84. Cruz J, Mane-Padros D, Zou Z, Raikhel A (2013) Distinct roles of isoforms of the heme-liganded nuclear receptor E75, an insect orthologue of the vertebrate Rev-Erb, in mosquito reproduction. 349(2):262–271.

85. Fujimoto K, et al. (2000) Identification and characterization of negative regulatory elements of the human telomerase catalytic subunit (hTERT) gene promoter: possible role of MZF-2 in transcriptional repression of hTERT. *Nucleic Acids Res* 28(13):2557–62.

86. Rosenfeld MG, et al. (1988) Response and binding elements for ligand-dependent positive transcription factors integrate positive and negative regulation of gene expression. *Cold Spring Harbor Symposia on Quantitative Biology*, pp 545–556.

87. Xu J, Thompson KL, Shephard LB, Hudson LG, Gill GN (1993) T3 receptor suppression of Sp1-dependent transcription from the epidermal growth factor receptor promoter via overlapping DNA-binding sites. *J Biol Chem* 268(21):16065–16073.

88. Docagne F, et al. (2004) Sp1 and Smad transcription factors co-operate to mediate TGF-beta-dependent activation of amyloid-beta precursor protein gene transcription. *Biochem J* 383(Pt 2):393–399.

89. Dostert A, Heinzel T (2004) Negative glucocorticoid receptor response elements and their role in glucocorticoid action. *Curr Pharm Des* 10(23):2807–16.

90. Nagae M, Nakata M, Takahashi Y (2008) Identification of Negative cis-Acting Elements in Response to Copper in the Chloroplastic Iron Superoxide Dismutase Gene of the Moss Barbula unguiculata. *PLANT Physiol* 146(4):1687–1696.

91. Turunen MM, Dunlop TW, Carlberg C, Väisänen S (2007) Selective use of multiple vitamin D response elements underlies the 1 α,25-dihydroxyvitamin D3-mediated negative regulation of the human CYP27B1 gene. *Nucleic Acids Res* 35(8):2734–2747.

92. Qi W, Wang Z (2012) Negative androgen-response elements in androgen target genes. *Androgen-Responsive Genes in Prostate Cancer: Regulation, Function and Clinical Applications*, pp 29–41.

93. Qi W, Gao S, Chu J, Zhou L, Wang Z (2012) Negative Androgen-Response Elements Mediate Androgen-Dependent Transcriptional Inhibition of TGF- 1 and CDK2 Promoters in the Prostate Gland. *J Androl* 33(1):27–36.

94. Unnikrishnan I, Miller S, Meinke M, LaPorte DC (2003) Multiple positive and negative elements involved in the regulation of expression of GSY1 in Saccharomyces cerevisiae. *J Biol Chem* 278(29):26450–7.

95. Jho SH, Radoja N, Im MJ, Tomic-Canic M (2001) Negative Response Elements in Keratin Genes Mediate Transcriptional Repression and the Cross-talk among Nuclear Receptors. *J Biol Chem* 276(49):45914–45920.

96. Dostert A, Heinzel T (2003) Negative glucocorticoid receptor response elements and their role in glucocorticoid action. *Negat glucocorticoid Recept response Elem their role glucocorticoid action*.

97. Bhattacharjee S, Renganaath K, Mehrotra R, Mehrotra S (2013) Combinatorial control of gene expression. *Biomed Res Int* 2013:407263.

98. Cavaillès V, et al. (1995) Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. *EMBO J* 14(15):3741–51.

99. Cohen RN, Wondisford FE, Hollenberg a N (1998) Two separate NCoR (nuclear receptor corepressor) interaction domains mediate corepressor action on thyroid hormone response elements. *Mol Endocrinol* 12(10):1567–1581.

100. Tran HT, et al. (2001) Requirement of co-factors for the ligand-mediated activity of the insect ecdysteroid receptor in yeast. *J Mol Endocrinol* 27(2):191–209.

101. Voss TC, et al. (2011) Dynamic Exchange at Regulatory Elements during Chromatin Remodeling Underlies Assisted Loading Mechanism. *Cell* 146(4):544–554.

102. Bernardo TJ, Dubrovskaya VA, Xie X, Dubrovsky EB (2014) A view through a chromatin loop: Insights into the ecdysone activation of early genes in Drosophila. *Nucleic Acids Res* 42(16):10409–10424.

103. Roy S, et al. (2015) Regulation of gene expression patterns in mosquito reproduction. *PLoS Genet* 11(8):e1005450.

104. Braun S, Azoitei A, Spindler-Barth M (2009) DNA-binding properties of Drosophila ecdysone receptor isoforms and their modification by the heterodimerization partner ultraspiracle. *Arch Insect Biochem Physiol* 72(3). doi:10.1002/arch.20328.

105. Shin SW, Kokoza V, Ahmed A, Raikhel AS (2002) Characterization of three alternatively spliced isoforms of the Rel/NF-kappa B transcription factor Relish from the mosquito Aedes aegypti. *Proc Natl Acad Sci U S A* 99(15):9978–9983.

106. Schauer S, Callender J, Henrich VC, Spindler-Barth M (2011) The N-terminus of ecdysteroid receptor isoforms and ultraspiracle interacts with different ecdysteroid response elements in a sequence specific manner to modulate transcriptional activity. *J Steroid Biochem Mol Biol* 124(3–5):84–92.

107. Dittmer NT, Sun G, Wang S, Raikhel AS (2003) CREB isoform represses yolk protein gene expression in the mosquito fat body. *Mol Cell Endocrinol* 210(1–2):39–49.

108. Seo S-JJ, Cheon H-MM, Sun J, Sappington TW, Raikhel AS (2003) Tissue- and Stage-specific Expression of Two Lipophorin Receptor Variants with Seven and Eight Ligand-binding Repeats in the Adult Mosquito. *J Biol Chem* 278(43):41954–41962.

109. Martín D, Wang SF, Raikhel AS (2001) The vitellogenin gene of the mosquito Aedes aegypti is a direct target of ecdysteroid receptor. *Mol Cell Endocrinol* 173(1–2):75–86.

110. Zhu J, Chen L, Raikhel AS (2003) Posttranscriptional control of the competence factor betaFTZ-F1 by juvenile hormone in the mosquito Aedes aegypti. *Proc Natl Acad Sci U S A* 100(23):13338–13343.

111. Li C, et al. (2000) Conserved molecular mechanism for the stage specificity of the mosquito vitellogenic response to ecdysone. *Dev Biol* 224(1):96–110.

112. Roy SG, Hansen IA, Raikhel AS (2007) Effect of insulin and 20-hydroxyecdysone in the fat body of the yellow fever mosquito, Aedes aegypti. *Insect Biochem Mol Biol* 37(12):1317–1326.

113. Saha TT, et al. (2016) Hairy and Groucho mediate the action of juvenile hormone receptor Methoprene-tolerant in gene repression. *Proc Natl Acad Sci* 113(6):E735–E743.

114. Dutko-Gwóźdź J, et al. (2008) The variety of complexes formed by EcR and Usp nuclear receptors in the nuclei of living cells. *Mol Cell Endocrinol* 294(1–2). doi:10.1016/j.mce.2008.07.021.

115. Raikhel AS, et al. (2002) Molecular biology of mosquito vitellogenesis: From basic studies to genetic engineering of antipathogen immunity. *Insect Biochem Mol Biol* 32(10):1275–1286.

116. Sun G, Zhu J, Chen L, Raikhel AS (2005) Synergistic action of E74B and ecdysteroid receptor in activating a 20-hydroxyecdysone effector gene. *Proc Natl Acad Sci* 102(43):15506–15511.

117. Helsen C, Claessens F (2014) Looking at nuclear receptors from a new angle. *Mol Cell Endocrinol* 382(1):97–106.

118. Geserick C, Meyer H-A, Haendler B (2005) The role of DNA response elements

as allosteric modulators of steroid receptor function. *Mol Cell Endocrinol* 236(1–2):1–7.

119. Wood JR, Greene GL, Nardulli AM (1998) Estrogen Response Elements Function as Allosteric Modulators of Estrogen Receptor Conformation. *Mol Cell Biol* 18(4):1927–1934.

120. Telorac J, et al. (2016) Identification and characterization of DNA sequences that prevent glucocorticoid receptor binding to nearby response elements. *Nucleic Acids Res* 44(13):6142–56.

121. Telorac J, et al. (2016) Identification and characterization of DNA sequences that prevent glucocorticoid receptor binding to nearby response elements. *Nucleic Acids Res* 44(13):6142–6156.

122. Lempiäinen JK, et al. (2017) Agonist-specific Protein Interactomes of Glucocorticoid and Androgen Receptor as Revealed by Proximity Mapping. *Mol Cell Proteomics* 16(8):1462–1474.

123. Klinge CM (2001) Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res* 29(14):2905–2919.

124. Millard CJ, Watson PJ, Fairall L, Schwabe JWR (2013) An evolving understanding of nuclear receptor coregulator proteins. *J Mol Endocrinol* 51(3):T23-36.

125. Peterson TJ, Karmakar S, Pace MC, Gao T, Smith CL (2007) The silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) corepressor is required for full estrogen receptor alpha transcriptional activity. *Mol Cell Biol* 27(17):5933–48.

126. Kimura S, et al. (2008) Drosophila arginine methyltransferase 1 (DART1) is an ecdysone receptor co-repressor. *Biochem Biophys Res Commun* 371(4):889–893.

127. Bian G, Shin SW, Cheon H-M, Kokoza V, Raikhel AS (2005) Transgenic alteration of Toll immune pathway in the female mosquito Aedes aegypti. *Proc Natl Acad Sci U S A* 102(38):13568–13573.

128. Shin SW, Zou Z, Saha TT, Raikhel AS (2012) bHLH-PAS heterodimer of methoprene-tolerant and Cycle mediates circadian expression of juvenile hormone-induced mosquito genes. *Proc Natl Acad Sci U S A* 109(41):16576–16581.

129. Bryant B, Raikhel AS (2011) Programmed autophagy in the fat body of Aedes aegypti is required to maintain egg maturation cycles. *PLoS One* 6(11):e25502.

130. Antonova Y, Alvarez KS, Kim YJ, Kokoza V, Raikhel AS (2009) The role of NF-kappaB factor REL2 in the Aedes aegypti immune response. *Insect Biochem Mol Biol* 39(4):303–314.

131. Shin SW, Kokoza VA, Raikhel AS (2003) Transgenesis and reverse genetics of mosquito innate immunity. *J Exp Biol* 206(Pt 21):3835–3843.

132. Zou Z, et al. (2011) Transcriptome analysis of Aedes aegypti transgenic mosquitoes with altered immunity. *PLoS Pathog* 7(11):e1002394.

133. Neira-Oviedo M, et al. (2011) The RNA-Seq approach to studying the expression of mosquito mitochondrial genes. *Insect Mol Biol* 20(2):141–152.

134. Zhao B, et al. (2014) Regulation of the gut-specific carboxypeptidase: A study using the binary Gal4/UAS system in the mosquito Aedes aegypti. *Insect Biochem Mol Biol* 54:1–10.

135. Nene V, et al. (2007) Genome sequence of Aedes aegypti, a major arbovirus vector. *Science* 316(5832):1718–1723.

136. Wilke ABB, Marrelli MT (2015) Paratransgenesis: a promising new strategy for mosquito vector control. *Parasit Vectors* 8(1):342.

137. Giraldo-Calderón GI, et al. (2015) VectorBase: an updated bioinformatics resource for invertebrate vectors and other organisms related with human diseases. *Nucleic Acids Res* 43(Database issue):D707-13.

138. Feitosa FM, et al. (2006) A transcriptome analysis of the Aedes aegypti vitellogenic fat body. *J Insect Sci* 6(1). doi:10.1673/1536-2442(2006)6[1:ATAOTA]2.0.CO;2.

139. Kistler KE, Vosshall LB, Matthews BJ (2015) Genome Engineering with CRISPR-Cas9 in the Mosquito Aedes aegypti. *Cell Rep* 11(1):51–60.

Chapter II

Regulation of gene expression patterns in mosquito reproduction

Sourav Roy, Tusar T. Saha, Lisa Johnson, Bo Zhao, Jisu Ha, Kevin P. White, Thomas Girke, Zhen Zou, and Alexander S. Raikhel

2.1 Abstract

In multicellular organisms, development, growth and reproduction require coordinated expression of numerous functional and regulatory genes. Although a large body of data is available concerning spatial and temporal gene expression patterns, regulatory pathways orchestrating these patterns are mainly unidentified. In addition to being the most specious animal group with enormous biological and economical significance, insects represent outstanding model organisms for studying regulatory mechanisms of synchronized gene expression due to their rapid development and reproduction. Disease-transmitting female mosquitoes have adapted uniquely for ingestion and utilization of the huge blood meal required for rapid reproductive events to complete egg development within a 72-h period. We investigated the network of regulatory factors mediating sequential gene expression in the fat body, a multifunctional organ analogous to the vertebrate liver and adipose tissue. Transcriptomic and bioinformatics analyses revealed that approximately 7500 genes are differentially expressed, most of this differential expression occurs in four sequential waves over the 72-h reproductive period, within the fat body. Using a combination of RNA interference gene silencing and an in-vitro organ culture, we identified the major regulators

responsible for up- and downregulation of the co-expressed gene sets. We detected the first wave of gene activation, regulated by amino acids (AAs), between 3 h and 12 h post-blood meal (PBM); genes within this set are later repressed by 20-hydroxyecdysone (20E), through its receptor EcR. During the second wave, between 12 h and 36 h PBM, most genes are highly upregulated by a synergistic action of AAs, 20E and EcR. The expressions of these genes tend to decrease with a decline in the 20E titer; the nuclear receptor (NR) HR3 augments the downregulation. Between 36 h and 48 h PBM, the third wave of gene activation—regulated mainly by HR3—occurs. These genes were found to be downregulated by 20E and EcR during the early period PBM, and by juvenile hormone (JH) through its receptor Methoprene-tolerant (Met) during the later stage PBM. JH and Met were found to be the major regulators for the final wave of gene activation between 48 h and 72 h PBM, and representatives of this gene set were found to be repressed by AAs during the early period PBM. We found that insulin has a limited role during this period—activating just the yolk protein precursor genes, which are a subset of the second co-expressed gene set—and it is active only in combination with AAs and 20E. Taken together, our study provides a better understanding of the complexity of the regulatory mechanisms responsible for the temporal coordination of gene expression during reproduction in the female Aedes aegypti mosquito.

2.2 Introduction

Numerous studies in model organisms have identified patterns of gene expression correlated with embryogenesis and development (White et al., 1997; Arbeitman et al., 2002; Li and White, 2003; Stolc et al., 2004; Hooper et al, 2007; Papatsenko et al., 2010; Short and Lazzaro, 2013; Chanut-Delalande et al., 2014; Potier et al., 2014). These studies have eloquently demonstrated the existence of a tight coordination between large gene cohorts and various stages of a developing organism on a spatiotemporal scale. In contrast, investigation of genomic profiles during reproduction has attracted much less attention. Bloodfeeding animals such as mosquitoes, in addition to being vectors of numerous devastating human diseases, represent outstanding models because their reproductive events are synchronized by the intake of blood and occur within a short time span. Moreover, their reproduction is cyclic, with each cycle of egg development linked to a separate blood-feeding event. Previous studies identified differential gene expression associated with blood feeding in the malaria mosquito Anopheles gambiae and the Dengue virus vector mosquito Aedes aegypti (Dana et al., 2005; Marinotti et al., 2006; Bonizzoni et al., 2011). However, temporal control of gene expression patterns during blood-meal-activated mosquito reproduction is not yet completely understood. The gonadotrophic cycle of a female mosquito is divided into two periods: pre- and post-blood meal. In the *A. aegypti* female, the pre-blood meal period, which in the first gonadotrophic cycle also includes post-eclosion (PE) development, lasts at least 72 h until a mosquito takes a blood meal. It is controlled by juvenile hormone (JH) and its receptor Methoprene-tolerant (Met) (Hagedorn, 2005; Zou et al., 2013). Both amino

acid/Target of Rapamycin nutritional signaling and insulin are essential for activating post-blood-meal (PBM) events in the gut, ovaries and the fat body (Hansen et al., 2004; Roy et al., 2007; Brown et al., 2008; Brandon et al., 2008; Gulia-Nuss et al., 2011). 20Hydroxyecdysone (20E) is the main regulator of PBM events in the fat body, an adipose tissue analogous to the mammalian liver, which produces yolk protein precursors (YPPs) for subsequent egg development (Hagedorn, 2005; Raikhel 2005). In the *A. aegypti* female, it takes 72 h to complete the entire PBM period. During each gonadotrophic cycle, the fat body undergoes dramatic changes, shifting its functions from acting as a storage depot for lipid and carbohydrate reserves to becoming an immense protein-producing factory (Raikhel et al., 2005). At the end of the gonadotrophic cycle, it undergoes programmed autophagy and transforms itself back to reserve storage (Bryant and Raikhel, 2011). Hence, this tissue is particularly useful for studies of temporal coordination of gene expression. Our previous study (Zou et al., 2013) revealed gene expression patterns in the fat body during the pre-blood-meal period of the first gonadotrophic cycle in the *A. aegypti* female. We have shown that while metabolic genes are expressed early, those encoding transcription and translation machineries get activated later during this period. Moreover, we demonstrated that while the former group of genes is repressed by JH and Met, the latter is activated by these factors (Zou et al., 2013). Here, we investigated the network of regulatory factors responsible for sequential gene expression in the PBM fat body. We show that systemic factors—JH, 20E and nutritional amino acids (AAs)—differentially regulate this gene-expression program. Moreover, our study has revealed that JH and 20E signaling in the PBM fat body is

mediated by Met, EcR and HR3. Importantly, we report the previously unidentified role of JH in controlling gene expression during the PBM period. Finally, in this study, we have demonstrated the repressive function of 20E, which downregulates large cohorts of PBM genes in this mosquito tissue. Furthermore, we have shown that EcR mediates this repressive function. Taken together, our study provides new insights into the complexity of regulatory mechanisms responsible for temporal coordination of gene expression during reproduction in the female *A. aegypti* mosquito.

2.3 Materials and methods

Mosquito rearing

Mosquitoes of the *A. aegypti* wild-type UGAL strain were raised at 27°C and 80% humidity, as described previously [16]. The larvae were raised in non-crowded conditions [37,38] (200 in 750ml distilled water per 9”x12” pan) and fed 0.125–0.900 ml vol. of standard diet (equal parts of rodent diet, Lactalbumin and active dry yeast) between Day 0 and Day 4. Four pans of pupae were combined into one adult cage. Adult mosquitoes were fed continuously on water and 10% (wt/vol) sucrose solution. All dissections were performed in Aedes physiological solution (APS) at room temperature [16]. Bloodfeeding of all adult mosquitoes other than the Met knocked-down ones, was done with white rats. Adult Met knocked-down mosquitoes were blood-fed using White Leghorn chickens. All procedures for using vertebrate animals were approved by the UCR animal care and use committee.

in vitro fat body culture (IVFBC)

For testing the effects of AAs, 20E and insulin. A total of 9–12 fat bodies were dissected from mosquitoes 72hPE and incubated in a complete culture medium [16] supplemented with amino acids [16] and increasing concentrations of 20E (Sigma, St. Louis, MO; 5x10−8 M for 4h and 10−6M for 4h) for 8h (S12 Fig), to check the effects of amino acids and 20E. To check the effects of lower concentration of 20E, tissues were incubated with AAs and increasing concentrations of 20E (1x 10−8 M for 4h and 5x10−8 M for 4h). Similar tissues were used to check the effects of insulin, using 17μM bovine insulin solution (Sigma, St. Louis, MO). Experiments were repeated three times under similar conditions. For testing the effects of JH: Tissues were collected from female mosquitoes at 24h PBM and were incubated with JH (Sigma, St. Louis, MO; 10 μg/mL JHIII) or solvent (acetone) added to the culture medium for 8h. Experiments were done in triplicate under the same conditions.

Total RNA extraction

For microarray transcriptome analysis, RNA samples were collected at nine time points, starting at 3h PBM. RNA was extracted from fat bodies of 9–12 female mosquitoes using the TRIzol method (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. It was concentrated using the RNeasy MinElute cleanup kit (Qiagen, Valencia,CA) for further processing. For qRT-PCR post IVFBC, RNA was extracted from 9–12 fat bodies of female mosquitoes post IVFBC, using the TRIzol method (Invitrogen) according to the manufacturer's protocol.

cDNA preparation

cDNAs were synthesized from 2μg total RNA using the SuperScriptIII Reverse Transcriptase kit (Invitrogen). RNA was treated with DNase I (Invitrogen) before cDNA synthesis. PCR was performed using the Platinum High Fidelity Supermix (Invitrogen).

dsRNA preparation and microinjection

For iEcR and iHR3: To synthesize EcR and HR3dsRNA, we followed a method described previously [16]. In brief, dsRNA of a specific gene template was synthesized using the MEGAscript T7 kit (Ambion, Austin, TX) and the luciferase gene was used to generate control iLuc dsRNA. After dsRNA synthesis, samples were subjected to phenol/chloroform extraction and ethanol precipitation. dsRNA then was suspended in RNase-free water to a final concentration of 5μg/μl. At 24h PE, female mosquitoes were injected with 300nl dsRNA into the thorax. The Picospritzer II (GeneralValve Corporation, Fairfield, NJ) was used to introduce corresponding dsRNAs into the thorax of CO2-anesthetized female mosquitoes. The knockdown efficiencies for EcR ranged between 53.3% and 62.7% whereas that of HR3 ranged between 55.6% and 64.1%. For iMet: To synthesize Met dsRNA, we followed the same method as for iEcR and iHR3, except that female mosquitoes were injected with 300nl dsRNA into the thorax at 72hPE, after the completion of the first preparatory cycle. The knockdown efficiencies of Met ranged between 40.3% and53.2%. Sequences of all primers used for dsRNA preparation are shown in S2Table.

qRT-PCR analysis

qRT-PCR was performed using the iCycler iQsystem (Bio-Rad, Hercules, CA and an IQ SYBR Green Supermix (Bio-Rad). Quantitative measurements were performed in triplicate and relative expression (RE) was measured as RE=2-ΔΔCt and normalized to the internal control of S7 ribosomal protein mRNA for each sample. Real-time data were collected from the software iCycler v3.0. Raw data were exported to Microsoft Excel and analyzed. P-values were calculated with the help of unpaired t test using the online version of GraphPad. Sequences of all primers used for qRT-PCR analyses are shown in S2 Table.

2.4 Results

Differential gene expression in the blood-meal-activated fat body of the female *Aedes aegypti*

The goal of this study was to obtain detailed information about differential gene expression dynamics and to elucidate the regulatory networks governing the complex gene expression patterns following blood meal activation of reproductive events in female mosquitoes. We addressed these issues using the fat body, because it constitutes a tissue critical for female reproduction and is more amenable for experimental studies in this organism, which lacks well established genetics (Raikhel et al., 2005). Custom-made Agilent microarray chips containing probe sets corresponding to 15,321 *A. aegypti* genes (Zou et. al, 2011, Zou et. al, 2013) were used to examine the fat-body tissue samples

collected at nine time points, spanning from 3 h to 72 h PBM. Differentially expressed gene (DEG) sets were established by comparing transcripts from each of the nine time points with that from the fat body of pre-blood-meal female mosquitoes, 72 h PE, using a minimum fold change of ≥1.75 (0.8 in a log2 scale) as the confidence threshold and a false-discovery rate (P value) of ≤0.01, similar to the criteria used by Zou et al, 2011, 2013. 7468 genes, which constituted almost half of the total number of genes probed in the *A. aegypti* genome, were found to be differentially expressed at least at one of the nine time points during the 72-h period PBM, within the fat body of female Aedes mosquitoes (Fig. 1A and Dataset S1). 3045 genes were found to be downregulated (genes downregulated by ≥1.75-fold and upregulated by <1.75-fold), 2938 upregulated (genes upregulated by ≥1.75-fold and downregulated by <1.75-fold) and 1485 genes were found to be both up- and downregulated (≥1.75-fold) during different time points within the 72-h period PBM (Fig. 1B and Dataset S1). Of these 1485 genes, 395 were downregulated to a greater extent than these were upregulated (a difference of >1.75 between the fold changes), 306 were more upregulated than downregulated (Fig. 1B and Dataset S1), and 784 were almost equally up- and downregulated at different time points (a difference of <1.75 between the fold changes). More than 2500 genes displayed differential expression of greater than 5-fold (Fig. 1B and Dataset S1). Unlike during the PE period, when the number of DEGs increased consistently to reach a maximum during late PE (60–66 h) (Zou et. al, 2013), the number of DEGs during the PBM period started to increase at around 12 h, reached a maximum between 18 h and 24 h, and then decreased sharply after 36 h PBM (Fig. S1). Hierarchical clustering of the DEGs resulted

in 12 different clusters (Fig. 1A). Genes within certain clusters displayed similar profiles, barring minor variations. As a result, most of the genes could be categorized into four broad sets, depending on their expression profiles and the time of their maximal expression: early genes (EGs), early-mid genes (EMGs), late-mid genes (LMGs) and late genes (LGs) (Fig. 1A). Transcript levels of EGs (Clusters 1, 11 and 6) were elevated by 3 h PBM, reached their maximum levels between 6 h and 12 h PBM, and declined between 18 h and 36 h, before getting slightly elevated again between 48 h and 72 h PBM (Fig 1C, Dataset S2). In comparison, EMG transcript levels (Clusters 8, 7, 4) did not show significant increase until 12 h PBM, reached the maximum levels between 18 h and 24 h, after which their expression declined by 36 h PBM (Fig 1C, Dataset S3). The genes that displayed a low expression prior to 24 h PBM followed by a sudden increase within 36-48 h PBM and a sharp decline post 48 h were grouped as the LMGs (Cluster 5; Fig 1C, Dataset S4). The LGs (Clusters 2 and 3) are those showing a decline in their expression following a blood meal, maintaining low expression during early-mid and late-mid periods, and showing maximum expression between 48 h and 72 h (Fig 1C, Dataset S5). Expression patterns of fat body genes were confirmed by means of quantitative real-time polymerase chain reaction (qRT-PCR) analysis; transcript levels of selected EGs, EMGs, LMGs and LGs, measured using qRTPCR, displayed good correlation with microarray data (Fig. S2 A-L). Overall, our microarray analysis revealed an extremely high level of transcriptional activity in the fat body during the PBM period of the gonadotrophic cycle. Moreover, we were able to identify four major sequential waves of gene expression over the 72-h period PBM in the female fat body.

