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Ecdysis Triggering Hormone: A Multifunctional Peptide Regulator of Reproductive Physiology in Yellow Fever Mosquito *Aedes aegypti*

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Yike Ding

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

The Dissertation of Yike Ding is approved:

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_____

_____

Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE DISSERTATION

Ecdysis Triggering Hormone: A Multifunctional Peptide Regulator of Reproductive Physiology in Yellow Fever Mosquito *Aedes aegypti*

by

Yike Ding

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Dr. Michael E. Adams, Chairperson

During development, insects perform ecdysis behaviors required for shedding of old cuticle at the end of each molt. These behaviors are orchestrated by circulating peptides known as ecdysis triggering hormones (ETHs). Secreted by endocrine Inka cells, ETH acts on target neurons in the CNS via two GPCR isoforms (ETHR-A and ETHR-B) to recruit downstream peptide signaling cascades. Recent work on *Drosophila melanogaster* shows that Inka cells and transcripts encoding ETH and ETHR receptors persist through metamorphosis into adulthood, a period during which no ecdysis occurs. This suggests possible regulation of reproductive functions by ETH.

Many studies about reproductive physiology have been conducted in the yellow fever mosquito *Aedes aegypti.* In *Aedes* species, egg development proceeds through previtellogenic and vitellogenic phases. During previtellogenesis, the follicles, which are about 45 μm long at eclosion, grow to 100-110 μm. The previtellogenic phase controlled by Juvenile hormone (JH) begins at eclosion and is completed in ~60 hr, leading to competence for vitellogenesis, which is triggered by the blood meal. During

vitellogenesis, the fat body produces the yolk protein precursor vitellogenin. At the same time, oocytes accumulate vitellogenin to form protein yolk, leading to egg maturation.

The main goal of my research is to demonstrate and characterize ETH signaling in adult mosquitoes, to study endocrine cross talk between the hormones JH, ETH and ecdysteroids, and to establish their functional significance in mosquito reproductive physiology, which will contribute to a more complete understanding of mosquito reproduction. Here I report that ecdysone (20E)-dependent ETH signaling persists during the adult stage of the yellow fever mosquito (*Aedes aegypti*). I find that ETH functions as an obligatory allatotropin to promote juvenile hormone (JH) production and previtellogenesis. ETH signaling deficits lead to reductions of follicle size and egg production by producing JH deficiency. My findings indicate that the ecdysteroids, ETH, and JH function as an endocrine network essential for reproductive success in *Aedes aegypti*.

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Chapter 1 General Introduction

1.1 Vector-borne diseases and mosquito biology

Vector-borne diseases (VBDs) are caused by pathogens transmitted by arthropods or other invertebrates during blood feeding. Such diseases are on the increase, contributing to major public health problems particularly in tropical and sub-tropical regions. VBDs account for more than 17% of all infectious diseases, causing more than 700,000 deaths every year (WHO, 2017). Mosquitoes are vectors of some of the world's most devastating human diseases.

Mosquitoes are two-winged flies that belong to the family Culicidae under the order Diptera. There are approximately 3,500 species of mosquitoes (Tolle et al., 2009). Like all flies, mosquitoes go through complete metamorphosis with four stages in their life cycles: egg, larva, pupa and adult. The first three stages—egg, larva, and pupa—are largely aquatic. By emerging with wings, adults typically feed on nectars and other sugar resources in a totally different ecological niche. Two main groups of mosquitoes can be distinguished based on their reproductive strategies: autogenous and anautogenous (Roubaud, 1929). Autogenous mosquitoes can produce their first batch of eggs without dependence on a blood meal by utilizing the nutrients accumulated during larval stage. Subsequent batches of eggs depend on the energy from blood. However, in obligatorily anautogenous mosquitoes, a blood meal is required to produce their first batches of eggs. Most human disease vectors belong to the anautogenous groups, due to their early and

frequent interaction with the host, which provides parasites greater opportunities to infect the vector (Hansen et al., 2014).