Temporal separation of functional gene groups expressed during the PBM period

To understand the functional identity of genes expressed during the PBM period in the fat body, the EGs, EMGs, LMGs and LGs were examined by searching the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa M and Goto S, 2000). This analysis revealed that dramatic changes occur in the functional identity of the fat body transcriptome over the duration of 72 h PBM. We observed that 40% of the EGs belong to cellular processes and signaling (CP&S) gene category, 30% to metabolism (MT) and 30% to information storage and processing genes (IS&P) (Fig 2A). In the case of the EMGs, the percentage of MT genes increases to ~45%, while the percentages of the other two groups decreases (~35% CP&S; ~20% IS&P) (Fig. 2A). This trend continues for the LMGs, for which almost 70% are MT genes, with about 10% IS&P genes and about 20% CP&S genes (Fig 2A). However, this trend completely reverses with the LGs, for which only about 15% of the total genes is represented by the MT genes, and both CP&S and IS&P constitute a little over 40% each (Fig 2A). It was observed that while most MT genes are active during the early-mid and late-mid PBM periods, there is an enrichment of CP&S and IS&P during the early and late PBM periods. To take a closer look at the functional dynamics on a finer scale, we further sorted these genes into more-specific functional categories within the KEGG database (Fig 2B-D). The results of this analysis revealed a remarkable temporal separation of major functional gene categories over the 72 h PBM. Separation of IS&P genes into finer functional categories revealed that more than 70% of EGs are transcription (TR) and translation, ribosome structure and biogenesis (TRB) genes, where TRB alone constitutes

more than 50% of the genes (Fig 2B). The percentage of TR genes increases in EMGs (>35%) and further in LMGs (55%), whereas, the TRB genes show an opposite trend, these genes seem to be significantly downregulated during the late-mid and late stages (Fig 2B). Similarly, a closer look at the CP&S groups showed that while signal transduction mechanism (STM) genes constitute a little over 10% of CP&S amongst EGs, the percentage increases to ~45% in LMGs and decreases slightly in LGs (Fig 2C). Conversely, intracellular trafficking, secretion and vesicular transport (ITS&VT) shows an exact opposite trend (Fig 2C). The percentage of genes related to posttranslational modification, protein turnover and chaperones (PMTC) decrease, whereas that of genes related to cytoskeleton increases over the course of the gonadotrophic cycle (Fig 2C). Although MT genes account for most of the EMGs and LMGs, genes that belong to different functional sub-categories seem to be more prevalent in each gene set. While genes related to inorganic ion transport and metabolism (IIT&M) make up 25% of the early-mid MT genes, lipid transport and metabolism (LT&M) accounts for a similar amount of the late-mid MT genes (Fig 2D). It is worth mentioning that in each of the four gene sets, a large proportion of genes belongs to diverse or unknown functional classes, defined as having either insufficient information or no significant matches to other organisms.

EGs activated by AAs and JH, but repressed by 20E

Next, we investigated the regulatory signaling network responsible for the temporal dynamics of gene expression in the fat body during the PBM period of the mosquito gonadotrophic cycle. Previous studies have identified involvement of AAs,

insulin and 20E in regulation of vitellogenic events in the mosquito fat body (Hansen et al., 2004; Attardo et al., 2006; Roy et al., 2007). We used a combination of RNA interference (RNAi) and in-vitro organ culture techniques to elucidate the regulation of the temporal gene expression program in the fat body. The average EG expression increased soon after a blood meal and reached its maximum between 6 h and 12 h PBM, just before the 20E titer started to peak (Fig 1C). The expression levels of these genes declined sharply with the increase in 20E titer; therefore, there seems to be an inverse correlation. We monitored the responses of three EGs (AAEL013818 – spliceosome associated protein; AAEL002488 – dead box ATP-dependent RNA helicase; and AAEL04345 – cysteinyl t-RNA synthetase) to treatments in in-vitro fat body culture (IVFBC) using qRT-PCR. The genes were selected on the basis of their high level of expression and similarity to the average profile (Fig 1C). All three genes were IS&P genes, with two from the RPM and one from the TRB subcategories. Each of these genes showed a differential expression of >3-fold. Tissues (fat bodies) collected at 72 h PE, when there is only a basal level of 20E, were placed in complete culture media and treated with either AAs alone or with AAs plus increasing concentrations of 20E (5x 10-8 M for 4 h and 10-6 M for 4h) for 8 h (Figs S1B-D); non-treated (NT) fat bodies in culture media served as the control. Total RNA was extracted, cDNA was made and qRT-PCR for the three EGs suggested that these genes were being upregulated by AAs and repressed by 20E (Figs 3A, S3A-B). To confirm the repression of these genes by 20E, we used the RNA interference (RNAi) technique. Double-stranded RNA (dsRNA) was injected at 24 h PE to knock down EcR (Fig S1E), the mosquitoes were blood fed 72 h

post injection, and tissue was collected 24 h PBM. If these genes are actually being repressed by 20E then knocking down its receptor should remove the effects of repression. qRT-PCR for these genes with cDNA made from tissues collected from the knocked-down mosquitoes confirmed that these genes are indeed repressed by 20E (Figs 3B, S3C-D). Injecting dsRNA for the Luciferase gene (iluc) served as the control. Although the elevation of JH titer during the late PBM period has been reported (Fig S1A; Shapiro et al, 1986; Hernandez-Martinez et al., 2014), the role of this hormone in regulating PBM events in the female mosquito has remained unclear. To understand whether JH could play any regulatory role in EG expression during the late PBM period, we examined the effect of JH on the same genes using IVFBC and found that it had a moderate activating effect (Figs 3C, S4E-F). The tissue used to check the effects of JH was collected at 24h PBM when the titer of JH was at the basal level, and incubated in a complete culture medium supplemented with either JH (10 μg/ml JH III) or the solvent (acetone) for 8 h. To confirm this JH action, we knocked down the JH receptor, Met, with the help of a dsRNA (Fig S1F). Injections were done at 72 h PE, after the completion of the first preparatory phase; mosquitoes were blood fed 72 h post-injections and tissue was collected 72 h PBM. qRT-PCR for the same genes corroborated the activation by JH through its receptor Met, when the results demonstrated a decline in expression of the genes as a result of the Met knockdown (Fig. 3D, S4G-H). Next, we checked whether these genes were being activated by insulin, which has been reported to have a regulatory effect along with AAs on certain genes PBM (Brown et al., 2008, Gulia-Nuss et al., 2011, Roy et al., 2007, 2011). Exogenous insulin along with 20E has been shown to enhance

AA-dependent activation of Vg expression in the isolated fat body (Roy et al., 2007). The results suggested that insulin was not involved in the activation of these genes during the early PBM period. Overall, our results have shown that the representatives of the EGs tested are activated by AAs at the early stage of the PBM period, are repressed by 20E/EcR in the mid stage and are activated again moderately by JH/Met at the end of the PBM phase. Our experiments suggest that insulin is not involved in regulation of EGs tested.

EMGs are activated by 20E and repressed by HR3

Transcript levels of the early-mid genes or EMGs started increasing by 12 h, reached their maximum between 18 h and 24 h, and then declined drastically to basal levels by 36 h, staying low thereafter (Fig. 1C). These genes show a positive correlation with the 20E titer during the PBM period. To examine the regulation of EMGs, we selected six genes, three well-known YPP genes (AAEL010434 - Vitellogenin, AAEL007585 - Cathepsin b and AAEL006563 - Vitellogenic carboxypeptidase) and three others (AAEL014671- protease S51 alpha-aspartyl dipeptidase; AAEL001433 – FGF receptor activating protein; AAEL004398 – G-protein-coupled receptor). The latter genes were chosen on the basis of high expression and their closeness to the average EMG profile. Similar to the YPP genes, these three genes belong to the CP&S functional group and were upregulated by >8-fold. The expressions of genes were tested in the IVFBC with either AAs alone or AAs plus increasing concentrations of 20E for 8 h. The results showed that there is either minimal or no effect of AAs alone on these genes; however, all of these genes were activated by 20E in the presence of AAs (Figs 4A, 5A,

S4A-B, S5A-B). To confirm the activation by 20E, these genes were tested using qRT-PCR with tissues (fat bodies) from EcR knockeddown mosquitoes (similar to those used for testing the EGs). The results corroborated that these genes are activated by 20E, as there was a decrease in transcript level for each of these genes in fat bodies from EcR-silenced mosquitoes (Figs 4B, 5B, S4C-D, S5C-D). The declines in these gene transcript levels correlate with the 20E titer drop in female mosquitoes, by about 30 h PBM. To test whether 20E was required for maintaining a high level of expression of EMGs, we modeled the 20E titer decrease in the IVFBC: the fat bodies from female mosquitoes 24h PBM were pre-incubated in the culture medium supplemented with AAs and changing concentration of 20E for 6 h (3 h each with two different concentrations, as described previously) and then incubated in a medium depleted of 20E for 3 h (three washes with complete culture medium every hour). The qRT-PCR results with cDNA made from these tissues showed a decline in the transcript levels of all six genes, further confirming the direct correlation between the 20E titer and the expression these genes (Figs 4C, 5C, S4E-F, S5E-F). Expression levels of EMGs decline to their lowest levels by 36 h PBM when the orphan NR HR3 has been reported to regulate transcriptional reprogramming of the fat body (Mane-Pedros et al., 2012, PLoS One). Therefore, we examined a possible effect of HR3 on these genes by its RNAi silencing. dsRNA was injected at 24 h PE to knock down the HR3 (Fig S1G), the mosquitoes were blood fed 72 h post-injection and tissue was collected 36 h PBM. This RNAi experiment showed that the transcript levels of all tested genes were elevated, suggesting that they were indeed repressed by HR3 (Figs 4D, 5D, 4SG-H, S5G-H). Therefore, as judged by tests of the six selected EMGs,

the decrease in 20E titer and repression by HR3 constituted conditions responsible for the programmed decline in the transcriptional activity of EMGs after 24 h PBM. It is has been shown that insulin activates the YPP gene Vitellogenin in the presence of AAs and 20E in the *A. aegypti* fat body (Roy et al., 2007). Our results confirmed a positive effect of insulin on expression of two other YPP genes, Vitellogenic Carboxypepetidase (Fig 4E), and Cathepsin b (S4I), along with that on the expression of Vitellogenin (S4J). Surprisingly, we could not detect any activation by insulin of the other EMGs tested.

Since the JH titer has been reported to rise again during the late PBM period, we wanted to check whether these genes are repressed by JH. The results suggested that JH has no repressive effect on these genes. In summary, our findings indicate that the representatives of the EMGs are activated by 20E and EcR, are downregulated by a declining 20E titer, and repressed by HR3. We also observed that insulin activated only a subset of EMGs, as tested here using the YPP genes.

LMGs are repressed by 20E and JH but activated by HR3

The LMGs are a group in which the expressions of most of the genes are at low levels until 24 h PBM, after which they increase sharply and reach the maximal level between 36 h and 48 h PBM, declining thereafter (Fig 1C). Therefore, these genes appear to have a high level of expression only within a window when the titers of both 20E and JH are at low levels. We selected three genes on the basis of their high level of expression and similarity to the average LMG profile. All three genes (AAEL003568 – threonine dehydratase, AAEL010075 - oxidoreductase and AAEL002638 – cytochrome 450) are

metabolism-related genes and >10-fold upregulated at 36 h PBM when compared with their expression levels at 72 h PE. The effect of AAs in-vitro on these genes was not entirely consistent, because two of three genes tested were not affected (Figs 6A, S6A), whereas the third (Fig S6B) showed activation by AAs. 20E repressed these genes in-vitro, which is consistent with their low level of expression up to 24 h PBM (Figs. 6A, S6A-B). EcR RNAi silencing (performed similarly to that described in the previous sections) confirmed the repression of these genes by EcR (Figs. 6B, S6C-D). Transcripts of the LMGs are elevated at the time when HR3 has been reported to be active in the mosquito fat body (Mane-Padros et al., 2012). We hypothesized that HR3 is the factor responsible for upregulation of this gene set. When we conducted the HR3 RNAi silencing (as in the previous section), it was indeed found that this NR is responsible for the activation of the LMG representatives in the fat body (Fig. 6C, S6E-F). The LMGs showed a low level of expression between 48 h and 72 h PBM. Therefore, we checked the effects of JH on the LMG representatives by IVFBC and found that they were repressed by JH (Figs. 6D, S6G-H). Met RNAi silencing revealed that these genes are repressed by the JH receptor Met (Figs 6E, S6I-J). Overall, the results suggest that the LMG expression peak between 36 h and 48 h can likely be defined by repressive actions of 20E during the early PBM part and JH during the late PBM period. LMGs are activated by the reprogramming factor HR3 when the titers of both hormones are at relatively low levels.

Activation of LGs by JH during the late PBM period

The expression level of LGs starts to decline after a blood meal, remaining low through the early-mid and late-mid phases PBM. Expression then rises after 36 h and reaches the maximal levels between 48 h and 72 h. This pattern of LG expression has a positive correlation with the reported titer of JH during the PBM period (Shapiro et al., 1986; Hernandez-Martinez et al., 2014). Our hypothesis was that 20E and JH determined temporal coordination of LG expression in which they are repressed by 20E during most of the PBM period and then activated by the rising titer of JH. To test this hypothesis, we selected three representative LGs (AAEL015143 – Glycine-rich ribosome binding protein; AAEL003352 – Ribosomal protein l7ae_E2; and AALE004328 – Origin recognition complex). We used the previously described criteria of high levels of expression and similarity with the average profile. All three genes were related to information storage and processing and were >20-fold downregulated when their minimum expressions were compared with their expressions at 72 h PE. Interestingly, IVFBC results showed that none of the genes tested were repressed by 20E (RNAi silencing of EcR corroborated these results), but were downregulated by AAs (Figs. 7A, S7A-B). In contrast, these genes were indeed activated by JH in-vitro (Figs. 7B, S7C-D). In-vivo RNAi confirmed their activation by JH through its receptor Met (Figs. 7C, S7E-F). Thus, our experiments have shown that JH and Met play roles of major regulators for LG activation during the late PBM period. The AA pathway appears to be a repressor of these genes. Surprisingly, we found no role for 20E in the regulation of the genes tested from this group.

Cyclical genes are regulated by JH through Met

A characteristic feature of female mosquito reproduction is that it is cyclical, with each egg developmental cycle tightly linked to a separate blood feeding. Hence, we investigated whether the same genes are expressed during the late PE and the late PBM periods, when the organism is preparing itself for a blood meal. We compared the LGs with those expressed during the late PE period and regulated by Met (LPE and iMet genes, Zou et al., 2013). We found that 112 late genes appeared in both LPE (i.e., these are upregulated by >1.75-fold during the LPE period) and iMet downregulated (i.e., knockdown of Met during the PE period results in downregulation of >1.75-fold) gene sets (Fig 8A). We call them cyclical genes (CGs). A comparison of functional groups constituting the LGs and CGs shows that, the enrichment of the functional subcategories in the two gene sets is markedly different (Fig 8B-D). We selected one gene each from the IS&P (AAEL001171- tRNA-dihydrouridine synthase), CP&S (AAEL002675 – arginase) and MT (AAEL001623 - proteasome subunit) functional groups from within the 121 CGs and checked the effects of AAs, 20E and JH using IVBFC. IVBFC with AAs and changing concentrations of 20E demonstrated that these genes are repressed by AAs in-vitro (Figs 8E, S8A-B), just like the PBM-specific LGs, whereas 20E might or (Fig S8A) might not (Figs 8E, S8B) have a repressive effect. The activation by JH was evident from the IVFBCs with JH (Figs. 8F, S8C-D) and was confirmed by the in-vivo RNAi knockdown of the JH receptor Met (Figs. 8G, S8E-F). It is worth mentioning that Met dsRNA was injected at 72 h PE, after the completion of PE preparatory phase, and

its effect was examined at 72 h PBM. We also found that one gene (AAEL003352) of the three, tested as PBM LGs, appeared in the list of LPE genes and was regulated by Met during the PE period.

Next, we checked the expression profiles of the representatives of CGs along with that of AAEL003352, post second blood meal, after the mosquitoes completed the first reproductive cycle and had laid eggs. The expression profiles (Figs. 8H, S8G-H, AAEL003352 profile not shown) demonstrated that these genes are indeed cyclical and are activated at the same time during the second egg maturation cycle. In summary, the results suggest that, similar to LGs, the CGs are activated by JH and Met, and their activation by this pathway occurs during both late PE and late PBM periods. These CG representatives appear to be repressed by AAs after the blood feeding.

2.5 Discussion

In this study, we have uncovered the key elements of the regulatory network mediating temporal gene expression in the fat body of a female mosquito during blood-meal-activated reproduction. Unlike previous studies of the *A. aegypti* fat body transcriptome (Price et al., 2011; Feitosa et al., 2006) that have identified the differentially expressed genes at a single time point (24 h PBM), in this study, we have not only looked at the changes in the transcripts over a 72-h period (nine different time points) PBM, but have also identified the factors responsible for the differential expression of the genes. Our results have revealed the complexity of gene regulation in

the fat body of female *A. aegypti* during this period, when the organism is undergoing massive physiological changes within a short time span. The KEGG analysis reflects changes in the expression of genes that belong to the different functional groups during the four different stages (early, early-mid, late-mid and late) within the PBM period, with MT genes being highly active during the early- and late- mid PBM periods, between 18 h and 48 h, when blood is digested, yolk proteins are made and the fat body reprogramming for the next egg developmental cycle begins. It has been well established that 20E is the major stimulus that upregulates YPP gene expression in mosquitoes, which, along with protein synthesis, positively correlates with 20E titers (Attardo et al., 2005; Raikhel et al., 2005). In this study, we were able to demonstrate 20E-mediated activation of not only the YPP genes but also representatives of a super-group (EMGs) within which the YPPs fall (Fig.9). We have also shown that 20E represses representatives from groups of genes that are activated before the rise of the 20E titer (EGs), and after the decline of the 20E titer (LMGs). To the best of our knowledge, this is the first study to report large-scale transcriptional repression by 20E and EcR during post-blood meal waves of gene expression. Functionally, this repression is significant, because exact orchestration of complex physiological processes requires that each individual process is turned off in a timely manner. While the molecular mechanism of gene activation by 20E hierarchy is well established, little is known about repressive 20E action. Although 20E is known to be the main regulator of vitellogenesis, it is not by itself sufficient to activate this process. Experiments have demonstrated that AAs are critical for the activation of YPP genes by 20E (Hansen et al., 2004; Attardo et al., 2006), and we have shown here that the AAs are

not only essential for the activation of YPP genes by 20E, but can activate and repress gene sets without the assistance of 20E. Representatives of EGs were found to be activated whereas those of LGs were found to be repressed by AAs (Fig. 9). At around 36 h PBM, when the titer of 20E has declined to basal levels and YPP synthesis has ceased, the fat body converts back to a nutrient storage and metabolism function until the next vitellogenic cycle is initiated (Raikhel, 1992). It has been demonstrated previously that HR3 targets genes in the termination phase of the vitellogenic cycle and plays an essential role in programmed termination of the first cycle as well as in the entry into the second (Mane-Padros et al., 2012). In this study, we demonstrated that the repressive effects of HR3 are not limited to vitellogenin or even the YPP genes. It inhibits a number of genes during the termination of vitellogenesis; mostly EMGs (Fig. 9) which are activated by 20E between 18 h and 24 h PBM. Similarly, the activation by HR3 is not restricted to the transcription factors in the 20E regulatory cascade, but affects a much larger set of genes (mostly LMGs) that are activated during the termination of vitellogenesis, between 36 h and 48 h (Fig. 9). Thus, the critical role played by HR3 during the termination of the first vitellogenic cycle by switching on and off certain gene sets has been well established in this study.

JH is associated with changes in the fat body during the pre-vitellogenic period, which allows the fat body to become responsive to signals that induce vitellogenesis (Flanagan and Hagedorn, 1977; Raikhel and Lea, 1990; Zhu et al., 2003). The fat body becomes competent to respond to the steroid hormone 20E and to synthesize the massive amounts of yolk protein required for egg maturation (Raikhel and Lea, 1983). In this

study, we have identified the role of JH during the late PBM (post vitellogenic) period and demonstrated that JH not only activates representatives of a group of genes (LGs), but also is responsible for repressing genes that are activated by HR3 during the termination of vitellogenesis (LMGs, Fig. 9). Moreover, apart from activating genes in the late PBM period, it also regulates a set of genes (CGs) that are cyclical, follows the JH titer and probably plays important roles in preparing the fat body for successive vitellogenic cycles. Thus, this work clearly demonstrates that the role of JH is not limited to the pre-vitellogenic stage; along with HR3, JH plays a key role in remodeling the fat body for the next egg developmental cycle, post vitellogenesis. In contrast to observed effects of AAs, 20E, HR3 and JH, we were unable to detect any large-scale effects of insulin, other than activation of the YPP genes, within the fat body, during the PBM period. We were able to detect a synergistic activating effect of 20E and insulin in the presence of amino acids (previously reported by Roy et al. 2007, IBMB) on genes tested other than Vitellogenin. Surprisingly, the other EMGs tested (of which YPPs is a subgroup) showed no significant activation by insulin. We also could not detect any activation of the EGs by insulin, either by itself or in the presence of amino acids and 20E, suggesting that the activation by insulin is limited to the YPP genes only during the early and early-mid PBM period. Therefore, we can summarize, based on the microarray transcriptomic analysis, that the PBM period can be divided broadly into four phases: early (012 h), early-mid (12-30h), late-mid (30–48h) and late (48-72h). Our study using in-vitro organ culture and RNAi depletion analyses also revealed the major regulators of gene expression during these four phases—AAs, 20E, HR3 and JH, respectively (Fig. 9).

Each of these factors is responsible for the activation and repression of different gene sets during the four distinct PBM phases, thereby successfully completing vitellogenesis, and the reprogramming of the fat body, for the next reproductive cycle (Fig. 9).

These results provide a clear insight into the complexity of gene regulation within this key mosquito tissue, thereby elucidating the coordination among the different key regulators in the orchestration of spatial and temporal gene expression patterns required during this critical phase of female mosquito reproduction. Although we have been able to identify the major regulators of the female mosquito gonadotrophic cycle, further study is required to identify other factors involved in the regulation of gene expression patterns during this period.

2.6 References

1. White KP, Hurban P, Watanabe T, Hogness DS (1997) Coordination of Drosophila metamorphosis by two ecdysone-induced nuclear receptors. Science. 276(5309):114-117.

2. Arbeitman MN, Furlong EE, Imam F, Johnson E, Null BH, Baker BS, Krasnow MA, Scott MP, Davis RW, White KP (2002) Gene expression during the life cycle of Drosophila melanogaster. Science. 297(5590):2270-2275.

3. Li TR, White KP (2003) Tissue-specific gene expression and ecdysone-regulated genomic networks in Drosophila. Dev Cell. 5(1):59-72.

4. Stolc V, Gauhar Z, Mason C, Halasz G, van Batenburg MF, Rifkin SA, Hua S, Herreman T, Tongprasit W, Barbano PE, et al. (2004) A gene expression map for the euchromatic genome of Drosophila melanogaster. Science. 306(5696):655-660.

5. Hooper SD, Boué S, Krause R, Jensen LJ, Mason CE, Ghanim M, White KP, Furlong EE, Bork P (2007) Identification of tightly regulated groups of genes during Drosophila melanogaster embryogenesis. Mol Syst Biol 3:72.

6. Papatsenko I, Levine M, Papatsenko D (2010) Temporal waves of coherent gene expression during Drosophila embryogenesis. Bioinformatics. 26(21):2731-2736.

7. Short SM and Lazzaro BP (2013) Reproductive status alters transcriptomic response to infection in female Drosophila melanogaster. G3 (Bethesda). 3(5):827-840.

8. Chanut-Delalande H, Hashimoto Y, Pelissier-Monier A, Spokony R, Dib A, Kondo T, Bohère J, Niimi K, Latapie Y, Inagaki S, et al. (2014) Pri peptides are mediators of ecdysone for the temporal control of development. Nat Cell Biol. 16(11):1035-1044.

9. Potier D, Davie K, Hulselmans G, Naval Sanchez M, Haagen L, HuynhThu VA, Koldere D, Celik A, Geurts P, Christiaens V, et al. (2014) Mapping gene regulatory networks in Drosophila eye development by large-scale transcriptome perturbations and motif inference. Cell Rep. 9(6):2290-2303.

10. Dana AN, Hong YS, Kern MK, Hillenmeyer ME, Harker BW, Lobo NF, Hogan JR, Romans P, Collins FH (2005) Gene expression patterns associated with blood-feeding in the malaria mosquito Anopheles gambiae. BMC Genomics. 6:5.

11. Marinotti O, Calvo E, Nguyen QK, Dissanayake S, Ribeiro JM, James AA (2006) Genome-wide analysis of gene expression in adult Anopheles gambiae. Insect Mol Biol. 1:1-12.

12. Bonizzoni M, Dunn WA, Campbell CL, Olson KE, Dimon MT, Marinotti O, James AA (2011) RNA-seq analyses of blood-induced changes in gene expression in the mosquito vector species, Aedes aegypti. BMC Genomics. 12:82.

13. Hagedorn HH (2005) Mosquito Endocrinology. In: Marquardt WC, editor. Biology of Disease Vectors. Elsevier Academic Press. p317-327.

14. Zou Z, Saha TT, Roy S, Shin SW, Backman TW, Girke T, White KP, Raikhel AS (2013) Juvenile hormone and its receptor, methoprenetolerant, control the dynamics of mosquito gene expression. Proc Natl Acad Sci U S A. 110(24):E2173-E2181.

15. Hansen IA, Attardo GM, Park JH, Peng Q, Raikhel AS (2004) Target of rapamycin-mediated amino acid signaling in mosquito anautogeny. Proc Natl Acad Sci U S A. 101(29):10626-10631.

16. Roy SG, Hansen IA, Raikhel AS (2007) Effect of insulin and 20hydroxyecdysone in the fat body of the yellow fever mosquito, Aedes aegypti. Insect Biochem Mol Biol. 37(12):1317-1326.

17. Brown MR, Clark KD, Gulia M, Zhao Z, Garczynski SF, Crim JW, Suderman RJ, Strand MR (2008) An insulin-like peptide regulates egg maturation and metabolism in the mosquito Aedes aegypti. Proc Natl Acad Sci U S A. 105(15):5716-5721.

18. Brandon MC, Pennington JE, Isoe J, Zamora J, Schillinger AS, Miesfeld RL (2008) TOR signaling is required for amino acid stimulation of early trypsin protein synthesis in the midgut of Aedes aegypti mosquitoes. Insect Biochem Mol Biol. 38(10):916-922.

19. Gulia-Nuss M, Robertson AE, Brown MR, Strand MR (2011) Insulin-like peptides and the target of rapamycin pathway coordinately regulate blood digestion and egg maturation in the mosquito Aedes aegypti. PLoS One. 6(5):e20401.

20. Raikhel AS (2005) Vitellogenesis of Disease Vectors, From Physiology to Genes. In: Marquardt WC, editor. Biology of Disease Vectors. Elsevier Academic Press. p329-345.

21. Bryant B, Raikhel AS (2011) Programmed autophagy in the fat body of Aedes aegypti is required to maintain egg maturation cycles. PLoS One. 6(11):e25502.

22. Zou Z, Souza-Neto J, Xi Z, Kokoza V, Shin SW, Dimopoulos G, Raikhel A. (2011) Transcriptome analysis of Aedes aegypti transgenic mosquitoes with altered immunity. PLoS Pathog. 7(11):e1002394.

23. Kanehisa M, Goto S (2000) KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 28(1):27-30.

24. Attardo GM, Hansen IA, Shiao SH, Raikhel AS (2006) Identification of two cationic amino acid transporters required for nutritional signaling during mosquito reproduction. J Exp Biol. 209(Pt 16):3071-3078.

25. Shapiro AB, Wheelock GD, Hagedorn HH, Baker FC, Tsai LW, Schooley DA (1986) Juvenile hormone and juvenile esterase in adult females in Aedes aegypti. J Insect Physiol. 32:867–877.

26. Hernández-Martínez S, Rivera-Perez C, Nouzova M, Noriega FG (2014) Coordinated changes in JH biosynthesis and JH hemolymph titers in Aedes aegypti mosquitoes. J Insect Physiol. 72:22-27.

27. Roy SG, Raikhel AS (2011) The small GTPase Rheb is a key component linking amino acid signaling and TOR in the nutritional pathway that controls mosquito egg development. Insect Biochem Mol Biol. 41(1):62-69

28. Mane-Padros D, Cruz J, Cheng A, Raikhel AS (2012) A critical role of the nuclear receptor HR3 in regulation of gonadotrophic cycles of the mosquito Aedes aegypti. PLoS One. 2012;7(9):e45019.

29. Price DP, Nagarajan V, Churbanov A, Houde P, Milligan B, Drake LL, Gustafson JE, Hansen IA (2011) The fat body transcriptomes of the yellow fever mosquito Aedes aegypti, pre- and post- blood meal. PLoS One. 6(7):e22573.

30. Feitosa FM, Calvo E, Merino EF, Durham AM, James AA, de Bianchi AG, Marinotti O, Capurro ML (2006) A transcriptome analysis of the Aedes aegypti vitellogenic fat body. J Insect Sci. 6:1-26.

31. Attardo GM, Hansen IA, Raikhel AS (2005) Nutritional regulation of vitellogenesis in mosquitoes: implications for anautogeny. Insect Biochem Mol Biol. 35(7):661-675.

32. Raikhel AS, Dhadialla TS (1992) Accumulation of yolk proteins in insect oocytes. Annu Rev Entomol. 37:217-251.

33. Hagedorn HH, Turner S, Hagedorn EA, Pontecorvo D, Greenbaum P, Pfeiffer D, Wheelock G, FlanaganTR (1977) Postemergence growth of the ovarian follicles of Aedes aegypti. J Insect Physiol. 23(2):203-206.

34. Raikhel AS, Lea AO (1990) Juvenile hormone controls previtellogenic proliferation of ribosomal RNA in the mosquito fat body. Gen Comp Endocrinol. 77(3):423-434.

35. Zhu J, Chen L, Raikhel AS (2003) Posttranscriptional control of the competence factor betaFTZ-F1 by juvenile hormone in the mosquito Aedes aegypti. Proc Natl Acad Sci U S A. 100(23):13338-13343.

36. Raikhel AS, Lea AO (1983) Previtellogenic development and vitellogenin synthesis in the fat body of a mosquito: an ultrastructural and immunocytochemical study. Tissue Cell. 15(2):281-99.

37. Zahurak M, Parmigiani G, Yu W, Scharpf RB, Berman D, Schaeffer E, Shabbeer S, Cope L (2007) Pre-processing Agilent microarray data. BMC Bioinformatics 8:142.

Acknowledgements: This work was supported by the Strategic Priority Research Program of the CAS (# XDB11030600) and NSF of China (# 31472008, 31272367), grants (to ZZ) and R01 AI036959 grant (to A.S.R.) from the National Institutes of Health.

Author Contributions Conceived and designed the experiments: SR, TTS, ZZ and ASR. Performed the experiments: SR, TTS, LJ and BZ. Analyzed the data: SR, LJ, JH, TG and ASR. Contributed reagents/materials/analysis tools: SR, TTS, LJ, KPW, TG and ZZ. Wrote the paper: SR and ASR

Figure 2.1

Expression dynamics of *A. aegypti* fat body genes post blood meal.

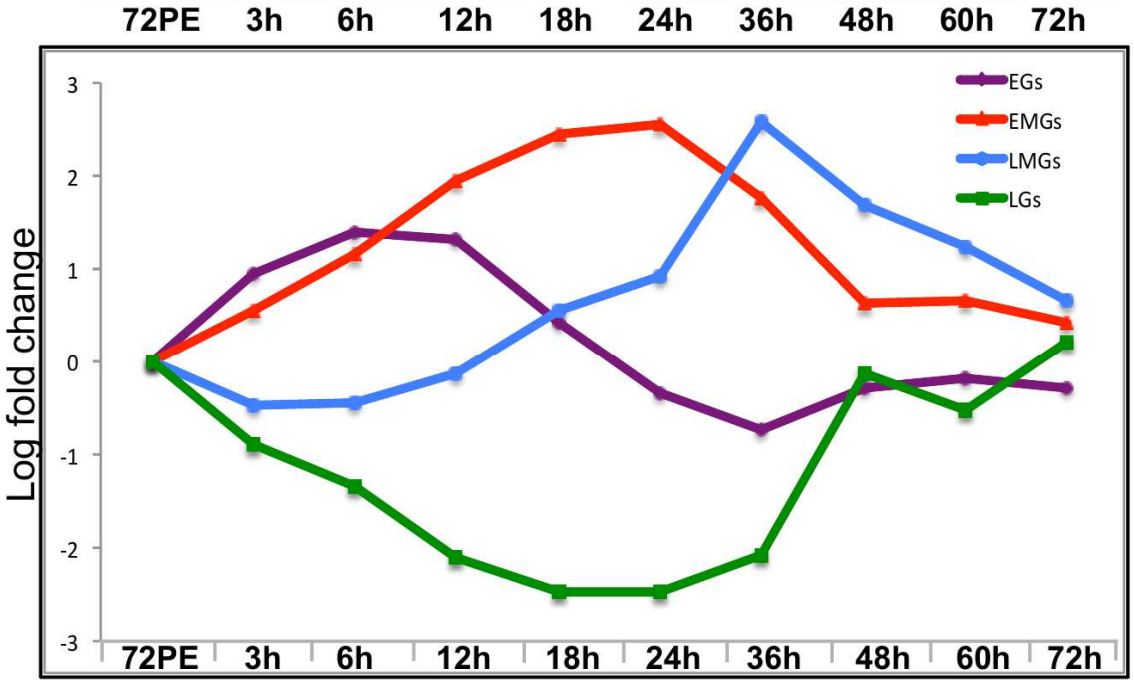


Figure 2.1

Average expression profiles of all EGs (purple line), EMGs (red line), LMGs (blue line) and LGs (green line) shown, X-axis shows the different time points while the Y-axis displays the average log fold change.

Figure 2.2

Functional group enrichment analysis of EGs, EMGs, LMGs and LGs

Figure 2.2

(A) Analyses of functional groups within the EG, EMG, LMG and LG groups using the KEGG database. The Y-axis of this 100% stacked
columns show the percentage of genes that fall within each functional group. (B-D) A more detailed analysis of the same gene cohorts
using the KEGG database. (B) The enrichment of IS&P genes within more specific functional categories determined. (C) The
enrichment of CP&S genes within more specific functional categories determined. (D) The enrichment of metabolism genes within more
specific functional categories determined.

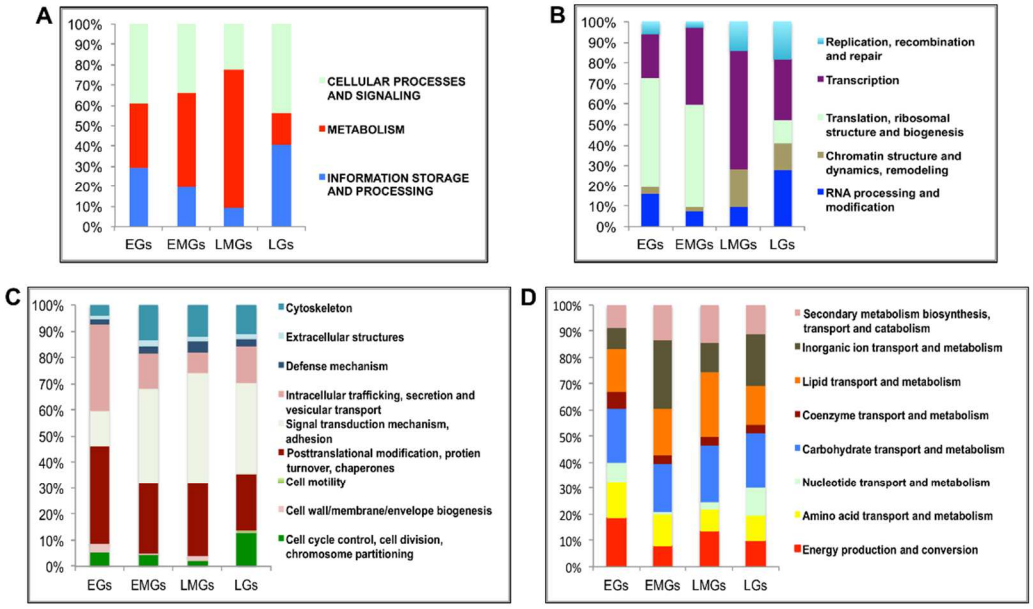


Figure 2.3

Effects of AAs, 20E and JH on representative EGs

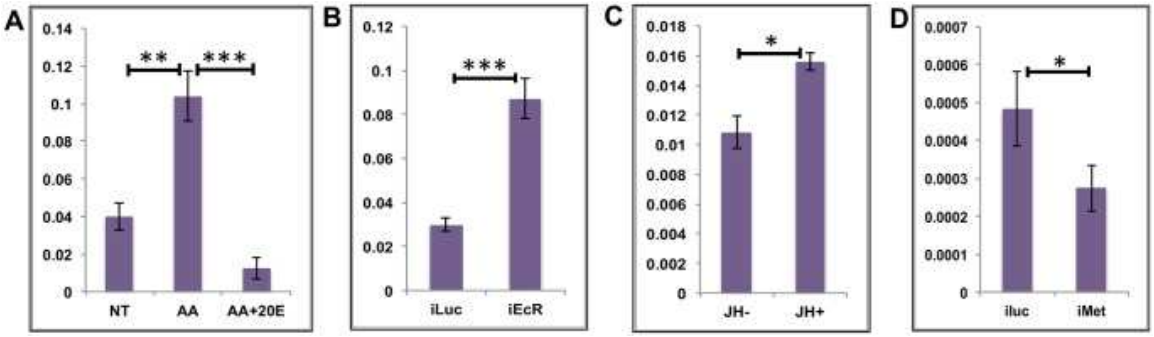


Figure 2.3

(A) Relative expression of AAEL002269, Purine nucleoside phosphorylase detected by qRT-PCR, in tissues subjected to *in vitro* fat body culture (IVFBC) in culture media without (NT) and with amino acids (AA) and with amino acid plus 20E (AA+20E). **(B)** Relative expression of the same gene detected by qRT-PCR, in fat body tissues collected from female mosquitoes after EcR knock-down (iEcR). **(C)** Relative expression of the same gene in tissues subjected to IVFBC in culture media without (JH-) and with (JH+) juvenile hormone. **(D)** Relative expression detected by qRT-PCR, in fat body tissues collected from female mosquitoes after knock-down of the JH receptor Met (imet). Injecting double stranded RNA for the Luciferase gene (iluc) served as the control in the RNAi experiments (**B** and **D**). All expressions calculated against housekeeping gene RPS7. Data representative of three biological replicates, with three technical replicates and are illustrated as average ± SD, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2.4

Effects of AAs, 20E, insulin and HR3 on YPP genes.

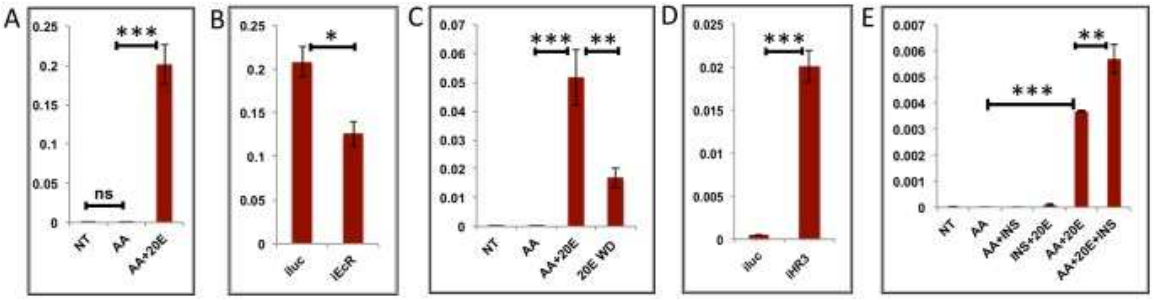


Figure 2.4

(A) Relative expression of the gene AAEL006563, (Carboxypeptidase), detected by qRT-PCR, in tissues subjected to *in-vitro* fat body culture (IVFBC) in culture media without (NT) and with amino acids (AA) and with amino acid plus 20E (AA+20E). **(B)** Relative expression of the same gene detected by qRT-PCR, in fat body tissues collected from female mosquitoes post EcR knock-down (iEcR). **(C)** Relative expression in tissues subjected IVFBC in culture media without (NT) and with amino acids (AA), with amino acids plus 20E (AA+20E) and after the withdrawal of 20E (20E WD). **(D)** Relative expression detected by qRT-PCR, in fat body tissues collected from female mosquitoes post HR3 knock-down (iHR3). **(E)** Relative expression of the same gene in tissues subjected to IVFBC in culture media without (NT) and with amino acids (AA), with amino acids and Insulin (AA+INS), Insulin and 20E (INS+20E), amino acids plus 20E (AA+20E), and amino acids plus 20E and Insulin (AA+20E+INS). Injecting double stranded RNA for the Luciferase gene (iluc) served as the control in the RNAi experiments (**B** and **D**). All expression calculated against housekeeping gene RPS7. Data representative of three biological replicates, with three technical replicates and are illustrated as average ± SD, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2.5

Effects of AAs, 20E and HR3 on EMGs.

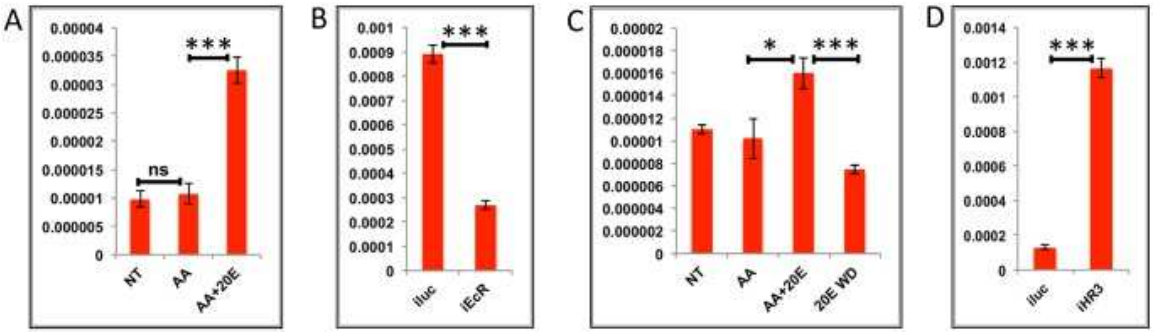


Figure 2.5

(A) Relative expression of gene AAEL001433, fgf receptor activating protein detected by qRT-PCR, in tissues subjected to *in- vitro* fat body culture (IVFBC) in culture media without (NT) and with amino acids (AA) and with amino acid plus 20E (AA+20E). **(B)** Relative expression of the same gene detected by qRT-PCR, in fat body tissues collected from female mosquitoes post EcR knock-down (iEcR). **(C)** Relative expression in tissues subjected to IVFBC in culture media without (NT) and with amino acids (AA), with amino acids plus 20E (AA+20E) and after the withdrawal of 20E (20E WD). **(D)** Relative expression detected by qRT-PCR, in fat body tissues collected from female mosquitoes post HR3 knock-down (iHR3). Injecting double stranded RNA for the Luciferase gene (iluc) served as the control in the RNAi experiments (**B** and **D**). All expression calculated against housekeeping gene RPS7. Data representative of three biological replicates, with three technical replicates and are illustrated as average ± SD, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Effects of AAs, 20E, JH and HR3 on representative LMGs.

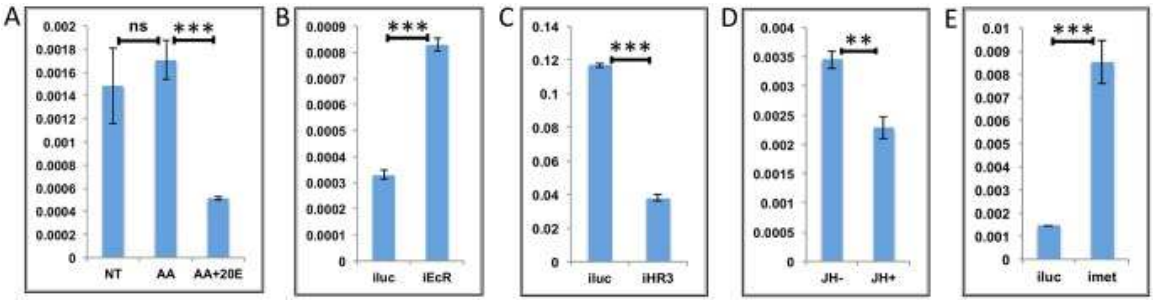


Figure 2.6

(A) Relative expression of gene AAEL003568, Threonine dehydratase detected by qRT-PCR, in tissues subjected to *in-vitro* fat body culture (IVFBC) in culture media without (NT) and with amino acids (AA) and with amino acid plus 20E (AA+20E). **(B)** Relative expression of the same gene detected by qRT-PCR, in fat body tissues collected from female mosquitoes post EcR knock-down (iEcR). **(C)** Relative expression detected by qRT-PCR, in fat body tissues collected from female mosquitoes post HR3 knock-down (iHR3). **(D)** Relative expression in tissues subjected to IVFBC in culture media without (JH-) and with (JH+) juvenile hormone. **(E)** Relative expression detected by qRT-PCR, in fat body tissues collected from female mosquitoes post Met knock-down (iMet). Injecting double stranded RNA for the Luciferase gene (iluc) served as the control in the RNAi experiments (**B**, **C** and **E**). All expression calculated against housekeeping gene RPS7. Data representative of three biological replicates, with three technical replicates and are illustrated as average ± SD, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2.7

Effects of AAs, 20E and JH on representative LGs

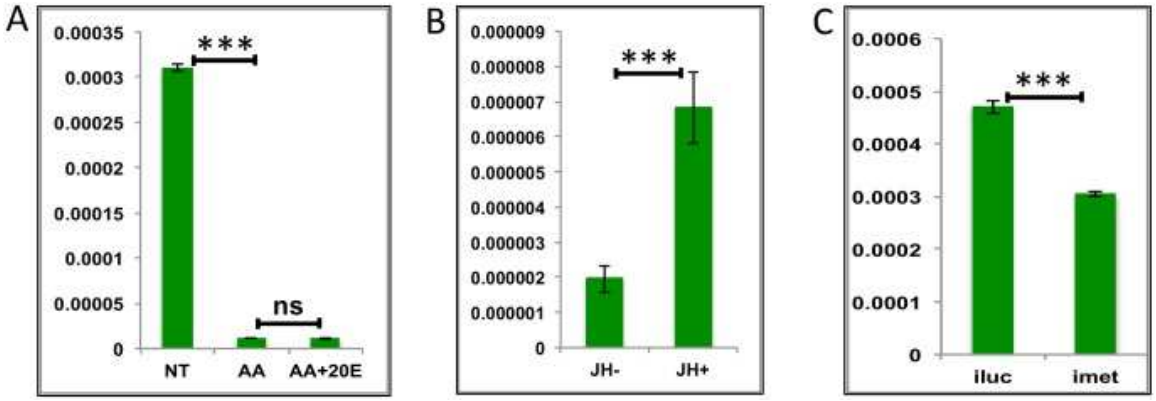
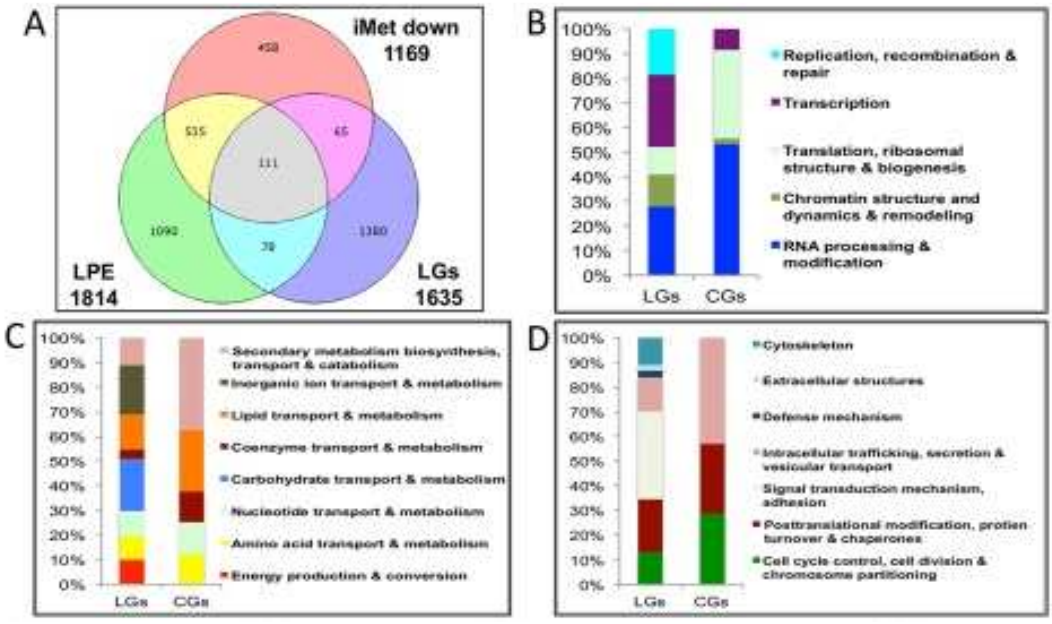


Figure 2.7

(A) Relative expression of gene AALE004328 –Origin recognition complex, detected by qRT-PCR, in tissues subjected to *in-vitro* fat body culture (IVFBC) in culture media without (NT) and with amino acids (AA) and with amino acid plus 20E (AA+20E). **(B)** Relative expression of the same gene in tissues subjected to IVFBC in culture media without (JH-) and with (JH+) juvenile hormone. **(C)** Relative expression detected by qRT-PCR, in fat body tissues collected from female mosquitoes post Met knock-down (iMet). Injecting double stranded RNA for the Luciferase gene (iluc) served as the control in the RNAi experiment. All expressions calculated against housekeeping gene RPS7. Data representative of three biological replicates, with three technical replicates and are illustrated as average ± SD, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2.8

Genes cyclically activated by JH through Met—Functional group enrichment and the effects of AAs, 20E and JH.



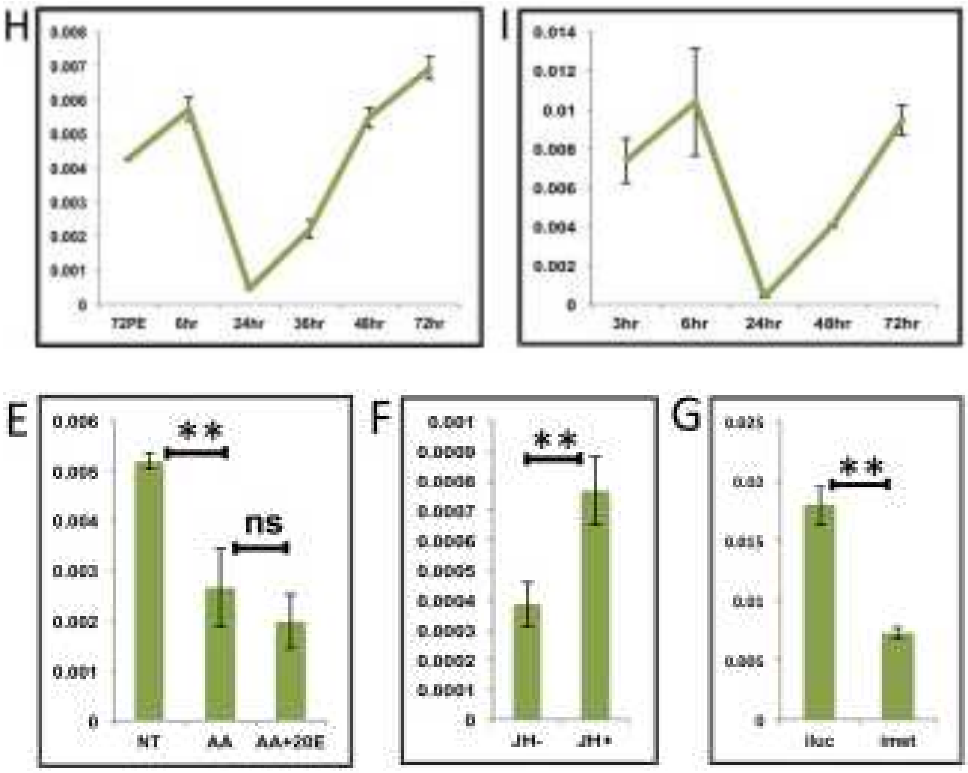


Figure 2.8

(A) Venn diagram showing genes that are up regulated in late post eclosion (LPE) and late post blood meal (LGs) periods and that are down regulated in Met knocked-down (iMet down) fat body tissues. **(B-D)** Comparison of functional categories viz. **(B)** Information storage and Processing, **(C)** Metabolism and **(D)** Cellular Processes and Signaling, that constitute the late genes (LGs) and cyclical genes (CGs) using the inNOG database. **(E-F)** Relative expression of gene AAEL001171, tRNA-dihydrouridine synthase detected by qRT-PCR, in tissues subjected to *in-vitro* fat body culture (IVFBC) in culture media, **(E)** without (NT) and with amino acids (AA) and with amino acid plus 20E (AA+20E); **(F)** without (JH-) and with (JH+) juvenile hormone. **(G)** Relative expression of the same gene detected by qRT-PCR, in fat body tissues collected from female mosquitoes post Met knock-down (iMet); injecting double stranded RNA for the Luciferase gene (iluc) served as the control. **(H)** Expression profile of the gene after the first blood meal. **(I)** Expression profile of the gene after the completion of the first reproductive cycle (egg laying) and post second blood meal. All expression calculated against housekeeping gene RPS7. Data representative of three biological replicates, with three technical replicates and are illustrated as average ± SD, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2.9

Genes cyclically regulated by JH through Met—the effects of AAs, 20E, EcR and Met on genes cyclically repressed by Met.