Human disease vectors fall into three genera: *Anopheles* (malaria, filariasis), *Aedes* (Yellow fever, Dengue, Chikungunya, Zika), and *Culex* (West Nile, Japanese encephalitis, Filariasis) (Tolle et al., 2009). Yellow fever, dengue, chikungunya and Zika transmitted by *Aedes aegypti* have caused the most human suffering over past centuries and continue to do so now. More than half of the world's population live in areas where this mosquito species is present (Bhatt, S. et al., 2013). Chikungunya virus emerged from Africa in the mid-2000s. It spread across India and entered the Americas in 2013. Chikungunya, even though rarely fatal, can cause long-term morbidity in the form of debilitating arthralgia (Aalst et al., 2017). At the meantime, Zika virus outbreaks occurred in the South Pacific and spread into the Americas in 2015 (Mayer, S. V et al., 2017). Dengue fever is the fastest growing VBD in the world, its occurrence increasing 30-fold over the last 50 years (Pang, T et al., 2017). Around 40% of world's population is at risk of dengue, with 50–100 million dengue infections occurring worldwide annually (Bhatt, S. et al., 2013). An estimated 500,000 people with severe dengue require hospitalization each year with a 2.5% mortality. With regard to Yellow Fever, even effective vaccines have failed to prevent, 84,000–170,000 severe cases and 29,000–60,000 deaths during 2013 in Africa (WHO 2016). A recent outbreak of yellow fever from Luanda (Angola) and Kinsahs (Democratic Republic of the Congo) to many other countries in 2016 has shown that that yellow fever poses a serious global threat requiring new strategic thinking (Bagcchi, S, 2017). With unprecedented global emergence of viruses transmitted by *A. aegypti*,

sustained mosquito control efforts are urgently needed to prevent future outbreaks from these diseases. Therefore, a thorough understanding of mechanisms governing mosquito reproductive physiology is of paramount importance for development of novel control methods to eliminate mosquito-borne disease in the future.

1.2 Three classes of insect hormones

The word hormone is derived from the Greek word "hormao" meaning 'I excite or arouse'. Hormones are signaling molecules that are transported through insect circulatory system. They carry messages from their point of synthesis to target cells. In 1922, the Polish scientist Stefan Kopec was the first to demonstrate that the brain contains a factor responsible for molting of the gypsy moth, *Lymantria dispar* from the final larval instar to the pupal stage (Kopec, 1922). The "brain hormone' described by Kopec is known today as prothoracicotropic hormone (PTTH), which was originally purified from *Bombyx mori* brain extracts as a substance that could stimulate ecdysone production in the prothoracic glands (Kataoka et al., 1991). Since then, three main classes of insect hormones have been defined: ecdysteroids, juvenile hormone and peptide hormones. Hormones are especially pervasive in insect systems, affecting a wide variety of physiological processes, including embryogenesis, postembryonic development, behavior, water balance, metabolism, caste determination, polymorphism, mating, reproduction, and diapause.

1.2.1 Ecdysteroids

Ecdysteroids, including ecdysone (E), 20-hydroxyecdysone (20E) and other related steroids, are critical regulators of insect development and reproduction. During larval stages, ecdysone is produced by the prothoracic gland (PG), and then converted into the active form 20E after release into the hemolymph (Yamanaka et al., 2013). 20E initiates molting behavior and regulates metamorphosis of insects. During the adult stage, ecdysone is the principle regulator of female reproduction in hymenopterans, lepidopterans and dipterans. Generally, ecdysteroid-induced cellular changes leading to molting, metamorphosis, and reproductive maturation are mediated by a heterodimer comprising the ecdysone receptor (EcR) and ultraspiracle (USP) (Thomas et al.,1993; Yao et al.,1992; Yao et al.,1993). The insect EcR is a distant relative of the vertebrate farnesol X receptor (FXR). The USP protein is orthologous to the retinoid X receptor (RXR), which in turn, is a heterodimeric partner of several other nuclear receptors (King-Jones K and Thummel CS, 2005). Both EcR (EcR-A, EcR-B) and USP (USPA and USPB) have two isoforms in most insect species, and these isoforms interact to form different heterodimers (Hill et al., 2013). Differential promoter usage and alternative splicing of the EcR gene produces two receptor isoforms, EcR-A and EcR-B1. *Drosophila* has an additional B2 isoform (Talbot et al., 1993). Each EcR isoform has a distinct trans-activation activity, expression, and function during insect development and reproduction. Following binding of 20E, the heterodimer EcR and USP recognizes ecdysone response elements (EcRE) in a sequence specific way, which directly induces the transcription of early genes. These early genes encode mainly transcription factors,

including E74, E75, and Broad-Complex (BR-C) that regulate numerous late ecdysone-responsive genes (Thummel, 1996, 2002).