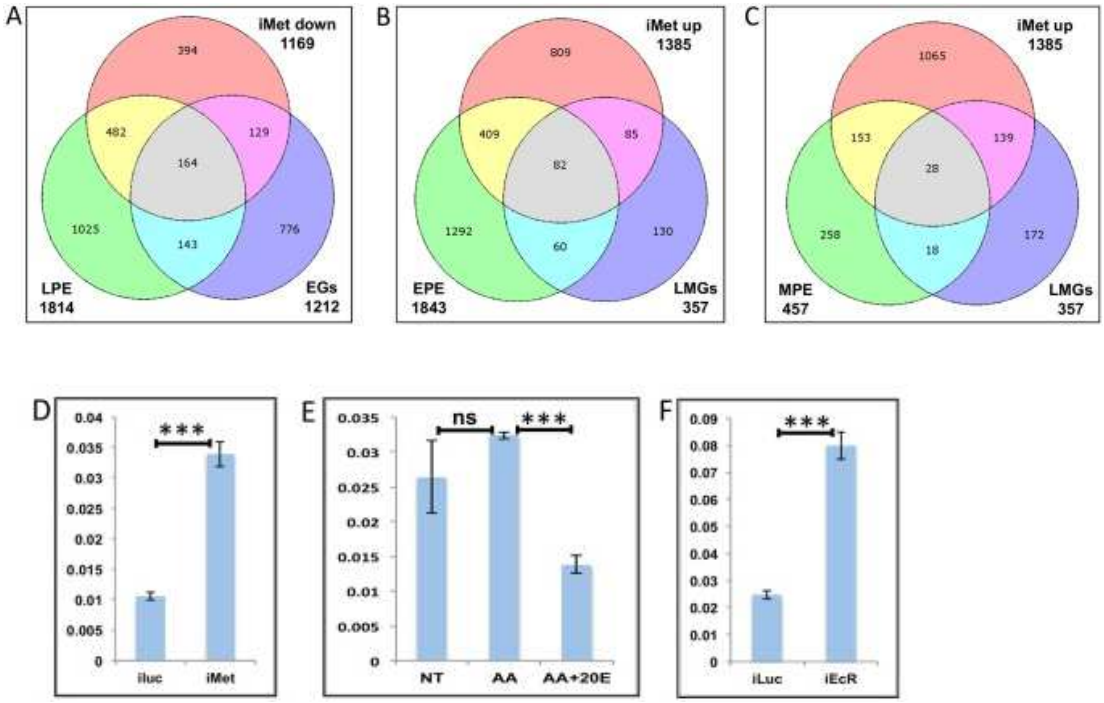


Figure 2.9

(A) Venn diagram showing genes that are up regulated in late post eclosion (LPE) and early post blood meal (EGs) periods and that are down regulated in Met knocked-down (imet down) fat body tissues. **(B)** Venn diagram showing genes that are up regulated in early post eclosion (EPE) and late-mid post blood meal (LMGs) periods and that are up regulated in Met knocked-down (imet up) fat body tissues. **(C)** Venn diagram showing genes that are up regulated in early post eclosion (EPE) and late-mid post blood meal (LMGs) periods and that are up regulated in Met knocked-down (imet up) fat body tissues. **(D)** Relative expression of the gene, AAEL002781, Galactokinase detected by qRT-PCR, in fat body tissues collected from female mosquitoes post Met knock-down (iMet); injecting double stranded RNA for the Luciferase gene (iluc) served as the control. **(E)** Relative expression of the same gene detected by qRT-PCR, in tissues subjected to *in-vitro*fat body culture (IVFBC) in culture media without (NT) and with amino acids (AA) and with amino acid plus 20E (AA+20E). **(F)** Relative expression of the same gene detected by qRT-PCR, in fat body tissues collected from female mosquitoes post EcR knock-down (iEcR).

Figure 2.10

The main regulators mediating transcription in four distinct waves of gene expression during the gonadotrophic cycle in the fat body of female *Aedes aegypti*.

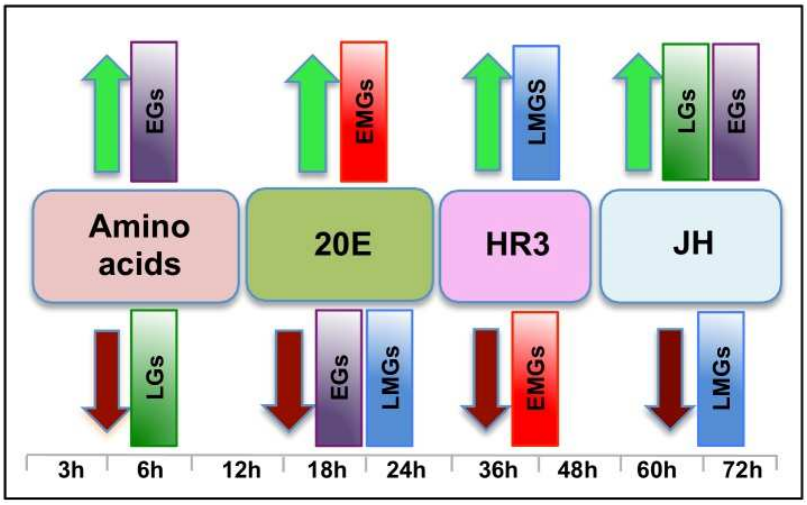


Figure 2.10

Amino acids are the main regulators of gene expression during the early hours post blood meal (PBM); found to up- and down-regulate representatives of early (EGs) and late genes (LGs) respectively. Around 12 h PBM, the effect of 20E becomes apparent. 20E activates early mid genes (EMGs) and represses EGs as well as late mid genes (LMGs) between 18 h-24 h. HR3 seems to be the main regulator between 36 h and 48 h, the expressions of EMGs decline under the influence of HR3 whereas that of the LMGs increase. JH regulates the terminal part of this cycle by up-regulating late genes (LGs) and down-regulating the LMGs. A moderate activating effect of JH can be detected in case of the EGs during this period.

CHAPTER III

Gene Repression by 20-Hydroxyecdysone and Ecdysone Receptor during the Gonadotrophic Cycles of the Disease Vector Mosquito, *Aedes aegypti*

3.1 Abstract

The acquisition of a blood meal to initiate egg development is the basis of disease transmission by female mosquitoes. Therefore, understanding the molecular events of reproduction is essential to our ability to control this important vector. In our previous study, transcriptomic and reverse genetic analysis revealed four distinct waves of gene expression during blood meal-activated reproduction in the female mosquito, each uniquely regulated by hormonal and nutritional factors (Chapter II). Genes that were upregulated at 18-24 hours post-blood meal (PBM), during the peak of vitellogenesis, are designated early-mid genes, and are activated by 20-hydroxyecdysone (20E). A different set of genes, designated late-mid genes, are highly expressed 36-48 hours PBM, after the peak of vitellogenesis but before its termination. This set of genes is presumably repressed by 20E, and their expression is lowest at 24h PBM during the peak of 20E titer. While 20E, acting through its cognate receptor, the ecdysone receptor (EcR), is known to be the master regulator of the vitellogenic phase of reproduction, its ability to simultaneously direct the activation and repression of two sets of target genes was previously unknown and the factors contributing to its bi-directional regulation remain unclear. 20E/EcR signaling is mediated by the activity of a group of transcription factors (TF) transcribed in response to a pulse of 20E. Although there are a limited number of

these early gene TFs, their role is expanded by the generation of multiple isoforms, and we hypothesized that isoform-specific regulation results in selective up- or down-regulation of different gene clusters. Here, using RNA interference (RNAi), *in vitro* fat body culture (IVFBC) techniques and cycloheximide (CHX) assays, we demonstrate that 20E, through EcR, has a dual role in both activating and repressing two clusters of differentially expressed genes and that repression of late-mid genes by EcR is indirect, mediated by isoforms of the early gene TFs in the 20E regulatory cascade. Importantly, we found that repression vs. activation during the peak of 20E titer is directed by EcR in an isoform-specific manner. In addition, we describe the epistatic relationships between the different isoforms of 20E regulatory factors. The results of this study indicate that differences in regulation of genes important to mosquito reproduction are due, at least in part, to isoform specificity, and may also exist at the level of downstream cis and trans interacting elements. The combinatorial control by a limited number of factors to achieve varied results is an innovative evolutionary adaptation, and essential to the normal progression of important life stages. The complexity involved in regulation of mosquito reproductive genes requires continued investigation but defining the role of receptor and TF isoforms is an essential first step. Understanding the role-reversal of a major regulator of gene expression is important in deciphering the fine-tuning of a transcriptional response.

3.2 Introduction

Anautogenous mosquitos transmit numerous diseases due to the requirement of a blood meal to initiate reproduction and ensure the success of their species (14). Factors derived from the intake of blood, responding to conditions of developmental competence, trigger a regulatory signaling pathway that results in the differential expression of sequential clusters of genes (64, 103). The normal succession of reproductive events from initiation to termination depends on the timely orchestration of gene expression. In fact, control of gene expression, mainly at the transcriptional level, is the basis of all physiological changes in organisms during development, growth and reproduction. These patterns of gene expression are induced by rising and falling hormone levels and driven by the activity of receptors. Successful execution of developmental transitions requires that groups of target genes are expressed or silenced on cue, but relatively few regulatory factors direct the varied expression of many genes. This feat of regulation is accomplished through combinatorial control, involving the interplay of receptors and transcription factors (TF) with variable cis and trans regulatory elements (97). Many receptors and TFs exist as isoforms that are distinct from each other not only in structure, but in spatial and temporal expression (59–61, 80–82, 104–108). Isoforms that are expressed in a cell- or tissue-specific manner may display a distinct functional role restricted to those tissues in which they are expressed. However, even within the same cells, different isoforms can direct different results, as variations in sequence can lead to preferential affinity for specific response elements.

During vitellogenesis in the female mosquito fat body (FB), differential expression of nearly half the genes in the mosquito genome is observed throughout four sequential waves (103). Dynamic and multifunctional, the FB is analogous to vertebrate liver and adipose tissue combined and is a primary reproductive tissue in female mosquitos, responsible for the massive production of yolk protein precursors (YPP) (52). A cluster of genes designated early-mid genes are highly upregulated between 18-24 hours post-blood meal (PBM) and are activated by 20-hydroxyecdysone (20E) (103). A key insect steroid hormone, 20E titer rises following a blood meal, and peaks between 18-24 hours PBM (19). The expression of the early-mid genes subsequently drops, repressed by the nuclear receptor (NR) hormone receptor 3 (HR3), as termination of vitellogenesis requires that key vitellogenic genes are turned off and genes responsible for physiological changes necessary to complete one cycle and prepare for the next are expressed (65, 103). A well-studied example of a gene from the early-mid group is the yolk protein precursor (YPP) gene, vitellogenin (vg), essential for egg production. A subsequent wave of gene upregulation occurs at 36 hours PBM in a cluster of genes designated late-mid genes. These genes are activated at this time by HR3 but show repression in response to 20E and EcR. The peak titer of 20E occurs around 24 hours PBM when the expression of this set of late-mid genes is lowest. Therefore, it appears that 20E acts as an activator and a repressor of two distinct gene sets simultaneously. This phenomenon of bidirectional regulation by a single signaling pathway and the mechanisms that are responsible are poorly understood.

Signaling by 20E is mediated by the ecdysone receptor (EcR) which, in its active form is heterodimerized with another NR, ultraspiracle (USP). Studies in *drosophila* have defined the activating role of 20E through its ligand-activated receptor EcR/USP. Likewise, in *Aedes aegypti,* transactivation by the 20E signaling pathway has been described in detail at the molecular level for the YPP gene vg (51–53, 59, 61, 65, 66, 69, 103, 109–111). Up-regulation of vg is closely correlated to the sharp increase in 20E titer between 18-24h PBM and it has been shown that activation of *vg* expression in the mosquito requires direct binding of EcR-USP to ecdysone response elements (EREs) in the *vg* promoter. EcR and USP both have two isoforms in *Aedes aegypti:* EcRA and EcRB, and USPA and USPB, respectively, and activation of vg expression by the ligand-bound receptor is isoform-specific. EcRB and USPB are preferentially expressed early during vitellogenesis, and the heterodimer of these isoforms is responsible for activation of *vg*.

Isoforms of early gene TFs in the 20E regulatory pathway also contribute to *vg* activation. E74B is expressed at the peak of vitellogenesis and acts synergistically with EcR-USP to contribute to a high level of this gene expression (52). E74A, which is expressed later at the termination phase, does not contribute to *vg* activation (52). The early gene broad has four isoforms in *Aedes aegypti,* and these isoforms display differential involvement as well (62). Broad Z2 is expressed early after the blood meal and stimulates *vg* expression (61). Broad Z1 and Z4, expressed at 24h PBM, are involved in repression of *vg* (61). Broad Z3 has no effect on *vg* expression (61).

Reference genes for this study were chosen from microarray-generated lists of genes that were differentially expressed at eight different timepoints during the PBM phase. Selected genes were from the late-mid gene cluster that displayed maximum expression at 36h PBM and minimum expression at 24h PBM during the peak titer of 20E, opposite to the profile of early-mid genes, including *vg*. One additional gene was included that was not detected by the microarray but has a similar profile. The lipophorin receptor (LpR) is a member of the low-density lipoprotein receptor family and mediates the accumulation of lipids derived from circulating lipophorin (Lp). There are two tissue-specific splice variants of LpR that localize to the ovary (LpRov) or the fat body (LpRfb). We included LpRfb because of the similarity in profile and its functional importance in reproduction.

We hypothesized that the opposing functions of EcR may be carried out, directly or indirectly, by differential involvement of isoforms of 20E signaling pathway components. Using RNA interference (RNAi) technology, *in vitro* fat body culture (IVFBC) technique and cycloheximide (CHX) assays we demonstrate that this cluster of late-mid genes are indirectly repressed by EcRA through the action of the early gene transcription factor E74B. Thus, positive and negative regulation of gene expression by 20E during the vitellogenic cycle is achieved through action of the ligand-bound EcR–USP and isoforms of early gene products in the 20E regulatory hierarchy. Nevertheless, characterization of the repression of a group of genes important to insect reproduction, including the roles of receptor and TF isoforms of the 20E signaling pathway is an

important step forward in deciphering poorly understood aspects of NR hormone signaling in general, and particularly the dual nature of control by EcR.

3.3 Materials and methods

Mosquito rearing

Wild type *Aedes aegypti* mosquitoes of the UGAL strain were reared at 27°C and 80% humidity as described previously (112). The larvae were raised in non-crowded conditions [37,38] (200 in 750ml distilled water per 9"x12" pan) and fed 0.125–0.900 ml vol. of standard diet (equal parts of rodent diet, Lactalbumin and active dry yeast) between Day 0 and Day 4. Four pans of pupae were combined into one adult cage. Adult mosquitoes were given unrestricted access to water and 10% (wt/vol) sucrose solution. All dissections were performed in Aedes physiological solution (APS) at room temperature. Blood feeding of all adult mosquitoes was performed with White Leghorn chickens. All procedures for using vertebrate animals were approved by the UCR Animal Care and Use Committee.

RNA extraction and cDNA synthesis

Fat body samples were dissected into Trizol (Invitrogen/Life Technologies) and processed immediately or after brief storage at -80˚C. Total RNA was extracted from fat body samples using the Trizol method (Invitrogen/Life Technologies) according to the manufacturer's protocol. Samples were treated with DNAseI (Invitrogen/Life Technologies), and cDNA was synthesized from 2μg total RNA using the Superscript First-strand cDNA Synthesis kit (Invitrogen/Life Technologies).

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

qPCR analysis of cDNA samples was performed using SYBR Green Master Mix (Qiagen) and primers designed for qPCR detection (Supplementary Table 3.1). Experiments were run using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). All experiments were performed in triplicate and results were normalized against the housekeeping gene S7 ribosomal protein (RPS7) as an internal control. Raw data were exported and analyzed using Microsoft Excel. Relative expression (RE) was calculated as $RE = 2^{-\Delta\Delta Ct}$. Graphs represent average experimental Ct values relative to wild-type or iLuc-injected control samples. Error bars are representative of SEM and p-value significance was set at <0.05. Statistical analysis was performed by applying student's t-test using Graphpad Prism.

RNA interference-mediated gene knockdown

Genes selected for knockdown were cloned into the pGemT-EZ vector. dsRNA was produced as previously described (112). Briefly, double-stranded RNA (dsRNA) was synthesized using the MEGAscript T7 kit (Ambion). The Luciferase gene was used to generate iLuc dsRNA that was used as a control. For knockdown of individual isoforms, dsRNA was designed to correspond to an isoform-specific region of each gene. Primers used for dsRNA synthesis are shown in Supplementary Table 3.2. After synthesis of dsRNA, samples were phenol/chloroform-extracted and ethanol-precipitated.

Microinjection into the thorax of CO2-anesthetized female mosquitoes was performed using the PicospritzerII (General Valve) at 12-24 hours post eclosion (PE) using 0.5ug (0.25ul of 2 ug/ul) dsRNA. Mosquitoes were allowed to recover for three days before blood feeding. Dissections of mosquito fat bodies were performed at 24 hours PBM in room temperature Aedes Physiological Saline (APS). Knockdown samples relative to controls were used to assess expression level of each gene of interest using qPCR.

in vitro Fat Body Culture (IVFBC)

Female mosquito fat bodies were dissected in room temperature Aedes Physiological Saline (APS) at 3 days PE. Fat bodies were then incubated in complete culture medium supplemented with amino acids (AA) in the presence or absence of 20E (Sigma). The concentration of 20E used was 1x10-8M for the first 4 hours and 1x10-6M in fresh media for an additional 4 hours to reflect biological conditions, as previously described (103). Following the incubation period, samples were collected in Trizol, RNA was extracted, and cDNA synthesized as described above, and gene expression was quantified by qPCR. The efficiency of the hormonal treatment was tested by induction of established 20E-activated genes (Supplementary Figure 3.2).

Cyclohexamide (CHX) assay

Using IVFBC methods, incubation medium was supplemented with the protein synthesis inhibitor CHX at a concentration of 20μM (dissolved in DMSO) as described previously (113). Results were quantified by qRT-PCR.

3.4 Results

Expression profiling

The expression profiles of late-mid reference genes used for this study were validated by qRT-PCR using time course samples from blood fed female mosquito fat bodies collected from intervals across the entire PBM reproductive period. Examination of the profiles showed that these genes were expressed at varying levels immediately following the blood meal, ranging from basal to moderate expression compared to overall levels throughout the PBM period. All genes exhibited low expression levels at 24 hours PBM followed by a steep spike in expression at 36 hours PBM and a decline in expression thereafter throughout the termination phase (Figure 3.1). Reference gene IDs and biological functions are shown in Table 3.1. Figures show results for LpRfb and Aael010075, which will serve as representatives of all reference genes in our experimental set. Remaining gene data can be found in Supplemental Figures.

Late-mid genes are repressed by 20E

The results of our previous study revealed that several genes from the late-mid gene cluster that are upregulated at 36h PBM are repressed at 24h PBM when the titer of 20E is high. We found that repression was directed by 20E through EcR. To confirm that this finding extends to other genes in the cluster we first used IVFBC to test the effect of 20E exposure on an expanded set of late-mid reference genes. FBs from female

mosquitoes were removed at 72 hours PE, when 20E levels are low, and incubated in APS supplemented with AAs. Samples were incubated in 20E presence (experiment) or its absence (control). 20E was added at low physiological concentration (1x10-8M) for four hours followed by high physiological concentration (1x10-6M) for an additional four hours to mirror physiological conditions following a blood meal. Fat bodies exposed to 20E exhibited significantly decreased expression of late-mid genes compared to controls (Figure 3.2) suggesting that their repression is directed by 20E. These results also confirmed that a larger group of late-mid genes than originally tested are similarly regulated by a repressive effect of 20E.

20E-directed repression of late-mid gene expression is mediated by EcR

To determine if 20E acts through EcR, RNAi was performed to knockdown EcR *in vivo*, and reduction of EcR was determined to be efficient (Supplemental Figure 3.3). This included knockdown of both isoforms as common-region dsRNA was used. Therefore, while involvement by EcR could be determined, this experiment could not reveal information about specific isoforms involved in repression. The expression of reference late-mid genes was significantly de-repressed in EcR-deficient samples (iEcR) when compared to control Luciferase RNAi samples (iLuc) (Figure 3.3). The abatement of repression by elimination of both forms of the functional receptor provides evidence of EcR involvement in the repression of late-mid genes in the mosquito FB.

Isoform-specific repression by EcR

Our previous study analyzed only the effect of EcR (common region) without investigating isoform-specificity in regulation. Since isoforms often display different functional roles, we considered the possibility that the opposing activity by EcR was isoform specific. EcRB acts as a transcriptional activator of vg expression and is expressed during the peak of vitellogenesis. EcRA levels increase by the termination phase, however, a low level of EcRA is present throughout the PBM phase. To confirm the identity of the EcR isoform directing repression of late-mid genes, we used an RNAi approach. Double-stranded RNA (dsRNA) corresponding to the isoform-specific regions of EcRA and EcRB was delivered to knock down the expression of each isoform individually in two groups of mosquitoes. dsRNA complementary to the luciferase (luc) gene were injected into a third group as a control. Following a blood meal, the mosquitoes were dissected at 24h PBM. Relative transcript levels of knockdown target genes were detected by qPCR and knockdowns were confirmed to be efficient (Supplemental figure 3.3). In iLuc control mosquitoes, expression of the late-mid genes was similar to untreated wild-type mosquitoes (UGAL). A significant increase in expression was observed in the fat bodies of EcRA-depleted mosquitoes indicating that EcRA is the negative regulator of late-mid genes under normal conditions *in vivo.* In EcRB-depleted mosquitoes however, there was a decrease in transcript levels compared to controls, suggesting that EcRB is an activator of these genes (Figure 3.4). These results show that EcRA and EcRB have isoform-specific functions at different phases of vitellogenesis, but that repression of late-mid genes is controlled by EcRA. Thus, EcR

displays isoform-specific bi-functional regulation, with each isoform directing opposing actions on two distinct groups of genes simultaneously.

Repression by EcR is indirect

Although activation by EcR has been worked out at the molecular level in detail, repressive action by the ligand-bound EcR is previously uncharacterized. Therefore, we asked whether the repressive effect is due to direct binding at target gene promoter sites or if it is the result of indirect action through other intermediate factors. CHX is a compound that blocks translation and therefore does not allow the formation of proteins that could act as intermediate transcription factors. IVFBC experiments were used in which fat bodies were incubated in media with various combinations of AAs, 20E and CHX. As previously reported (citation), we observed activation of gene expression by AAs (complete media) compared to media alone, and addition of 20E caused repression to lower levels than media alone. Exposure to the protein synthesis inhibitor CHX abolished the observed repression of late-mid genes even in response to 20E (Figure 3.5). These results indicate that the repressive action of EcR is indirect and requires the participation of additional factors.

TF Isoforms of the 20E/EcR pathway display differential involvement in late-mid gene repression

Since repression of late-mid genes by EcR was shown to be indirect, RNAi depletion experiments were conducted to determine the factors that mediate the repressive effect and to examine the roles of 20E regulatory pathway components, including all isoforms, in repression. This included knockdown of USPA and B, E74A and B, E75A, B and C, Broad Z1-4, and HR3, and each factor was examined individually. Isoform-specific knockdown samples were created by targeting the unique region of each isoform. Knockdowns were validated for efficiency by qPCR (Supplementary Figure 3.3) and used to quantify reference gene expression in each deficient background. Expression of reference genes was determined by qPCR using knockdown samples compared to iLuc injected controls. Our results show that late-mid gene repression by 20E-bound EcRA is mediated primarily through the strong repressive action of the early gene E74B (Figure 3.6). EcRB (Figure 3.4) and E74A both displayed activating effects on the expression of the late-mid genes, as did the nuclear receptor HR3 (Figure 3.7). Activation of these genes by HR3 is in accordance with our results from the previous chapter. No significant difference in expression of late-mid genes was observed upon KD of E75 or Broad isoforms (Supplemental figure 3.8, additional gene data not shown).

Epistatic interactions of 20E/EcR pathway component isoforms

To gain a more in-depth understanding of the relationships between EcR and other members of the EcR regulatory pathway, we investigated the epistatic interaction of key components of the signaling cascade. The expression of each factor implicated in the regulatory pathway of our repressed gene set was examined in various RNAi backgrounds (Figure 3.8). This included elucidation of the roles of different isoforms. EcRA activates E74B, but EcRB activates E74A and represses E74B. Hr3 was shown to activate E74A.

3.5 Discussion

The results of this study suggest complex regulation of gene expression during blood meal-induced reproduction in the female mosquito. Ecdysteroid activation of key vitellogenic genes has been described in detail. However, the simultaneous repression of a different set of genes is remarkable because opposing roles for a ligand-bound NR is a phenomenon that does not follow the canonical paradigm of NR signaling and about which we have very little information. We hypothesized that bi-directional regulation could be attributed to isoform specificity involving EcR or other components of the 20E regulatory pathway. Thus, we analyzed the roles of individual receptor and TF isoforms in down-regulation of gene expression. In addition, isoform-specific interaction of 20E-pathway components was determined through epistatic analysis.

The transcript levels of all mid-late genes included in this study are low during the peak of vitellogenesis at 24h PBM but are sharply elevated after the drop in 20E titer, peaking at 36h PBM. It has been shown that declining levels of hormone can signal changes in gene expression patterns that trigger biological responses essential to normal patterns of life stages. Furthermore, while it is known that 20E and transcription factors associated with the 20E cascade are essentially recycled to induce different events throughout the lifespan of the mosquito, the particular interplay or rearrangement of these factors to regulate genes necessary to complete a reproductive cycle are unknown. In addition, the ability of these factors to both activate and repress distinct sets of genes at different times to achieve different results is a phenomenon that remains poorly understood. The objective of this chapter is to elucidate the epistatic relationships of transcription factor isoforms that direct expression patterns of this set of genes. In addition, this study attempts to understand the relationship between different isomers of the 20E family of transcription factors in transcriptional regulation of a group of genes downregulated during the PBM period. In understanding the nuances of gene expression patterns that regulate different phases of reproduction, we can better understand the intricacies of hormonal regulation as well as reproductive cycles in the mosquito, leading to the development of novel methods of vector control.

In vitro incubation of mosquito fat bodies in the presence of 20E caused decreased expression of late-mid genes. In addition, FBs depleted of EcR through RNAi were not responsive to 20E treatment. When EcR-depleted mosquito fat bodies were incubated in the presence of 20E, the repressive effect on mid-late gene expression was eliminated.

These data clearly show that 20E through its receptor EcR represses the expression of a set of mid-late genes. Therefore, the ligand-bound receptor plays a dual role during mosquito vitellogenic cycles functioning as an activator of genes like vg yet repressing the expression of others and preventing their premature activation. Vg is known to be activated by the influx of 20E which binds EcR/USP and forms a complex at EcR response elements (EcRE) in the vg promoter. The liganded receptor complex recruits coactivators, including E74B, that act synergistically to enhance activation. The vg promoter has multiple binding sites for these receptors and transcription factors that result in extremely high levels of gene expression. Formerly, our understanding of NR action was based on the classical model that states that activation of target genes results from ligand binding to its receptor and creating a conformational change that allows eviction of existing co-repressors and recruitment of co-activators to the receptor complex at a response element in a target gene promoter. Absence of ligand maintains occupancy of REs by the inactivated receptor and associated co-repressors. However, recent studies have revealed that ligand-bound NRs frequently direct down-regulation of target genes (72, 73, 87). The sequence or organization of REs can profoundly affect transcriptional outcome (74, 78, 86, 95)While the activation of vg by 20E and transcription factors in the 20E-regulatory cascade has been the subject of much research, other gene expression patterns during reproductive cycles and the ways in which they are directed are still unclear.

This study has revealed the differential role of EcR and E74 isoforms in the repression of late-mid genes: EcRA and E74B as strong repressors and EcRB and E74A

as activators of gene expression. Remarkably, we have shown for the first time that an EcR isoform other than that which is responsible for *vg* activation is simultaneously responsible for the repression of a different gene set. Isoform-specific RNAi experiments combined with organ culture assays, CHX assays and epistatic analysis clearly show that EcRA acts as the repressor of late-mid genes through the synergistic activity of E74B. These data suggest that differential regulation of different gene sets is due in part to isoform specificity, but likely also occurs through differences in downstream components such as the sequence or organization of REs. Furthermore, we have shown that E75 isoforms do not contribute to repression of late-mid genes. During developmental transitions in drosophila, E75 has been found to repress gene transcription in response to declining 20E levels by outcompeting and replacing EcR (57). Our results show that this alternate mechanism of 20E-directed gene repression is not responsible for the observed repression of late-mid genes in mosquito vitellogenesis. Epistatic analysis of 20E pathway components further corroborated our results. These findings are in agreement with previous studies in *Aedes aegypti,* but this more extensive study of an expanded group of components and their isoforms reveals new subtleties in the regulatory program. Regulation by each factor was largely consistent between reference genes.

Insects represent ideal models for studying developmental transitions, reproductive cycles and the molecular mechanisms by which these changes are governed. Hormonal cues directly signal the onset of molting, metamorphosis, and egg development, life stages that are easily observed. Mosquitoes are especially useful for the study of gonadotrophic events, because reproduction is initiated synchronously by intake

of blood and proceeds through clearly defined stages in rapid succession. A reproductive cycle is short, taking only six days to complete. Mosquito colonies are easily reared and contained in a laboratory environment. In the case of *Aedes* aegypti, eggs can be desiccated and stored until needed. The complete genome of the *Aedes* aegypti mosquito has been sequenced. However, advanced molecular tools like those developed for genetic manipulation in the model organism *Drosophila melanogaster* have been limited for mosquito studies, confining researchers to more traditional methods, such as reverse genetic analysis. Recently emerging technologies such as CRISPR that can be applied to mosquito research should accelerate necessary progress in understanding the biology of this important vector.

This study has provided valuable insight into the bifunctionality of NR-mediated gene regulation, a phenomenon that is not well-understood. Future work will include promoter analysis of target genes to identify binding sites for receptors and co-regulators, cell transfection assays and mutation analysis of binding regions. However, characterization of the roles of 20E pathway components and each of their isoforms represents an essential step in understanding the dual nature of NR signaling during mosquito reproduction.

3.6 References

1. Attardo GM, Hansen IA, Raikhel AS (2005) Nutritional regulation of vitellogenesis in mosquitoes: Implications for anautogeny. *Insect Biochem Mol Biol* 35(7):661–675.

2. Li C, et al. (2000) Conserved Molecular Mechanism for the Stage Specificity of the Mosquito Vitellogenic Response to Ecdysone. *Dev Biol* 224(1):96–110.

3. Roy S, et al. (2015) Regulation of gene expression patterns in mosquito reproduction. *PLoS Genet* 11(8):e1005450.

4. Bhattacharjee S, Renganaath K, Mehrotra R, Mehrotra S (2013) Combinatorial control of gene expression. *Biomed Res Int* 2013:407263.

5. Zhu J, Chen L, Raikhel AS (2007) Distinct roles of Broad isoforms in regulation of the 20-hydroxyecdysone effector gene, Vitellogenin, in the mosquito Aedes aegypti. *Mol Cell Endocrinol* 267(1–2):97–105.

6. Braun S, Azoitei A, Spindler-Barth M (2009) DNA-binding properties of Drosophila ecdysone receptor isoforms and their modification by the heterodimerization partner ultraspiracle. *Arch Insect Biochem Physiol* 72(3). doi:10.1002/arch.20328.

7. Shin SW, Kokoza V, Ahmed A, Raikhel AS (2002) Characterization of three alternatively spliced isoforms of the Rel/NF-kappa B transcription factor Relish from the mosquito Aedes aegypti. *Proc Natl Acad Sci U S A* 99(15):9978–9983.

8. Hiruma K, Riddiford LM (2004) Differential control of MHR3 promoter activity by isoforms of the ecdysone receptor and inhibitory effects of E75A and MHR3. *Dev Biol* 272(2):510–521.

9. Abel ED, et al. (1999) Divergent roles for thyroid hormone receptor β isoforms in the endocrine axis and auditory system. *J Clin Invest* 104(3):291–300.

10. Schauer S, Callender J, Henrich VC, Spindler-Barth M (2011) The N-terminus of ecdysteroid receptor isoforms and ultraspiracle interacts with different ecdysteroid response elements in a sequence specific manner to modulate transcriptional activity. *J Steroid Biochem Mol Biol* 124(3–5):84–92.

11. Dittmer NT, Sun G, Wang S, Raikhel AS (2003) CREB isoform represses yolk protein gene expression in the mosquito fat body. *Mol Cell Endocrinol* 210(1–2):39–49.

12. Cruz J, Mane-Padros D, Zou Z, Raikhel AS (2012) Distinct roles of isoforms of the heme-liganded nuclear receptor E75, an insect ortholog of the vertebrate Rev-erb, in mosquito reproduction. *Mol Cell Endocrinol* 349(2):262–271.

13. Bleicher L, et al. (2008) Structural basis of GC-1 selectivity for thyroid hormone receptor isoforms. *BMC Struct Biol* 8(1):8.

14. Seo S-JJ, Cheon H-MM, Sun J, Sappington TW, Raikhel AS (2003) Tissue- and Stage-specific Expression of Two Lipophorin Receptor Variants with Seven and Eight Ligand-binding Repeats in the Adult Mosquito. *J Biol Chem* 278(43):41954–41962.

15. Schroeder A, Jimenez R, Young B, Privalsky ML (2014) The ability of thyroid hormone receptors to sense t4 as an agonist depends on receptor isoform and on cellular cofactors. *Mol Endocrinol* 28(5):745–57.

16. Sun G, Zhu J, Chen L, Raikhel AS (2005) Synergistic action of E74B and ecdysteroid receptor in activating a 20-hydroxyecdysone effector gene. *Proc Natl Acad Sci* 102(43):15506–15511.

17. Hagedorn HH, et al. (1975) The ovary as a source of alpha-ecdysone in an adult mosquito. *Proc Natl Acad Sci U S A* 72(8):3255–9.

18. Mane-Padros D, Cruz J, Cheng A, Raikhel AS (2012) A Critical Role of the Nuclear Receptor HR3 in Regulation of Gonadotrophic Cycles of the Mosquito Aedes aegypti. *PLoS One* 7(9):e45019.

19. Martín D, Wang SF, Raikhel AS (2001) The vitellogenin gene of the mosquito Aedes aegypti is a direct target of ecdysteroid receptor. *Mol Cell Endocrinol* 173(1–2):75–86.

20. Zhu J, Chen L, Raikhel AS (2003) Posttranscriptional control of the competence factor betaFTZ-F1 by juvenile hormone in the mosquito Aedes aegypti. *Proc Natl Acad Sci U S A* 100(23):13338–13343.

21. Sun G, Zhu J, Li C, Tu Z, Raikhel AS (2002) Two isoforms of the early E74 gene, an Ets transcription factor homologue, are implicated in the ecdysteroid hierarchy governing vitellogenesis of the mosquito, Aedes aegypti. *Mol Cell Endocrinol* 190(1–2):147–157.

22. Sun G, Zhu J, Raikhel AS (2004) The early gene E74B isoform is a transcriptional activator of the ecdysteroid regulatory hierarchy in mosquito vitellogenesis. *Mol Cell Endocrinol* 218(1–2):95–105.

23. Li C, et al. (2000) Conserved molecular mechanism for the stage specificity of the mosquito vitellogenic response to ecdysone. *Dev Biol* 224(1):96–110.

24. Kapitskaya MZ, Li C, Miura K, Segraves W, Raikhel AS (2000) Expression of the early-late gene encoding the nuclear receptor HR3 suggests its involvement in regulating the vitellogenic response to ecdysone in the adult mosquito. *Mol Cell Endocrinol* 160(1–2):25–37.

25. Zhu J, Chen L, Sun G, Raikhel AS (2006) The competence factor beta Ftz-F1

potentiates ecdysone receptor activity via recruiting a p160/SRC coactivator. *Mol Cell Biol* 26(24):9402–12.

26. Chen L, Zhu J, Sun G, Raikhel AS (2004) The early gene broad is involved in the ecdysteroid hierarchy governing vitellogenesis of the mosquito Aedes aegypti. *J Mol Endocrinol* 33(3):743–761.

27. Roy SG, Hansen IA, Raikhel AS (2007) Effect of insulin and 20-hydroxyecdysone in the fat body of the yellow fever mosquito, Aedes aegypti. *Insect Biochem Mol Biol* 37(12):1317–1326.

28. Saha TT, et al. (2016) Hairy and Groucho mediate the action of juvenile hormone receptor Methoprene-tolerant in gene repression. *Proc Natl Acad Sci* 113(6):E735–E743.

29. Surjit M, et al. (2011) Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. *Cell* 145(2):224–241.

30. Xu J, Thompson KL, Shephard LB, Hudson LG, Gill GN (1993) T3 receptor suppression of Sp1-dependent transcription from the epidermal growth factor receptor promoter via overlapping DNA-binding sites. *J Biol Chem* 268(21):16065–16073.

31. Santos GM, Fairall L, Schwabe JWR (2011) Negative regulation by nuclear receptors: a plethora of mechanisms. *Trends Endocrinol Metab* 22(3):87–93.

32. Rosenfeld MG, et al. (1988) Response and binding elements for ligand-dependent positive transcription factors integrate positive and negative regulation of gene expression. *Cold Spring Harbor Symposia on Quantitative Biology*, pp 545–556.

33. Jho SH, Radoja N, Im MJ, Tomic-Canic M (2001) Negative Response Elements in Keratin Genes Mediate Transcriptional Repression and the Cross-talk among Nuclear Receptors. *J Biol Chem* 276(49):45914–45920.

34. Vander Kooi BT, et al. (2005) The Glucose-6-Phosphatase Catalytic Subunit Gene Promoter Contains Both Positive and Negative Glucocorticoid Response Elements. *Mol Endocrinol* 19(12):3001–3022.

35. Dostert A, Heinzel T (2004) Negative glucocorticoid receptor response elements and their role in glucocorticoid action. *Curr Pharm Des* 10(23):2807–16.

36. Johnston DM, et al. (2011) Ecdysone- and NO-Mediated Gene Regulation by Competing EcR/Usp and E75A Nuclear Receptors during Drosophila Development. *Mol Cell*. doi:10.1016/j.molcel.2011.07.033.

Figure 3.1

Expression profiles of mid-late reference genes

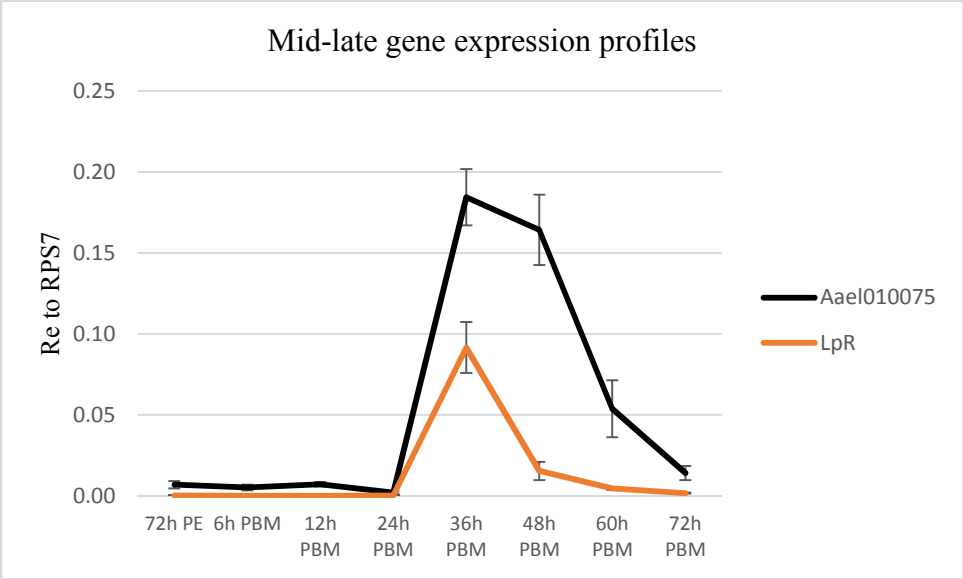


Figure 3.1

Expression profiles of late-mid reference genes (Aael010075 and LpR) in the female mosquito fat body from 72 hours post-eclosion (72h PE) to 72 hours post blood meal (72h PBM) as generated by qPCR. Expression is calculated against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

Figure 3.2

Late-mid genes are repressed by 20E

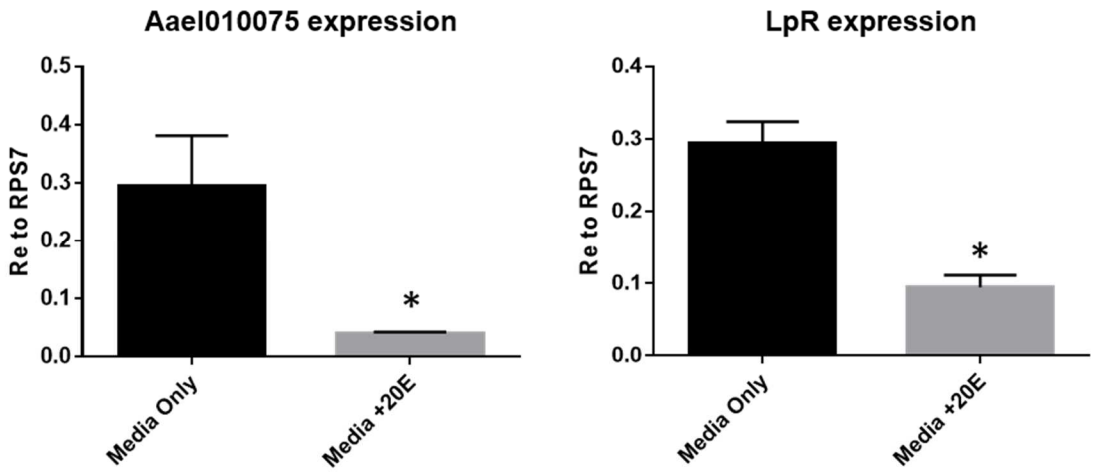


Figure 3.2

Relative expression of late-mid reference genes compared to control iLuc samples using qRT-PCR after IVFBC. Expression is calculated against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

Figure 3.3

Late-mid genes are repressed by EcR

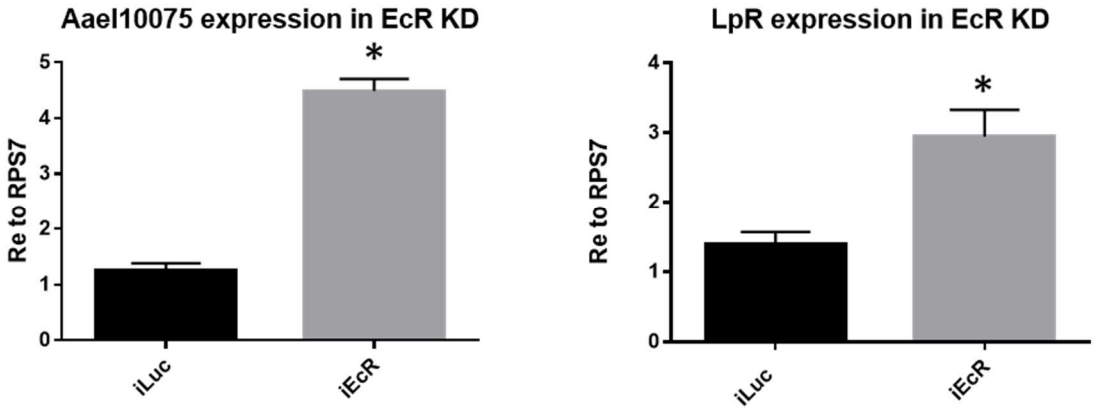


Figure 3.3A

Relative expression of late-mid reference genes in the female mosquito fat body following RNAi KD of EcR compared to iLuc (control). Expression normalized against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

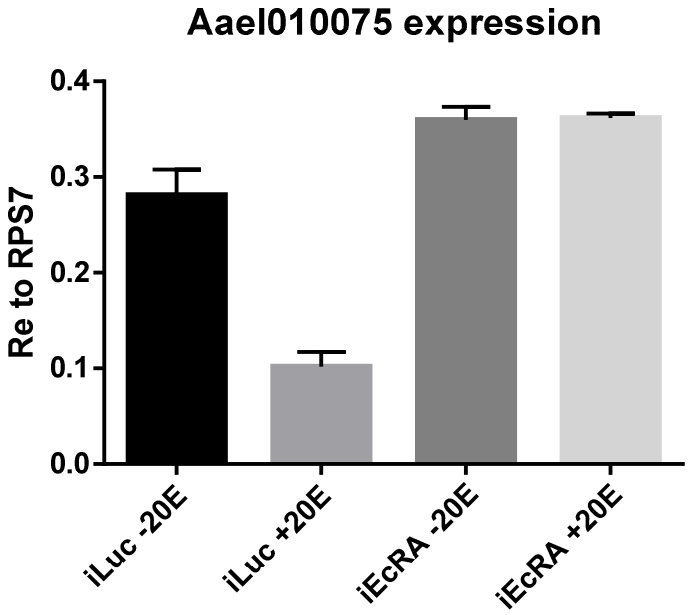


Figure 3.3B

IVFBC using fat bodies depleted of EcR through RNAi in presence and absence of of 20E. Expression normalized against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

Figure 3.4

Reference mid-late gene expression in EcR isoform A or B deficient background

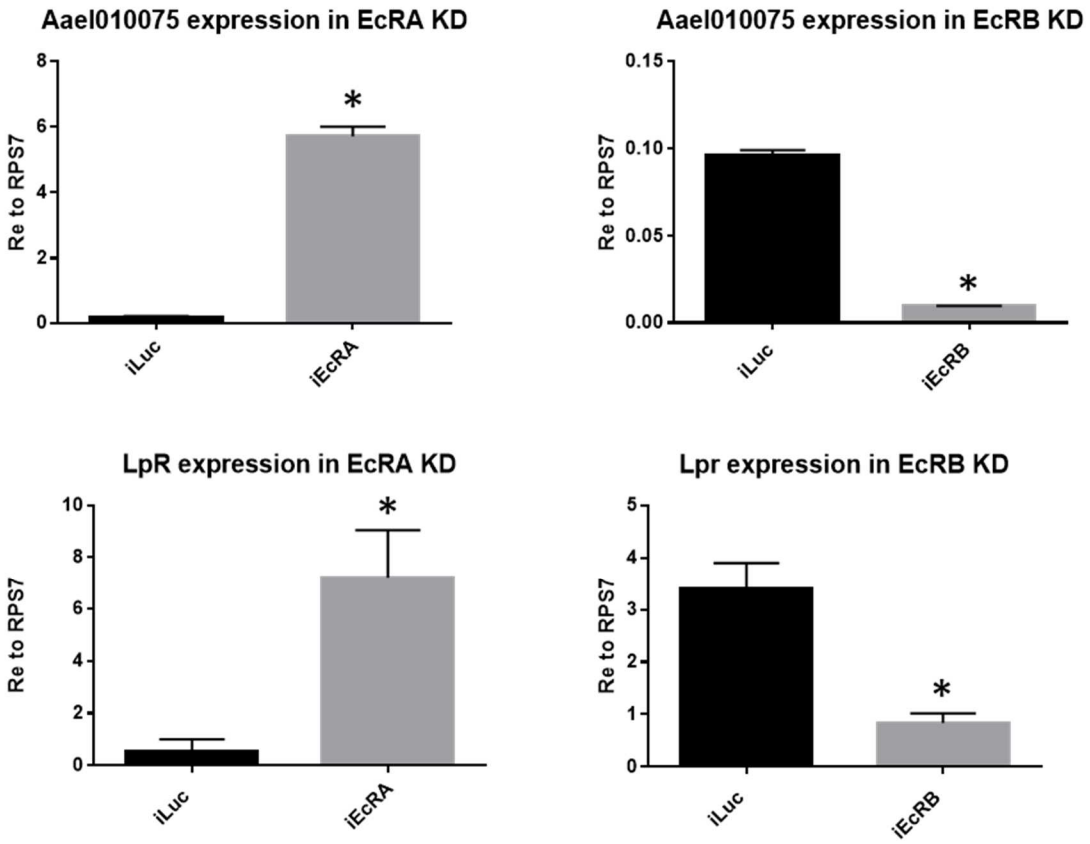


Figure 3.4

Expression is calculated using either EcRA- or EcRB-specific knockdown samples quantified by qPCR and normalized against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

Figure 3.5

Repression by EcR is indirect

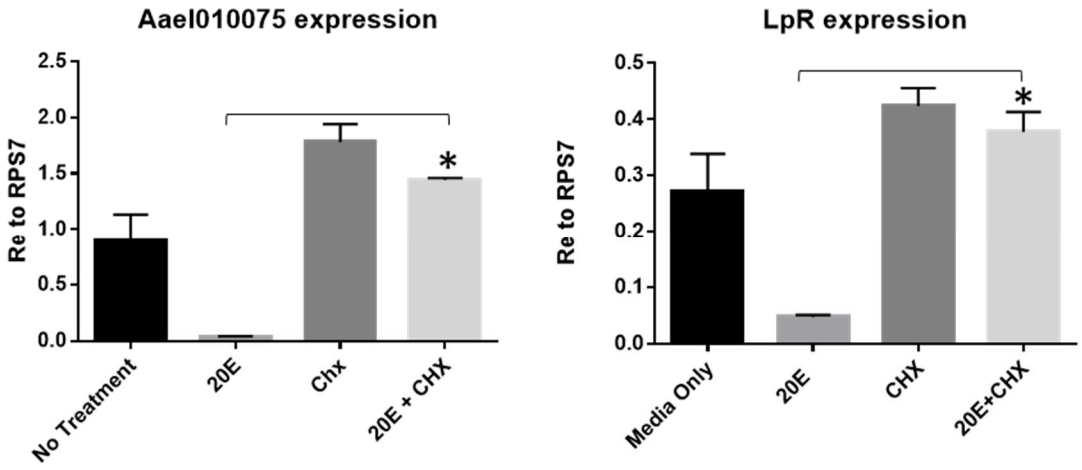


Figure 3.5

Graphs represent the effect of CHX exposure after IVFBC. Expression is calculated against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

Figure 3.6

Reference mid-late gene expression in E74B-deficient background

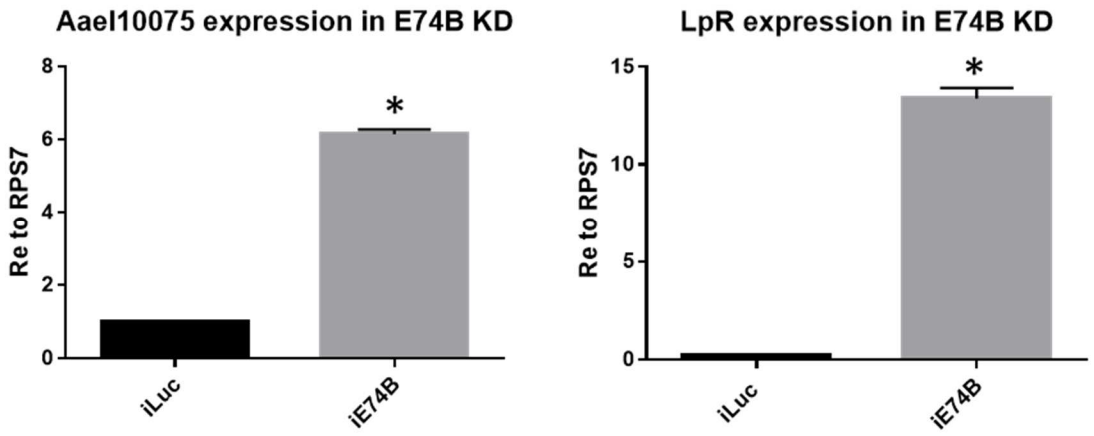


Figure 3.6

Expression is calculated against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

Figure 3.7

Reference gene expression in E74A- and Hr3-deficient background

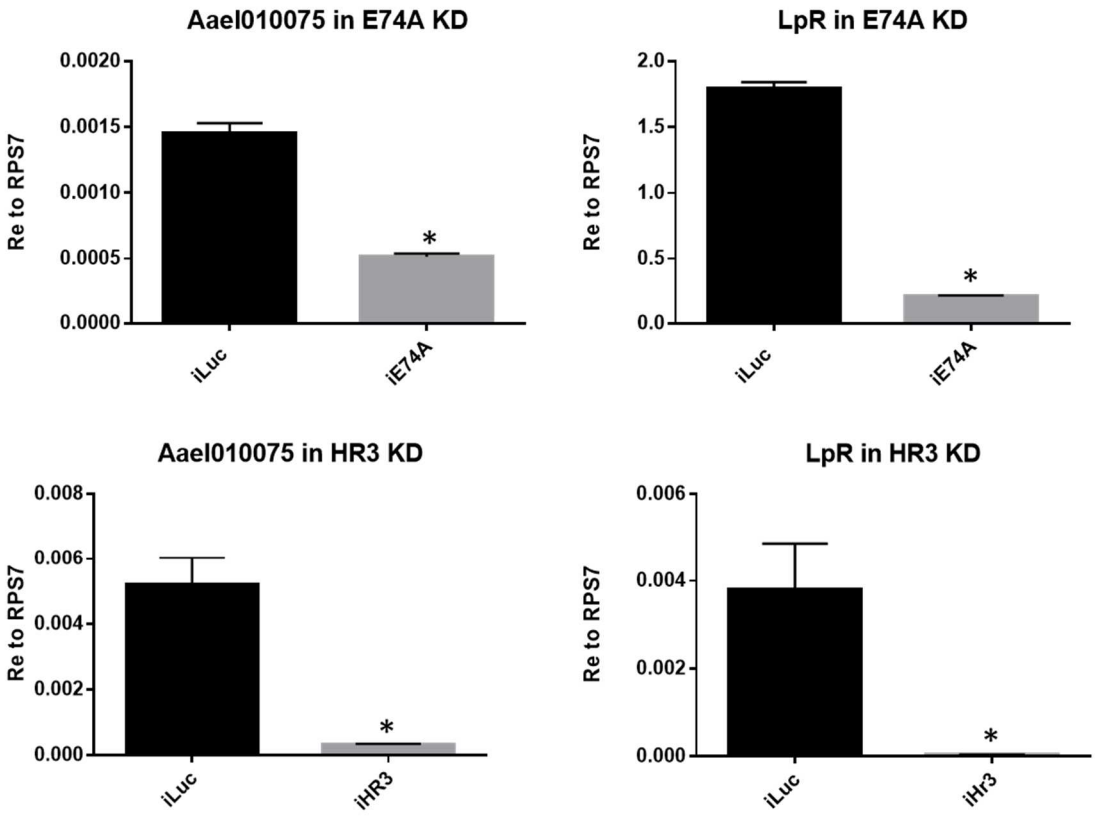
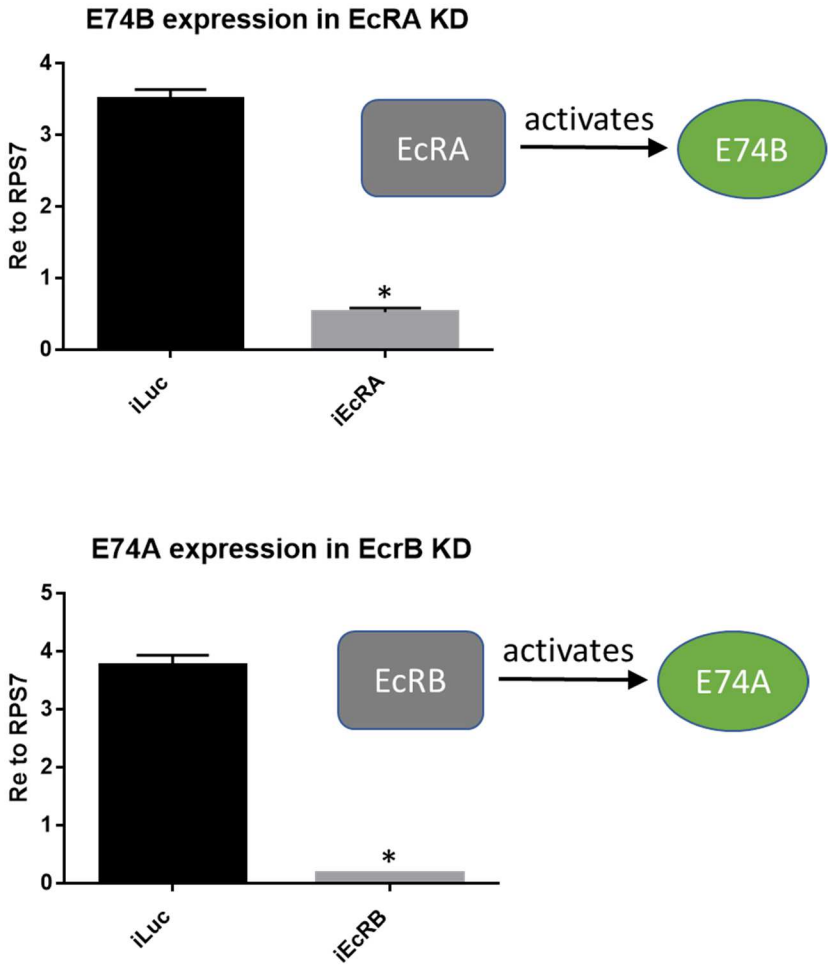


Figure 3.7

Expression is calculated against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

Figure 3.8

Epistatic analysis of 20E pathway components involved in repression of late-mid genes



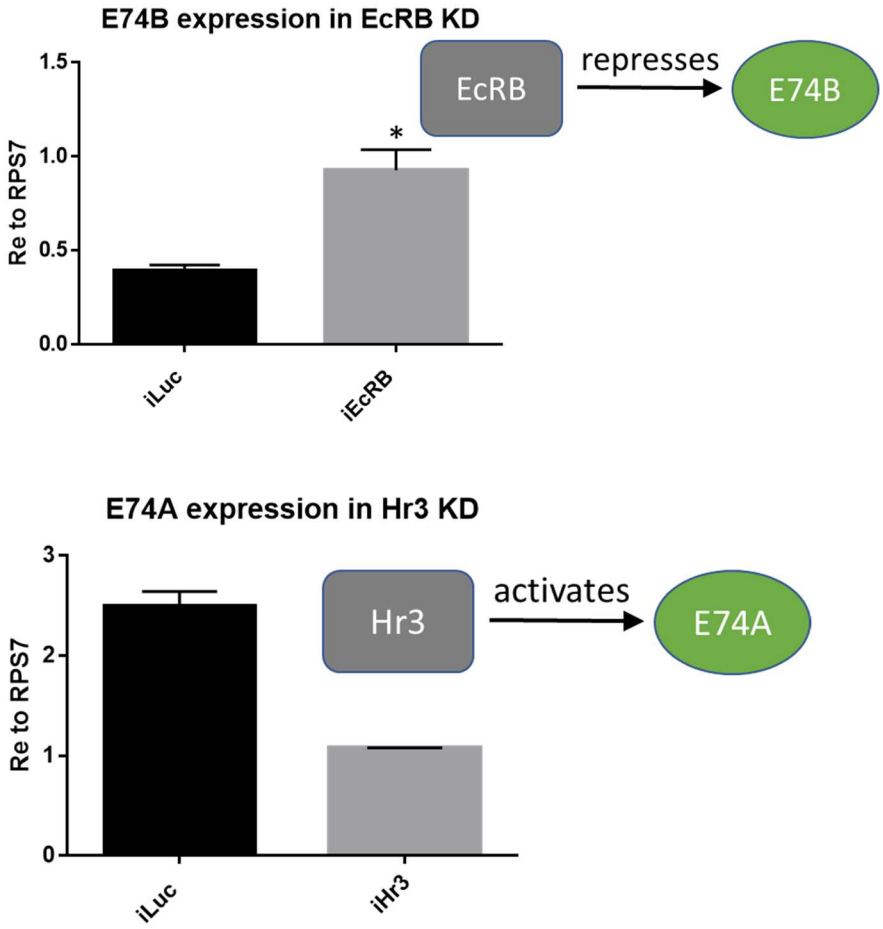


Figure 3.8

EcRA activates E74B. E74B is repressed by EcRB. EcRB has an activating effect only on E74A, which is also activated by HR3. Expression is calculated against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

Figure 3.9

Diagram depicting a model of mid-late gene regulation by 20E

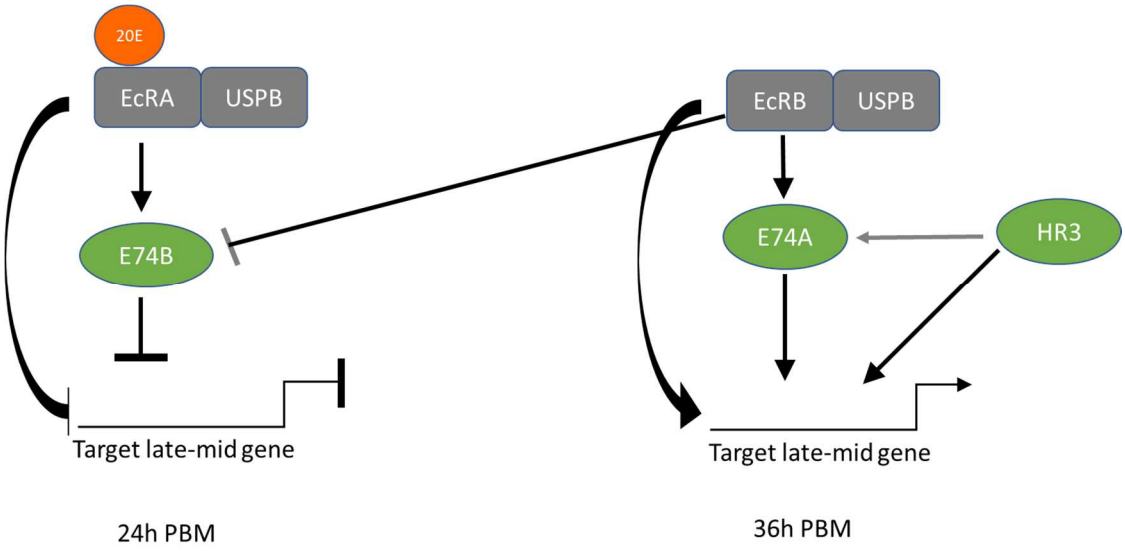


Figure 3.9

Target mid-late genes are repressed at 24 hours post-blood meal by the synergistic action of EcRA and by the EcRA-mediated upregulation of E74B. At 36 hours post-blood meal, target genes are activated by EcRB, Hr3, and EcRB-upregulated E74A.

Table 3.1

Selected gene IDs and biological functions:

LpRfb Aael012251	Lipophorin Receptor (fat body specific) Lipid transport and uptake
Aael010075	Oxidoreductase
Aael011890	Cytochrome p450
Aael001289	Permease
Aael000629	Adenylate kinase 3
Aael012687	Juvenile hormone-inducible protein

Supplemental Figure 3.1

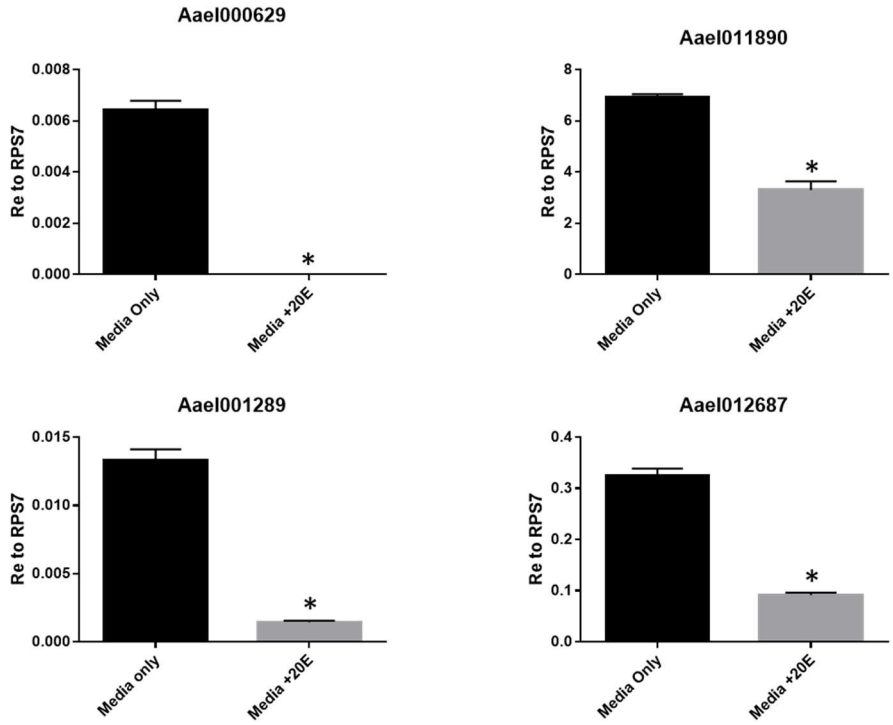
Expression profiles of additional mid-late genes

Supplemental Figure 3.1

Expression profiles of late-mid reference genes in the female mosquito fat body as generated by qPCR. Expression is calculated against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

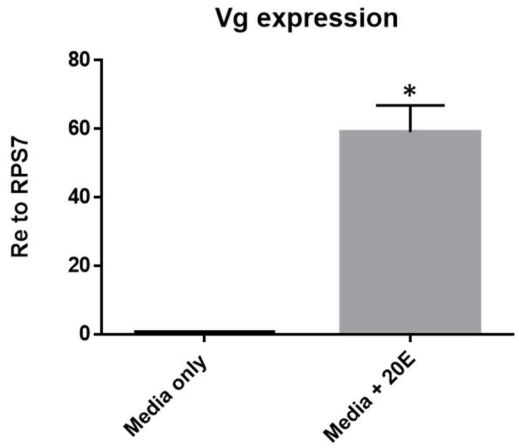
Supplemental Figure 3.2

Late-mid genes are repressed by 20E



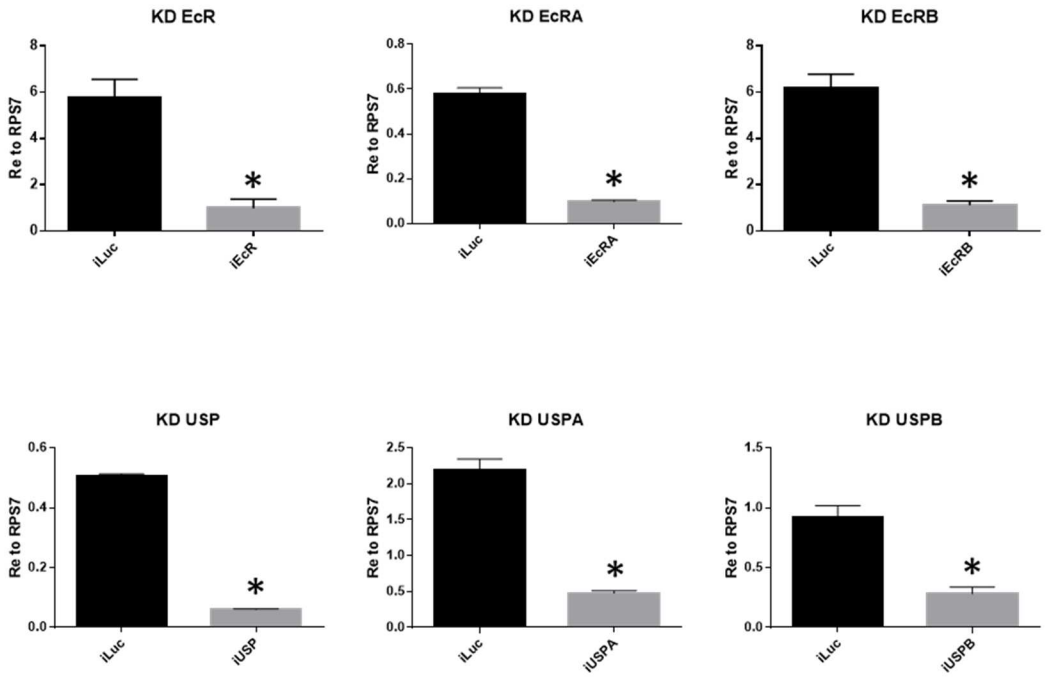
Supplemental Figure 3.2

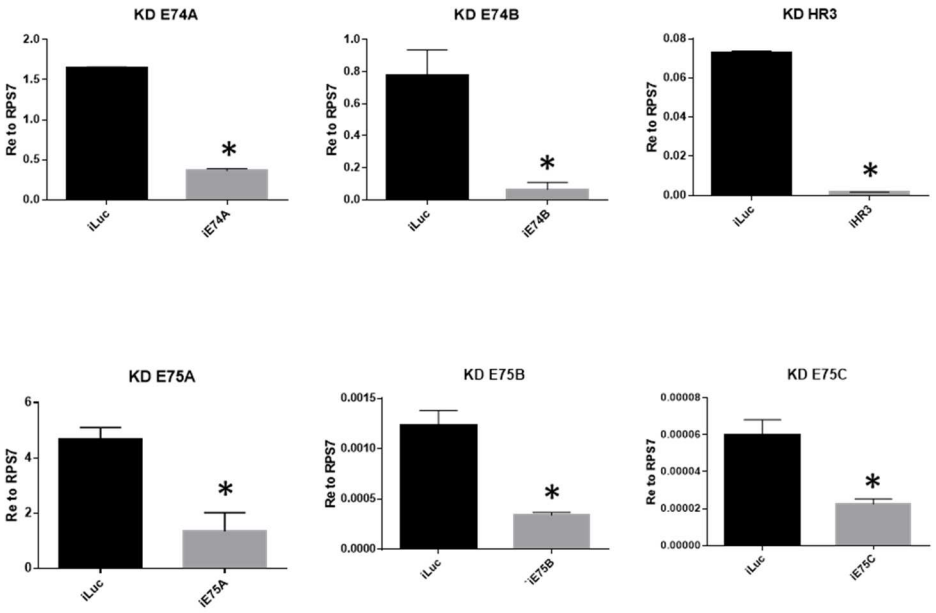
Relative expression of late-mid genes compared to control iLuc samples using qRT-PCR after IVFBC. Vg expression is shown as positive control. Expression is calculated against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.


Vg expression
Re to RPS7
80
60
40
20
0
*
Media only
Media + 20E

Supplemental Figure 3.3

Validation of knockdowns using qRT-PCR



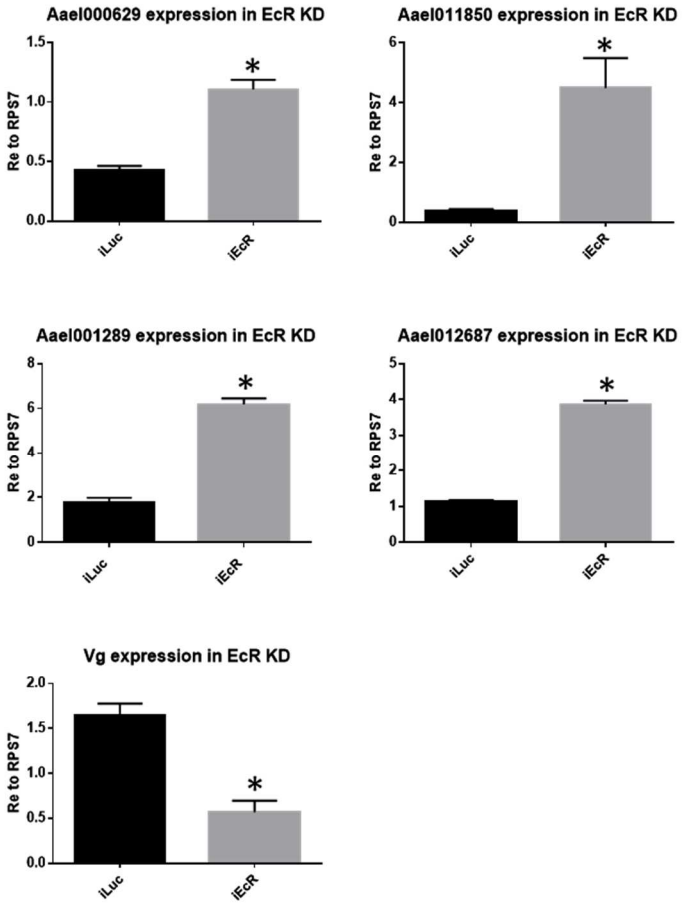


Supplemental Figure 3.3

Expression is calculated against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

Supplemental Figure 3.4

Late-mid genes are repressed by EcR

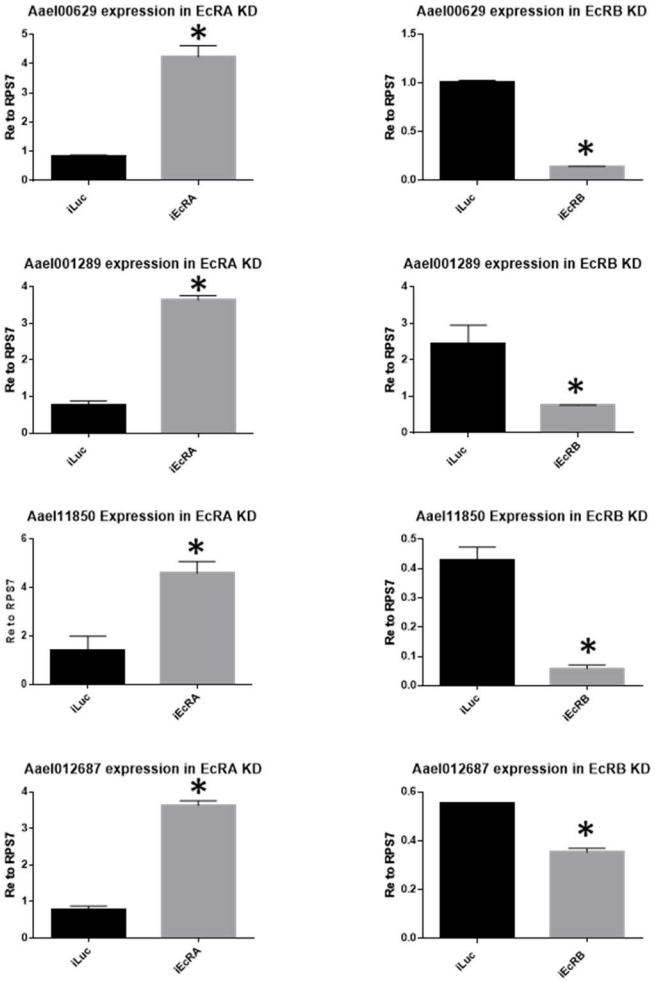


Supplemental Figure 3.4

Relative expression of late-mid genes in the female mosquito fat body following RNAi KD of EcR compared to iLuc (control). Vg expression is shown for comparison. Expression normalized against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

Supplemental Figure 3.5

Mid-late gene expression in EcR isoform A or B deficient background

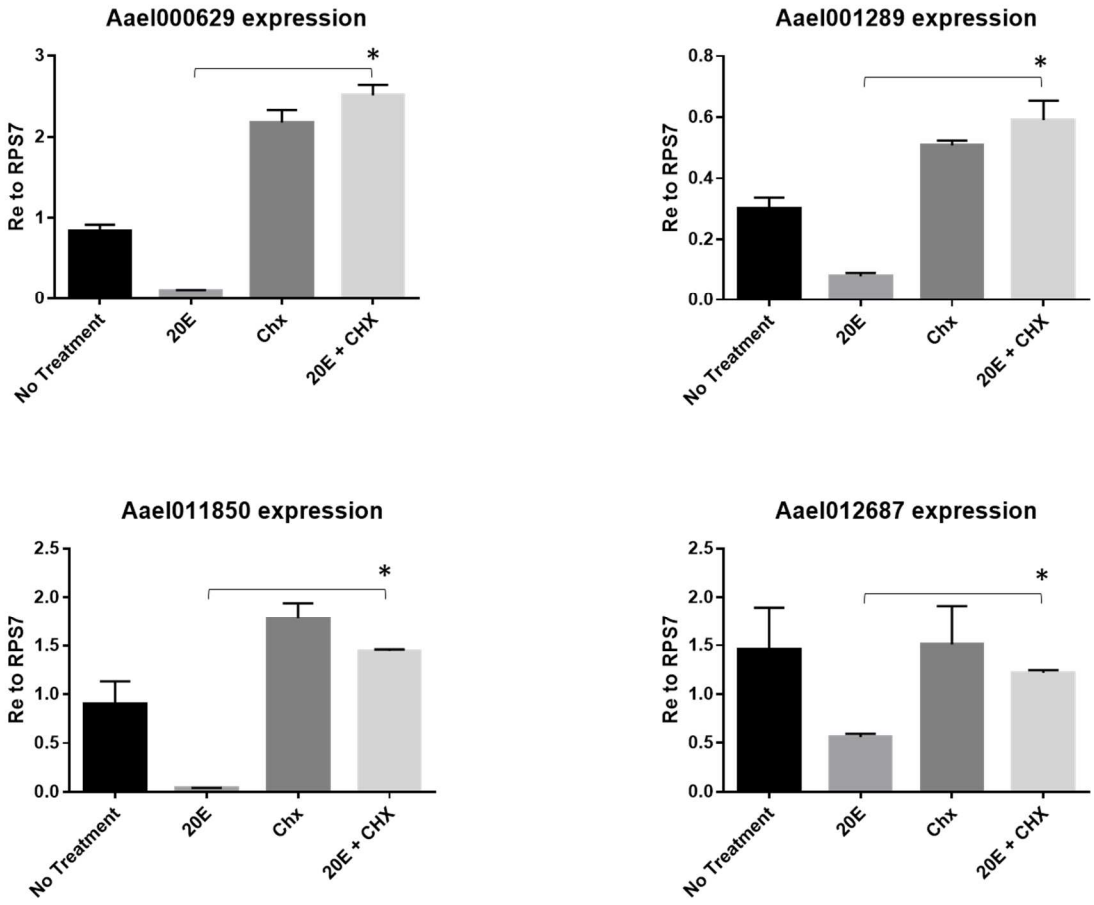


Supplemental Figure 3.5

Expression is calculated using either EcRA- or EcRB-specific knockdown samples quantified by qPCR and normalized against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

Supplemental Figure 3.6

Repression by EcR is indirect

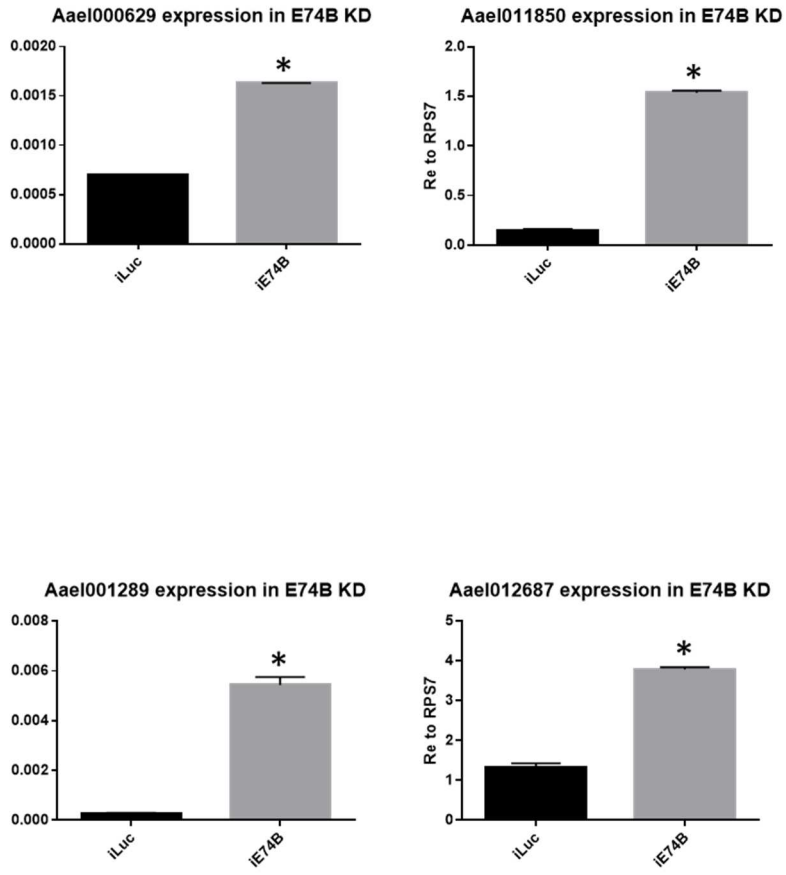


Supplemental Figure 3.6

Graphs represent the effect of CHX exposure after IVFBC. Expression is calculated against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

Supplemental Figure 3.7

Mid-late gene expression in E74B-deficient background

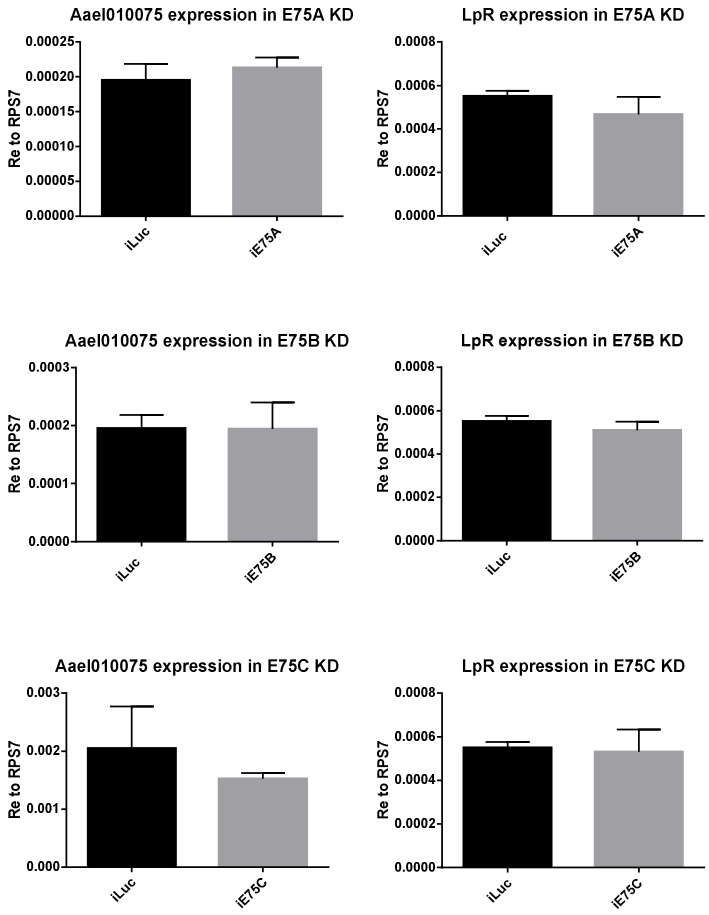


Supplemental Figure 3.7

Expression is calculated against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

Supplemental Figure 3.8

Mid-late gene expression in E75A-, B- and C-deficient background



Supplemental Figure 3.8

Expression is calculated against housekeeping gene RPS7. Data represent three biological replicates with three technical replicates and are illustrated as average ± SEM, * $P < 0.05$.

CHAPTER IV

Characterization of 20-Hydroxyecdysone- and Ecdysone Receptor-Mediated Gene Repression During a Gonadotrophic Cycle of the Female Disease Vector Mosquito, *Aedes aegypti*

4.1 Abstract

The negative regulation of gene expression by nuclear receptors (NRs) is a concept that is poorly understood and merits further investigation. Chapter III defined the negative regulation of a set of genes by 20-hydroxyecdysone (20E) and ecdysone receptor (EcR) in the female mosquito fat body during the late-mid phase of vitellogenesis. These genes were found to be repressed by the activated EcR in an isoform-specific and indirect manner, facilitated by the transcription factor E74B. Chapter IV explores the mechanism underlying this negative regulation, including analysis of gene regulatory regions and the involvement of co-factors. Bioinformatic analysis of gene promoters used in this study revealed multiple putative binding sites for EcR and E74B, and EMSA assays confirmed protein-DNA interaction. Cell transfection assays were performed in order to analyze gene promoter regions responsible for the negative regulation of these genes. In addition, the involvement of potential co-regulatory factors was explored using RNAi techniques. These results confirm that negative regulation of gene expression is a complex phenomenon that will require further study and will benefit from application of emerging technologies.

4.2 Introduction

A central topic of cellular and molecular biology is transcriptional control. Differential gene expression is fundamental to the success of organisms. Development, growth and reproduction result from the shifting expression of distinct sets of genes in a temporally precise and spatially specific context. Deviations from a transcriptional program can cause developmental defects or lead to disease or death of the organism. Hormones are the master regulators that initiate and ultimately orchestrate the proper expression of genes. Exertion of transcriptional control occurs through hormone/receptor interaction combined with the activity of intermediaries, and the convergence of these trans-acting elements with gene-specific cis-acting regulatory elements. Combinatory control results in a diversity of distinct outcomes even with relatively few regulatory factors.

The mechanisms that direct the activation of gene transcription by 20-hydroxyecdysone (20E) have been thoroughly studied for the *vitellogenin (vg)* gene (6, 53, 61, 64, 109). During the reproductive period in the fat body (FB) of the female mosquito, *Aedes aegypti,* up-regulation of the vitellogenin gene (*vg*) leads to the production of copious amounts of yolk protein precursor (YPP). This is a fundamental step in the reproductive process that occurs following a blood meal. The acquisition of blood triggers the action of several converging pathways involving insulin signaling, amino acid/TOR pathway nutrient signaling and ecdysteroid hormone signaling, which we focus on here (5). The active hormone 20-hydroxyecdysone (20E) binds to the ligand-binding domain (LBD) of its cognate receptor. The functional ecdysone receptor is a

heterodimer of two nuclear receptors: ecdysone receptor (EcR) and ultraspiracle (USP), and binding of 20E results in a conformational change in the receptor complex that allows interaction of the DNA-binding domain (DBD) with a stretch of DNA (48, 114). This occurs at multiple ecdysone response elements (EREs) in the *vg* promoter, contributing to its extremely high level of activation (115). REs for additional 20E-inducible genes are also found in the *vg* promoter, pointing to the importance of combinatorial control (21). The recruitment of the transcription factor (TF) E74B and the synergistic action of E74B with EcR/USP result in conditions favorable for the high-level transcription of *vg* (116) The protein βFTZ-F1, bound by the histone acetyltransferase p160-SRC (FISC) is also recruited to the receptor complex, resulting in epigenetic modulation of local histones, increasing chromatin accessibility for transcription of *vg* (66) This is a classic example of positive regulation in which ligand-bound and DNA-docked receptor recruitment of TFs and enzymatically active co-regulators results in modification of chromatin and transcriptional activation. Repression of gene transcription is traditionally thought to occur in the absence of ligand, where the receptor remains in a closed conformation that is unfavorable for complexing at the response element with co-activators. However, negative regulation in which gene output is reduced in the presence of hormone has increasingly been observed, yet the underlying mechanisms remain poorly understood.

As seen in the preceding example, the response of specific sets of genes to a transcriptional program centers around trans-acting elements including receptors, TFs and co-regulators that interact with cis-acting elements directly or indirectly through

associated complexes. We will examine both types of elements here with a focus on negative regulation.

Response elements (REs), typically located within the 2kb region upstream of a given gene's transcription start site, are composed of nucleotide sequence complimentary to DNA-binding domains (DBDs) within specific transcription factors (TFs) This assures that the correct set of regulatory factors associate with a given gene promoter. In the case of nuclear receptors, dimers often bind to two half-sites that can be arranged as direct or inverted palindromic repeats with variable numbers of spacer nucleotides between (117). The ecdysone receptor (EcR) is of this type, heterodimerizing with another nuclear receptor ultraspiracle (USP). The nucleotides that make up a RE for a protein can be degenerate to varying degrees and EcR/USP is characteristically flexible, recognizing a number of imperfect REs. More than simple docking sites, REs act as allosteric modulators of transcriptional output as slight differences in sequence can alter the conformation of transcription factors thereby modulating the activity of target genes (117–119). In addition to variability within cis-acting REs, the number or organization of REs can be important sources of variation in the transcriptional output of genes (74, 120). A growing number of transcription factors have been shown to bind to significantly different REs in cases of negative regulation, and these have been called negative response elements (nRE) (73). Repression of gene transcription has been shown to result from nRE interaction with hormone receptors including the glucocorticoid receptor (89, 96, 121), thyroid receptor (75, 79, 87), androgen receptor (92, 93, 118, 122) and estrogen

receptor (77, 119, 123). Still, relatively few nREs have been characterized and submitted to databases, but that number is increasing.

Co-regulators are trans-acting factors that are recruited to complexes assembled at regulatory regions of target genes and play a major role in mediating the control of gene expression by nuclear receptors. Upon recruitment by DNA-binding transcription factors, they act by stabilizing interactions between components of an assembled complex thereby altering the rate of transcription (71). This frequently involves enzymatically altering the chromatin environment to facilitate or hinder gene transcription (124). Increasingly, co-regulators have been found to defy simple classification as activators or repressors. RIP140, SRC1 and NCoR/SMRT are several of the co-regulators that have been found to act in an opposing fashion to their original classification. In some cases this is a response to the cellular environment, where a shift from an activating to repressing role is cell-type specific. For example, SMRT is a co-activator for ERalpha activation in HeLa and MCF-7 cells, but acts as a co-repressor in HepG2 cells (125). In other cases, the properties of co-regulator proteins can be changed by modifications to the proteins themselves, including alternative splicing or proteolytic processing. Modifications to phosphorylation sites on RIP140 influences the recruitment of HDACs (98). Phosphorylation of SMRT by the kinase ERK2 causes its eviction from receptor complex (71, 100). Despite these recent discoveries, co-receptor action is not fully understood.

This research examines several of the general factors that have been shown to influence the negative regulation of gene transcription as they relate to late-mid reference genes that are down-regulated in response to 20E during vitellogenesis.

4.3 Materials and methods

Data Analysis and bioinformatic promoter analysis

Geneious version 8.0.5 (http://www.geneious.com, Kearse et al., 2012) was used for sequence analysis, primer design and *in silico* cloning. Data analysis was done with Microsoft Excel and Graphpad. Statistical analysis was performed using Graphpad. Statistical significance was defined as $p < 0.05$. Data is shown as mean and SEM. JASPAR was used to identify cis-regulatory motifs. MEME and Weeder were used for bioinformatic identification of putative cis-regulatory sites.

EMSA

Chemiluminescent EMSA was performed using the Lightshift EMSA kit (Thermo Scientific) according to the manufacturer's protocol. Oligos were ordered from IDT with 3' biotin labeling. Nuclear protein was derived from fractionation using the NE-PER kit (Thermo Scientific) of S2 cells or by nuclear extraction from mosquito fat body cells harvested at 24h PBM. Total protein quantitation was accomplished by comparison to a BSA calibration curve using Bradford assays. Visualization and image processing was done with Chemidoc Touch (Bio-Rad).

Mosquito rearing

Wild type *Aedes aegypti* mosquitoes of the UGAL/Rockefeller strain were reared at 27°C and 80% humidity as described previously (Roy 2007). Adult mosquitoes were given unrestricted access to water and 10% sucrose solution. Blood feeding was

performed with White Leghorn chickens. All procedures for using vertebrate animals were approved by the UCR Animal Care and Use Committee.

RNA extraction and cDNA synthesis

Fat body samples were dissected into Trizol (Invitrogen/Life Technologies) and processed immediately or after brief storage at -80˚C. Total RNA was extracted from fat body samples using the Trizol method (Invitrogen/Life Technologies) according to the manufacturer's protocol. Samples were treated with DNAseI (Invitrogen/Life Technologies), and cDNA was synthesized from 2µg total RNA using the Superscript First-strand cDNA synthesis kit (Invitrogen/Life Technologies).

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

qPCR analysis of cDNA samples was performed using SYBR Green Master Mix (Qiagen) and primers designed for qPCR detection (Table #). Experiments were run using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). All experiments were performed in triplicate under identical conditions and results were normalized against the housekeeping gene S7 ribosomal protein (RPS7) as an internal control. Raw data were exported and analyzed using Microsoft Excel. Relative expression (RE) was calculated as $RE = 2^{-\Delta\Delta Ct}$. Graphs represent average experimental Ct values relative to wild-type or iLuc-injected control samples. Error bars are representative of SEM and p-value significance was set at <0.05. Statistical analysis was performed by applying student's t-test using Graphpad Prism.

RNA interference-mediated gene knockdown

Genes selected for knockdown were cloned into the pGemT-EZ vector. dsRNA was produced as previously described (Roy 2007). Briefly, double-stranded RNA (dsRNA) was synthesized using the MEGAscript T7 kit (Ambion). The Luciferase gene was used to generate iLuc dsRNA that was used as a control. After synthesis of dsRNA, samples were phenol/chloroform extracted and ethanol precipitated. Microinjection into the thorax of CO2 anesthetized female mosquitoes was performed using the PicospritzerII (General Valve) at 12-24h PE using 0.5ug (0.25ul of 2 ug/ul) dsRNA. Mosquitoes were allowed to recover for 3d before blood feeding. Dissections of mosquito fat bodies were performed at 24 or 36h PBM in room temperature Aedes Physiological Saline (APS). Knockdowns were confirmed to be efficient and quantified by qPCR. Knockdown samples relative to controls were used to assess expression level of each gene of interest using qPCR.

5'RACE

5'RACE (Clontech) was performed according to manufacturer's protocol. In brief: Gene specific nested primers were designed. Female fat bodies were collected at 36 hours PBM and total RNA (1ug) was extracted from and converted into RACE-Ready first-strand cDNA. The RACE touchdown PCR reaction was performed using the Universal Primer Mix (UPM) and two gene specific primers (GSP). The RACE products of interest were purified using the Nucleotrap Gel Extraction Kit (Clontech) and cloned

into the pRACE vector. Clones were sequenced by the UCR Genomics Core Facility and reads were mapped to reference genome and analyzed using Geneious software.

Cell culture and luciferase reporter assay

The 2kb promoter region of each mid-late target gene was amplified by pcr using primers with restriction site overhangs, subcloned into the multiple cloning region upstream of the Luciferase gene within the pGL4.17 reporter vector (Promega) and sequences were confirmed by the UCR Genomics Core Facility. Transient transfections were carried out in Aag2 cells and L57 cells that were maintained at 28˚C and constant humidity in Schneider's drosophila medium (Gibco, Life Technologies) containing 10% fetal bovine serum (Gibco, Life Technologies) and 0.05% penicillin/streptomycin (Gibco, Life Technologies). Each reporter plasmid was co-transfected with Renilla luciferase reporter vector pTKRL (Promega) as an internal control along with selected expression vectors or empty vector to normalize the final concentration of DNA in each well. Transfections were carried out using Fugene transfection reagent (Promega) according to the manufacturer's protocol and cells were harvested after 24 hours incubation. Luciferase activity was measured using the dual luciferase assay kit (Promega) according to the manufacturer's directions.

4.4 Results

Bioinformatic characterization of the 5' regulatory regions of mid-late reference genes

We analyzed the 5' regulatory region of each mid-late reference gene to identify cis-regulatory elements potentially implicated in the repression by 20E (Figure 4.1). The promoter of Aael010075 contained multiple putative EcREs in the region from -1067bp to -945bp. In addition, a number of putative E74 REs were found scattered throughout the regulatory region. The LpR promoter contained several putative EcREs: an imperfect direct repeat with 5bp spacing (DR5) in the region from -246bp to -231bp and an imperfect inverted repeat with 5bp spacing (IR5) in the region from -591bp to -573bp. A putative RE for E74 was located at -504bp to -498bp. Each of the additional reference gene promoters similarly contained multiple putative binding sites for both EcR/USP and E74 (Supplementary figure 4.1). The presence of *cis* regulatory elements for EcR/USP and E74 suggests that the 20E hierarchy is involved in repression of the set of mid-late reference genes.

Validation of putative binding sites in the promoters of representative mid-late genes

EMSA assays were used to validate the putative binding sites for EcR-USP in the upstream region of the *LpRfb* gene. The nuclear protein fraction of S2 cells overexpressing EcR and USP as well as nuclear extract derived from *Aedes aegypti* female fat bodies at 24h PBM were both tested for binding to biotin-labeled sequences corresponding to each of three putative EcR-USP response elements within the region 2kb upstream of the LpR gene. EcR-USP displayed strong binding to one of the three sites, consisting of three imperfect tandem repeats of the EcR motif. Addition of 50-fold

excess of unlabeled specific nucleotide drastically reduces binding to the labeled probe (Figure 4.2).

Actions of EcR/USP and E74 isoforms on the promoters of mid-late reference genes *in vitro*

Regulation of mid-late gene promoters by 20E was tested in transfection assays. The 2kb regulatory region of each reference gene was cloned into luciferase reporter vectors (pGL4.17). Where necessary, 5'RACE was performed to identify the correct 5'UTR and 2kb promoter region. Transfection of reporter vectors alone into *D. Melanogaster* L57 cells resulted in significantly increased expression compared to cells transfected with empty vector (Supplementary Figure 4.1) Cells were then co-transfected with reporter vectors together with EcR, USP and E74B expression plasmids. No significant difference was seen between samples without or with the addition of 1x10-6M 20E (data not shown). Therefore, we considered the possibility of co-regulator requirement for repression of mid-late genes.

Art1 corepressor RNAi

Since repression of mid-late genes occurs during the peak titer of 20E, we conducted a literature review of ligand-dependent EcR-associated co-repressors. Art1, the mosquito homolog of dART1 in *D. Melanogaster* is known to be an EcR-associated ligand-dependent co-repressor. Therefore, we asked whether knockdown of dART1 would inhibit the repressive effect 20E. Microinjection of dsRNA into 12h PE female

mosquitos effectively knocked down the expression of art1, as shown by qPCR analysis (Supplementary Figure 4.2). However, expression of our reference mid-late genes was unchanged in art1-depleted samples, indicating that art1 is not involved in repression of these genes (Figure 4.2).

4.5 Discussion

Activation of *vg* gene expression by 20E during mosquito vitellogenesis has been extensively studied ((52, 59, 62, 66, 109, 115). However, the role of 20E in repression of genes differentially regulated during the late-mid period of a gonadotrophic cycle is poorly understood. Our previous study (Chapter 3) reported that a cluster of genes highly up-regulated following the peak of vitellogenesis are repressed during the high titer of 20E at 24 hours PBM. Furthermore, we demonstrated using *in vitro* fat body culture and RNAi techniques that repression of these genes occurs in response to 20E and is mediated by specific isoforms of EcR and E74, namely EcRA and E74B. In this study, we attempted to uncover the factors that contribute to the switch from activation by EcRB to repression by EcRA by examination of promoter regions of mid-late repressed genes. Through bioinformatic analysis, we were able to find a number of putative binding sites for EcR and E74 in all gene (2kb) promoters in our repressed set, suggesting that these factors are indeed involved in regulation of expression of these genes. Since the recognized response elements for both EcR and E74 bind their targets in an isoform-indiscriminate manner and distinct nREs were not apparent, we decided to proceed with transfection/luciferase assays to identify the region of the promoters responsible for

repressive action. Our discovery that, at least in an *in vitro,* artificial system, co-transfection of the primary elements found to contribute to gene repression in our *in vivo* experiments, combined with the application of 20E, is not sufficient to display repression, it is likely that additional factors are required for specifying the repressive action of E74B in synergy with EcR on the promoters of this set of late-mid genes. Since a bloodmeal triggers a network of signaling pathways including those involving ecdysteroids, insulin and amino acids, crosstalk between these pathways could result in positive or negative feedback mechanisms that cannot be easily replicated *in vitro.* In addition, negative regulation could require the synergistic action of an additional factor such as a co-repressor. This possibility led us to consider co-repressors known to associate with EcR in a ligand-dependent manner. *D. Melanogaster* dART1 is an arginine methyltransferase that acts on histone H4 and is a homolog of mammalian PRMT1, a co-regulator of the estrogen receptor. dART1 has been shown to interact with EcR as a ligand-dependent co-repressor (126). After review, RNAi was used to knockdown the expression of dART1, but expression of each gene in our late-mid gene set was unchanged indicating that dART1 may not be involved in this gene repression. It is also possible that a partial knockdown does not result in a phenotype and that complete knockout may be required to see an effect. The involvement of other potential co-repressors should be addressed in future work, as well as the more complicated issue of crosstalk between regulatory networks. Additional future directions should involve the identification of negative response elements as well as the molecular mechanism of a repressive response.

4.6 References

1. Sun G, Zhu J, Raikhel AS (2004) The early gene E74B isoform is a transcriptional activator of the ecdysteroid regulatory hierarchy in mosquito vitellogenesis. *Mol Cell Endocrinol* 218(1–2):95–105.

2. Raikhel A (2004) Vitellogenesis of disease vectors, from physiology to genes. *Biology of Disease Vectors*, ed WC M (Elsevier - Academic Press), pp 329–346.

3. Zhu J, Chen L, Raikhel AS (2007) Distinct roles of Broad isoforms in regulation of the 20-hydroxyecdysone effector gene, Vitellogenin, in the mosquito Aedes aegypti. *Mol Cell Endocrinol* 267(1–2):97–105.

4. Li C, et al. (2000) Conserved Molecular Mechanism for the Stage Specificity of the Mosquito Vitellogenic Response to Ecdysone. *Dev Biol* 224(1):96–110.

5. Martín D, Wang SF, Raikhel AS (2001) The vitellogenin gene of the mosquito Aedes aegypti is a direct target of ecdysteroid receptor. *Mol Cell Endocrinol* 173(1–2):75–86.

6. Hansen IA, Attardo GM, Rodriguez SD, Drake LL (2014) Four-way regulation of mosquito yolk protein precursor genes by juvenile hormone-, ecdysone-, nutrient-, and insulin-like peptide signaling pathways. *Front Physiol* 5 MAR(March):1–8.

7. Cronauer M V., Braun S, Tremmel C, Kröncke KD, Spindler-Barth M (2007) Nuclear localization and DNA binding of Ecdysone receptor and ultraspiracle. *Archives of Insect Biochemistry and Physiology* doi:10.1002/arch.20184.

8. Dutko-Gwóźdź J, et al. (2008) The variety of complexes formed by EcR and Usp nuclear receptors in the nuclei of living cells. *Mol Cell Endocrinol* 294(1–2). doi:10.1016/j.mce.2008.07.021.

9. Raikhel AS, et al. (2002) Molecular biology of mosquito vitellogenesis: From basic studies to genetic engineering of antipathogen immunity. *Insect Biochem Mol Biol* 32(10):1275–1286.

10. Raikhel AS, Roy S, Saha TT, Zou Z (2017) Regulatory Pathways Controlling Insect Reproduction. *Annu Rev Entomol* 63(1).

11. Sun G, Zhu J, Chen L, Raikhel AS (2005) Synergistic action of E74B and ecdysteroid receptor in activating a 20-hydroxyecdysone effector gene. *Proc Natl Acad Sci* 102(43):15506–15511.

12. Zhu J, Chen L, Sun G, Raikhel AS (2006) The competence factor beta Ftz-F1 potentiates ecdysone receptor activity via recruiting a p160/SRC coactivator. *Mol Cell Biol* 26(24):9402–12.

13. Helsen C, Claessens F (2014) Looking at nuclear receptors from a new angle. *Mol*

Cell Endocrinol 382(1):97–106.

14. Geserick C, Meyer H-A, Haendler B (2005) The role of DNA response elements as allosteric modulators of steroid receptor function. *Mol Cell Endocrinol* 236(1–2):1–7.

15. Wood JR, Greene GL, Nardulli AM (1998) Estrogen Response Elements Function as Allosteric Modulators of Estrogen Receptor Conformation. *Mol Cell Biol* 18(4):1927–1934.

16. Telorac J, et al. (2016) Identification and characterization of DNA sequences that prevent glucocorticoid receptor binding to nearby response elements. *Nucleic Acids Res* 44(13):6142–56.

17. Vander Kooi BT, et al. (2005) The Glucose-6-Phosphatase Catalytic Subunit Gene Promoter Contains Both Positive and Negative Glucocorticoid Response Elements. *Mol Endocrinol* 19(12):3001–3022.

18. Surjit M, et al. (2011) Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. *Cell* 145(2):224–241.

19. Dostert A, Heinzel T (2003) Negative glucocorticoid receptor response elements and their role in glucocorticoid action. *Negat glucocorticoid Recept response Elem their role glucocorticoid action*.

20. Telorac J, et al. (2016) Identification and characterization of DNA sequences that prevent glucocorticoid receptor binding to nearby response elements. *Nucleic Acids Res* 44(13):6142–6156.

21. Dostert A, Heinzel T (2004) Negative glucocorticoid receptor response elements and their role in glucocorticoid action. *Curr Pharm Des* 10(23):2807–16.

22. Takeda T, Nagasawa T, Miyamoto T, Hashizume K, DeGroot LJ (1997) The function of retinoid X receptors on negative thyroid hormone response elements. *Mol Cell Endocrinol* 128(1–2):85–96.

23. Xu J, Thompson KL, Shephard LB, Hudson LG, Gill GN (1993) T3 receptor suppression of Sp1-dependent transcription from the epidermal growth factor receptor promoter via overlapping DNA-binding sites. *J Biol Chem* 268(21):16065–16073.

24. Wondisford FE, et al. (1989) Thyroid hormone inhibition of human thyrotropin beta-subunit gene expression is mediated by a cis-acting element located in the first exon. *J Biol Chem* 264(25):14601–4.

25. Lempiäinen JK, et al. (2017) Agonist-specific Protein Interactomes of Glucocorticoid and Androgen Receptor as Revealed by Proximity Mapping. *Mol Cell Proteomics* 16(8):1462–1474.

26. Qi W, Wang Z (2012) Negative androgen-response elements in androgen target genes. *Androgen-Responsive Genes in Prostate Cancer: Regulation, Function and Clinical Applications*, pp 29–41.

27. Qi W, Gao S, Chu J, Zhou L, Wang Z (2012) Negative Androgen-Response Elements Mediate Androgen-Dependent Transcriptional Inhibition of TGF- 1 and CDK2 Promoters in the Prostate Gland. *J Androl* 33(1):27–36.

28. Klinge CM (2001) Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res* 29(14):2905–2919.

29. Swami S, Krishnan A V., Peng L, Lundqvist J, Feldman D (2013) Transrepression of the estrogen receptor promoter by calcitriol in human breast cancer cells via two negative vitamin D response elements. *Endocr Relat Cancer* 20(4):565–577.

30. Szapary D, Huang Y, Simons SS (1999) Opposing Effects of Corepressor and Coactivators in Determining the Dose-Response Curve of Agonists, and Residual Agonist Activity of Antagonists, for Glucocorticoid Receptor-Regulated Gene Expression. *Mol Endocrinol* 13(12):2108–2121.

31. Millard CJ, Watson PJ, Fairall L, Schwabe JWR (2013) An evolving understanding of nuclear receptor coregulator proteins. *J Mol Endocrinol* 51(3):T23-36.

32. Peterson TJ, Karmakar S, Pace MC, Gao T, Smith CL (2007) The silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) corepressor is required for full estrogen receptor alpha transcriptional activity. *Mol Cell Biol* 27(17):5933–48.

33. Cavaillès V, et al. (1995) Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. *EMBO J* 14(15):3741–51.

34. Tran HT, et al. (2001) Requirement of co-factors for the ligand-mediated activity of the insect ecdysteroid receptor in yeast. *J Mol Endocrinol* 27(2):191–209.

35. Cruz J, Mane-Padros D, Zou Z, Raikhel AS (2012) Distinct roles of isoforms of the heme-liganded nuclear receptor E75, an insect ortholog of the vertebrate Rev-erb, in mosquito reproduction. *Mol Cell Endocrinol* 349(2):262–271.

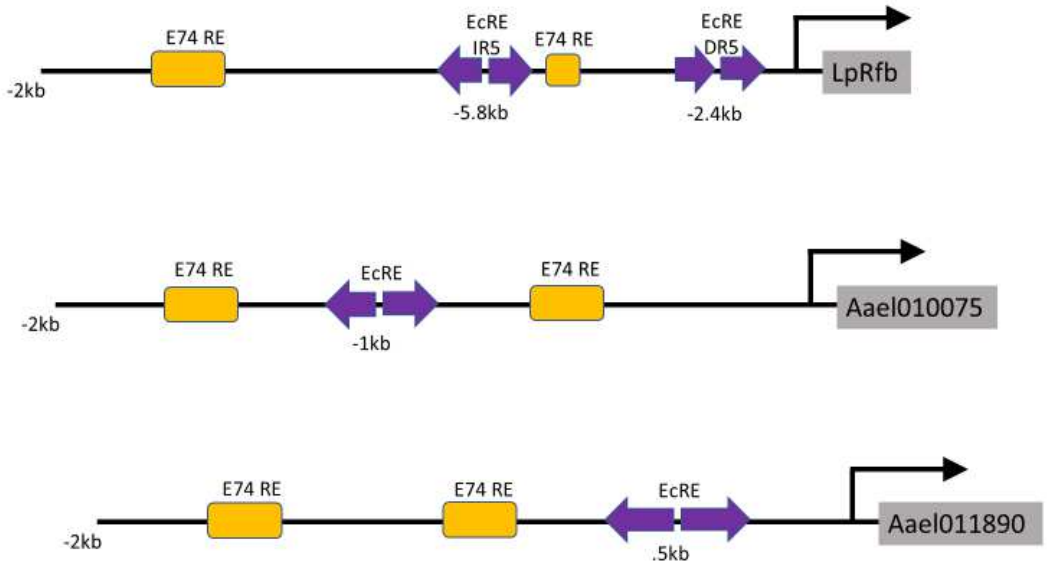
36. Chen L, Zhu J, Sun G, Raikhel AS (2004) The early gene broad is involved in the ecdysteroid hierarchy governing vitellogenesis of the mosquito Aedes aegypti. *J Mol Endocrinol* 33(3):743–761.

37. Sun G, Zhu J, Chen L, Raikhel AS (2005) Synergistic action of E74B and ecdysteroid receptor in activating a 20-hydroxyecdysone effector gene. *Proc Natl Acad Sci* 102(43):15506–15511.

38. Kimura S, et al. (2008) Drosophila arginine methyltransferase 1 (DART1) is an ecdysone receptor co-repressor. *Biochem Biophys Res Commun* 371(4):889–893.

Figure 4.1

Putative binding sites for EcR/USP and E74 identified by bioinformatic analysis of representative mid-late gene promoters.



E74RE 1	CCGGAAG
EcRE IR5	TCCCCGAAAGTCAGTGCAAGTTCATCAGG
E74RE 2	CCGGAAG
EcRE DR5	AAATAAATTGCAGTTAACTGCCCAACTAGA

Figure 4.1

Relative position of putative response elements found in the 2kb promoter regions of LpRfb, Aael010075 and Aael011890. Response elements for LpRfb subjected to functional analyses are given in the table.

Figure 4.2

Validation of protein binding to response element in LpRfb promoter region

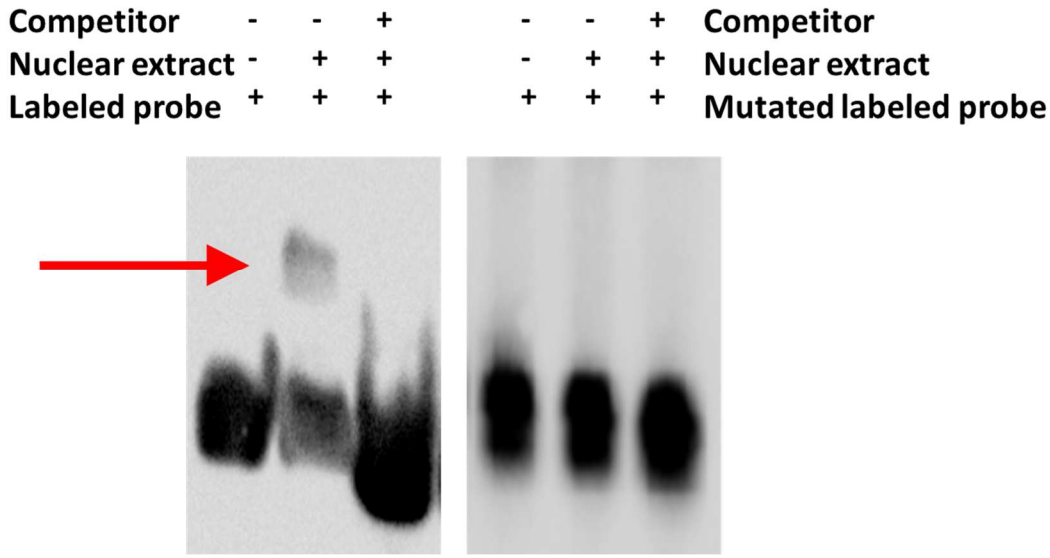


Figure 4.2

Chemiluminescent EMSA illustrating DNA-protein binding interaction at LpR promoter EcR response element in S2 cell nuclear extract, and with mutated EcRE labeled probe.

Figure 4.3

The corepressor Art1 is not involved in repression of reference genes

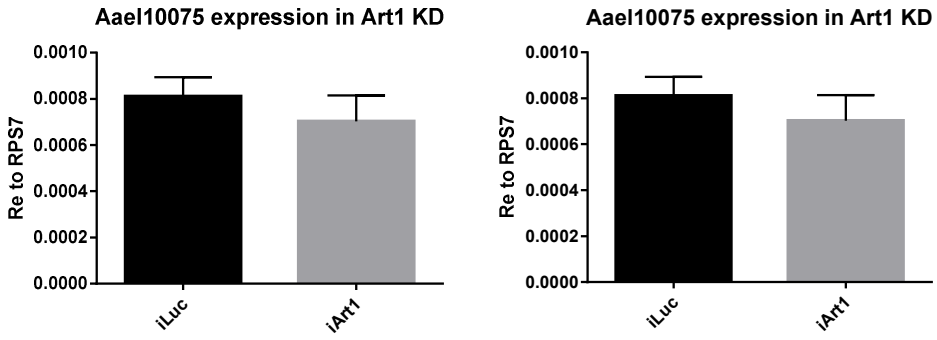
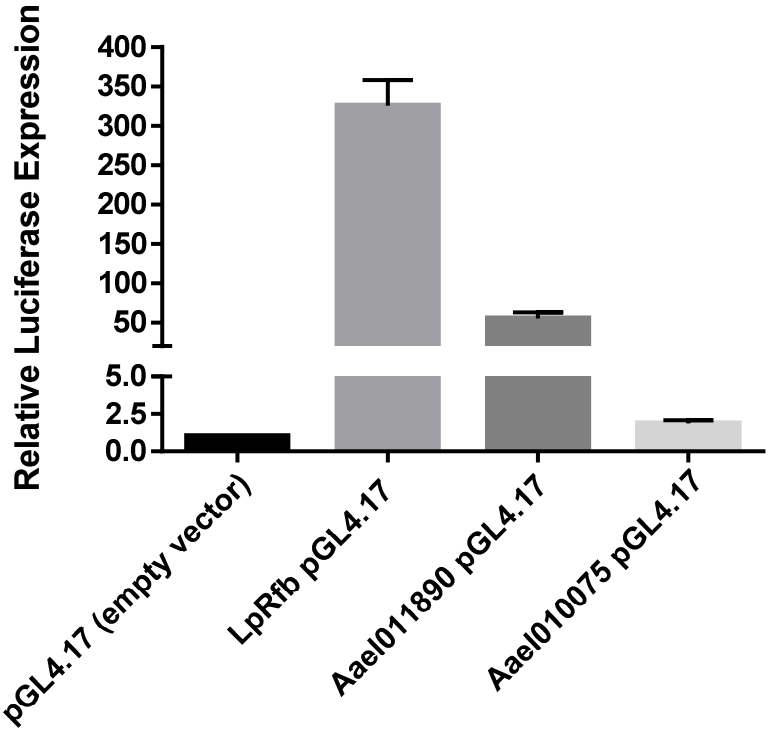


Figure 4.3

Knockdown of art1 does not affect expression of genes as shown by qPCR. Relative expression compared to RPS7/Expression normalized to RPS7. Samples done in triplicate, showing average and SEM. $p<0.05$ (Student's t-test).

Supplemental Figure 4.1

Relative luciferase expression of promoter constructs

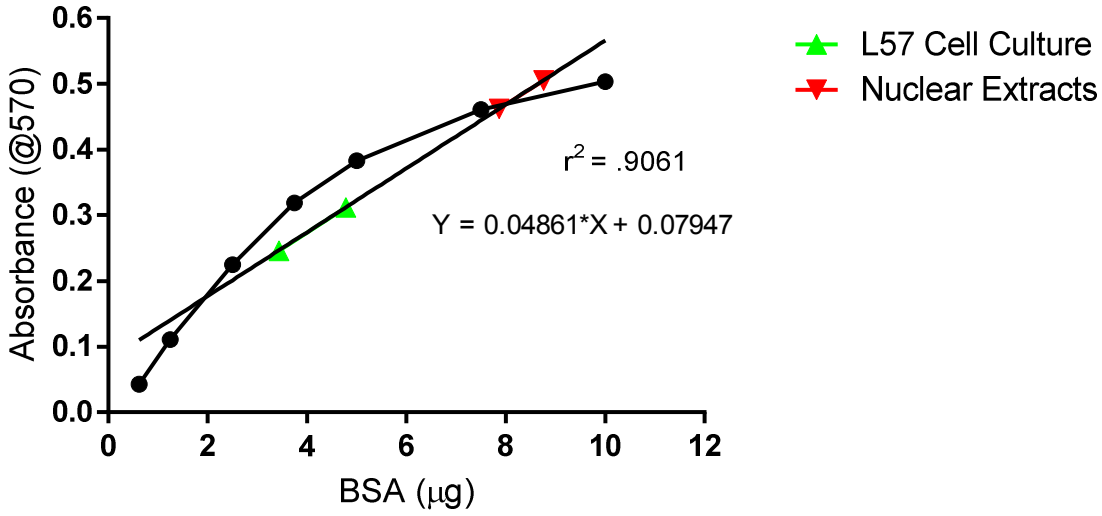


Supplemental Figure 4.1

The expression of three mid-late gene promoter constructs in pGL4.17 compared to the empty pGL4.17 vector as quantified by luciferase assay. Values are average expression normalized by an internal Renilla control.

Supplemental Figure 4.2

Protein quantitation for EMSA and Western assays

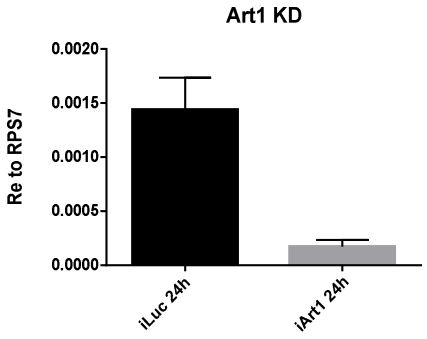


Supplemental Figure 4.2

Total protein content of L57 cell and nuclear extract samples was quantified by means of Bradford assay. A calibration curve was derived by plotting absorbance at 570 nm against known concentrations of bovine serum albumin (BSA), prepared from a 1 mg/mL stock solution in deionized water and added to Bradford assay reagent in graduated volumes. A linear equation (y=mx+b) was used to calculate total protein content where y is the absorbance value, m is slope, x is concentration and b is the y-intercept. The co-efficient of determination (r^2) is equal to 0.9061.

Supplemental Figure 4.3

Knockdown of Art1 was effective



Supplemental Figure 4.3

Knockdown of art1 expression quantified by qPCR. Individual samples after RNAi showed a 72-90% decrease in expression compared to control (iLuc) samples. Relative expression compared to RPS7/Expression normalized to RPS7. Samples done in triplicate, showing average and SEM. $p<0.05$ (Student's t-test).

Supplemental Table 4.1

Primer sequences used for 5'RACE.

RACE10075_R1	CTTCACGGGCACACAAGATCAGACCGATTACGCCAAGCTT
RACE10075_R2	CCCGTTACTGCGGCTACACGATTCTGATTACGCCAAGCTT
RACE6829_R1	CGACGCACCGCTTACAAGAAGAACAGATTACGCCAAGCTT
RACE6829_R2	CCGATCACGAAGTAGGGCAGGATGTGATTACGCCAAGCTT
RACE11850_R1	ATCGTCCCTTCGTAACCACCTTTGCGATTACGCCAAGCTT
RACE11850_R2	CAATGGCGAGGGATTCTGGCACTATGATTACGCCAAGCTT
RACE12687_R1	CACCAATCGGTTCGGAAAGCCACTAGATTACGCCAAGCTT
RACE12687_R2	TGAACCCTTCCATCGTCGTCACTTGGATTACGCCAAGCTT

Supplemental Table 4.2

Primer sequences for cloning and sequencing of pGL41.17 constructs.

Aael011890F	AAAAAAGGTACCTGGATCAGCGAAACCTCCAGT
Aael011890R	AAAAAAAAGCTTGTGCGGCTTTATTACTTTGCTTGCG
Aael001289F	CATTGCCTCGAGAACCAGTTCGAACTATTTATTTCA
Aael001289R	CATTGCAGATCTTATCGTTGCGACTGCGCAATAAAT
RVprimer3	CTAGCAAAATAGGCTGTCCC
pGL4.17R2	GAGTGGGTAGAATGGCG
Aael000629F	AAAAAAGGTACCGACCATTTTTGGCGTTACGAAA
Aael000629R	AAAAAAGAGCTCGATGATTCTCGGTTTTTCCTTCA

Supplemental Table 4.3

Primer sequences for dsRNA synthesis used for art1 knockdown.

art1 RNAi #1 F	**CTGATTGTGAAGCGAAACGA**
art1 RNAi #1 R	**TTCGTGAGCGAATTGTCAAC**
art1 RNAi #2 F	**TTTTCTCGGCGATTAGTGCT**
art1 RNAi #2 R	**TCTCCATGTTCACGTCGGTA**

CHAPTER V

Conclusion of the Dissertation

5.1 Concluding remarks

Mosquitoes are the world's most dangerous animal, responsible for millions of deaths annually and an enormous economic burden worldwide. Blood-sucking female mosquitoes bite and therefore transmit disease. Amino acids in a protein-rich vertebrate blood meal are required for the development of eggs during a gonadotrophic cycle, intimately linking reproduction with disease transmission. Furthermore, reproduction is cyclic, with each blood meal potentially serving as an impetus for human infection. The threat of mosquito-borne diseases must be addressed by the implementation of novel methods of control. Research aimed at understanding the molecular basis of reproduction in this important disease vector is essential to the development of new and effective strategies for preventing the spread of mosquito-borne diseases. This work uncovers regulators of each phase of a gonadotrophic cycle, as well as characterization of the negative regulation of a cluster of genes by the ecdysone signaling pathway.

Chapter II reveals the unique combinations of hormonal and nutritional factors that direct the up- and down-regulation of temporally distinct gene clusters throughout a gonadotophic cycle in the female mosquito fat body. Data from microarray analysis revealed approximately 7500 differentially expressed genes during the reproductive period that can be grouped into four sequential regulatory waves. RNAi and IVFBC techniques were used to determine the major regulators that contribute to each wave of

gene expression. The first wave, occurring between 3-12h after a blood meal, was found to involve activation of early gene expression by amino acids. This same set of early genes is subsequently downregulated by 20E, the titer of which begins to rise around 12h PBM and reaches a peak between 18-24h PBM. The second wave of gene expression is upregulated during this period by rising 20E levels that activate the ecdysone receptor (EcR) and is then repressed by the nuclear receptor HR3 by 36h PBM. A subset of this early-mid group of genes includes yolk protein precursor (YPP) genes such as vitellogenin which has been shown to be essential for egg development. Our study revealed that insulin signaling contributes to the activation of the YPP subset of genes but does not play a role in activation of other up-regulated gene sets during reproduction. The third wave of gene up-regulation occurs between 36-48h PBM, and these genes are activated by HR3 at this time, following earlier repression by the high titer of 20E and the action of EcR, in addition to the synergistic action of amino acids. The final wave of late gene expression from 48-72h PBM occurs concurrently with the termination of vitellogenic events and is activated by JH through its receptor Met. These genes are repressed earlier by amino acids. This study revealed information about the major regulators of gene expression at each phase throughout a gonadotrophic cycle, deepening our understanding of mosquito reproductive events.

The cluster of mid-late genes identified in Chapter II were shown to be repressed by the steroid hormone 20E through EcR. Activation of gene expression by the ligand-bound EcR has been studied in detail, but the simultaneous ability of this activated

nuclear receptor to up- and down-regulate the expression of different gene sets had not been previously characterized.

The canonical model of nuclear receptor action proposes that unbound by its ligand, a receptor maintains conditions that are unfavorable for gene transcription, including the presence of additional co-repressors and an inhibitory chromatin landscape. Binding of hormone causes an allosteric change in the receptor that allows association with gene regulatory elements, replacement of co-repressors with co-activators, and an environment that favors activation of target genes. The initial group of genes activated by a pulse of ecdysone are known as the early genes of the 20E regulatory hierarchy, including E74, E75 and Broad. As the hormone titer increases, the products of these genes regulate the transcription of the next set of 20E-targeted genes, including the yolk protein precursor gene, vitellogenin (vg). At high 20E titer, the ligand-bound receptor complex and E74 act synergistically through direct contact with response elements in the vg promoter to activate transcription at a massive level. Negative regulation is less straightforward, and despite the efforts of researchers, has not yet fit any one tidy model. Decades ago, the hierarchical nature of the ecdysone pathway suggested that late genes are repressed as early genes are activated, a concept that has since been confirmed. Nuclear hormone receptors, conserved across all metazoans, have been repeatedly shown to repress genes while activated by hormone. The revisiting of the canonical model is complicated by the often opposing actions of multiple isoforms of receptors and co-regulators. Even a single factor can produce different results depending on stage or cell

type, or varying hormone levels. These factors, combined with the combinatorial nature of transcriptional regulation underlies the complexity of negative regulation.

In Chapter III, we characterize the negative regulation of a set of mid-late genes down-regulated in expression by 20E and EcR through RNAi and IVFB experiments. We show that this gene regulation is isoform-specific with regard to EcR by techniques involving knockdown of individual isoforms of the receptor. These experiments indicated that in contrast to activation of vg by EcRB, repression of a group of late-mid genes is accomplished through the action of EcRA in response to 20E. To our knowledge, this is the first study to demonstrate that differences in gene regulation are due, at least in part to the action of specific isoforms of ligand-bound EcR. Also, through cycloheximide experiments combined with IVFBC, we show that it occurs indirectly, mediated by specific isoforms of early gene product transcription factors, namely E74B. Furthermore, we extended the isoform-specific RNAi experiments to other transcription factors central to ecdysone signaling to define the epistatic relationship between components of the 20E pathway. Together, these experiments have expanded our understanding of negative gene regulation by 20E and EcR, as well as the orchestration of gene expression during the reproductive period in the mosquito.

Chapter IV is a report of our examination of the possible mechanisms underlying the observed negative regulation by 20E. This included identification of response elements for EcR and E74 in gene regulatory regions through bioinformatic methods as well as experimental validation of protein-DNA interaction. We also performed *in vitro* cell transfection and luciferase assays to investigate the ability of regulatory factors to

repress the set of mid-late genes from their promoters. Finally, we conducted an investigation of potential co-regulators known to associate with hormone-bound EcR through RNAi experiments.

The results of these studies, while advancing our knowledge of negative gene regulation in general and during gonadotrophic cycles of mosquitoes in particular, illuminate the complexity of hormone-directed nuclear receptor signaling and regulation of gene expression. Future work should investigate differences in response elements for EcR and EcR-related transcription factors that direct differences in gene expression levels. In addition, the additional factors, such as co-regulatory elements, that are present at DNA response elements or as members of a scaffold should be identified, and the underlying mechanisms that they direct, such as epigenetic modifications, should be resolved. Finally, perhaps the most complex area of future research that must be addressed is the unraveling of crosstalk between the different regulatory networks at play during mosquito reproductive cycles to gain a more complete understanding of the intricate fine-tuning that biological systems have evolved to accomplish precise and timely genetic regulation. Arguably, among the most essential and urgent subjects of study are mosquito reproductive cycles, which are the vehicle for the spread of devastating diseases but could also be the key to novel and effective methods to combat these very diseases and thus reduce human suffering. In addition to their importance to human health, mosquitoes represent outstanding model organisms for studying regulatory mechanisms of synchronized gene expression because in anautogenous mosquitoes, vitellogenesis is tightly controlled and does not occur until the acquisition of a blood

meal. In addition, mosquitos are amenable to experimentation because of their rapid development and reproduction. Much information regarding mosquito reproduction at the molecular level has been obtained through the use of reverse genetic techniques such as RNAi (32, 33, 35, 37, 65, 112, 128–130). However, recent advances in genetic, molecular and bioinformatic analytic techniques, such as high-throughput sequencing and the availability of genomic information, transgenic manipulation, large-scale transcriptomic analyses, and a rapidly expanding molecular toolkit will profoundly expand our understanding of the mechanisms controlling mosquito reproduction and promise to revolutionize mosquito biology (15, 34, 47, 103, 131–139).

5.2 References

1. Roy SG, Hansen IA, Raikhel AS (2007) Effect of insulin and 20-hydroxyecdysone in the fat body of the yellow fever mosquito, Aedes aegypti. *Insect Biochem Mol Biol* 37(12):1317–1326.
2. Attardo GM, Hansen IA, Shiao S-H, Raikhel AS (2006) Identification of two cationic amino acid transporters required for nutritional signaling during mosquito reproduction. *J Exp Biol* 209(Pt 16):3071–3078.
3. Hansen IA, et al. (2011) AaCAT1 of the yellow fever mosquito, Aedes aegypti: a novel histidine-specific amino acid transporter from the SLC7 family. *J Biol Chem* 286(12):10803–10813.
4. Attardo GM, Higgs S, Klingler KA, Vanlandingham DL, Raikhel AS (2003) RNA interference-mediated knockdown of a GATA factor reveals a link to anautogeny in the mosquito Aedes aegypti. *Proc Natl Acad Sci U S A* 100(23):13374–13379.
5. Shin SW, Zou Z, Saha TT, Raikhel AS (2012) bHLH-PAS heterodimer of methoprene-tolerant and Cycle mediates circadian expression of juvenile hormone-induced mosquito genes. *Proc Natl Acad Sci U S A* 109(41):16576–16581.
6. Bryant B, Raikhel AS (2011) Programmed autophagy in the fat body of Aedes aegypti is required to maintain egg maturation cycles. *PLoS One* 6(11):e25502.
7. Roy SG, Raikhel AS (2011) The small GTPase Rheb is a key component linking amino acid signaling and TOR in the nutritional pathway that controls mosquito egg development. *Insect Biochem Mol Biol* 41(1):62–69.
8. Mane-Padros D, Cruz J, Cheng A, Raikhel AS (2012) A Critical Role of the Nuclear Receptor HR3 in Regulation of Gonadotrophic Cycles of the Mosquito Aedes aegypti. *PLoS One* 7(9):e45019.
9. Antonova Y, Alvarez KS, Kim YJ, Kokoza V, Raikhel AS (2009) The role of NF-kappaB factor REL2 in the Aedes aegypti immune response. *Insect Biochem Mol Biol* 39(4):303–314.
10. Shin SW, Kokoza VA, Raikhel AS (2003) Transgenesis and reverse genetics of mosquito innate immunity. *J Exp Biol* 206(Pt 21):3835–3843.
11. Zou Z, et al. (2011) Transcriptome analysis of Aedes aegypti transgenic mosquitoes with altered immunity. *PLoS Pathog* 7(11):e1002394.
12. Kokoza VA, Raikhel AS (2011) Targeted gene expression in the transgenic Aedes aegypti using the binary Gal4-UAS system. *Insect Biochem Mol Biol* 41(8):637–644.
13. Neira-Oviedo M, et al. (2011) The RNA-Seq approach to studying the expression of mosquito mitochondrial genes. *Insect Mol Biol* 20(2):141–152.

14. Zou Z, et al. (2013) Juvenile hormone and its receptor, methoprene-tolerant, control the dynamics of mosquito gene expression. *Proc Natl Acad Sci* 110(24):E2173–E2181.

15. Roy S, et al. (2015) Regulation of gene expression patterns in mosquito reproduction. *PLoS Genet* 11(8):e1005450.

16. Zhao B, et al. (2014) Regulation of the gut-specific carboxypeptidase: A study using the binary Gal4/UAS system in the mosquito Aedes aegypti. *Insect Biochem Mol Biol* 54:1–10.

17. Carpenter VK, et al. (2012) SLC7 amino acid transporters of the yellow fever mosquito Aedes aegypti and their role in fat body TOR signaling and reproduction. *J Insect Physiol* 58(4):513–22.

18. Nene V, et al. (2007) Genome sequence of Aedes aegypti, a major arbovirus vector. *Science* 316(5832):1718–1723.

19. Wilke ABB, Marrelli MT (2015) Paratransgenesis: a promising new strategy for mosquito vector control. *Parasit Vectors* 8(1):342.

20. Giraldo-Calderón GI, et al. (2015) VectorBase: an updated bioinformatics resource for invertebrate vectors and other organisms related with human diseases. *Nucleic Acids Res* 43(Database issue):D707-13.

21. Feitosa FM, et al. (2006) A transcriptome analysis of the Aedes aegypti vitellogenic fat body. *J Insect Sci* 6(1). doi:10.1673/1536-2442(2006)6[1:ATAOTA]2.0.CO;2.

22. Kistler KE, Vosshall LB, Matthews BJ (2015) Genome Engineering with CRISPR-Cas9 in the Mosquito Aedes aegypti. *Cell Rep* 11(1):51–60.