1.2.2 Juvenile hormone

Juvenile Hormone (JH), a key signaling molecule in metamorphosis and reproduction, is synthesized by the corpora allata (CA). The CA is a paired gland situated just posterior to the brain as part of the retrocerebral complex (Jindra et al., 2013). The receptor for JH is the basic helix-loop-helix Per/Arnt/Sim (bHLH-PAS) protein Methoprene tolerant (Met) (Ashok M et al., 1998; Charles JP et al., 2011; Jindra M et al., 2015), which requires dimerization with another bHLH-PAS protein to form a functional transcription factor. The best-studied Met binding partner is the insect homolog of vertebrate steroid receptor coactivator (SRC), variably termed Taiman in *D. melanogaster* (Miura K et al., 2005), FISC in *A. aegypti* (Li M et al.,2011), and SRC in some other insects (Kayukawa T et al.,2012; Zhang Z et al.,2011).

Insect molting and metamorphosis are orchestrated by ecdysteroids from prothoracic glands, with timing of secretion regulated by PTTH from the brain as well as by insulin-like peptides (ILPs) released in response to nutritional signals. Presence of JH ensures that the molt will produce another immature instar. Attainment of critical size/weight in the final instar leads to cessation of JH synthesis and a rise in JH metabolism. Under these conditions, ecdysteroids induce metamorphosis. JH reappears in the adult stage to regulate reproduction. The JH also is involved in caste differentiation, diapause, stress response, diapause and several polyphenisms (H.F. Nijhout, 1994).

1.2.3 Peptide hormones

Peptide hormones are produced by endocrine cells and neurosecretory cells, specialized neurons that produce chemical messengers. They typically signal via cell surface receptors and second messenger pathways. Some 300 distinct insect peptides have been identified (Gäde et al., 1997).

The first hormone demonstrated in insects is now known as prothoracicotropic hormone (PTTH) (Kopec, 1922). PTTH is synthesized in the two bilaterally-paired neurosecretory cells in the brain and stored in the corpora cardiaca–corpora allata complex (CC–CA). After release, PTTH activates the PGs to produce the ecdysteroid hormones (Gilbert et al., 1997).

A prominent group of peptide hormones is insulin-like peptides (ILPs), which are encoded by multigene families that are expressed in the brain and other tissues. The first confirmations of ILPs in invertebrates were in the silkworm *Bombyx mori* and eight ILPs for *Aedes aegypti*. Different subtypes of ILPs regulate insect physiology in different ways. ILP3 and ILP4 regulates ecdysteroid production by acting on the ovary (Brown et al., 1998). ILP5 disruption significantly increases body size and elevates lipid stores, while ILP2 or ILP6 has exactly the opposite effect on growth and metabolism in *Aedes* (Ling and Raikhel, 2018). ILP7 and ILP8 function in lipid homeostasis and ovarian development (Ling et al., 2017).

Eclosion hormone (EH) is a highly conserved 62 amino acid peptide with three internal disulfide bonds. *In situ* hybridization in embryos, larvae, and developing adults, along with Northern blots of different larval ganglia confirmed that the EH gene is expressed

only in the brain VM cells (Horodyski et al., 1989). EH is one of the hormones involving in triggering the shedding of the old cuticle at the end of each molt.

Most peptide hormones activate G protein-coupled receptors (GPCRs) (Brody and Cravchik, 2000; Ewer, 2005; Hewes and Taghert, 2001), while ILPs and PTTH target receptor tyrosine kinases (Fernandez et al., 1995; Rewitz et al., 2009) and eclosion hormone (EH) activates a receptor guanylate cyclase (Chang et al., 2009). Among their many functional roles, peptides regulate homeostasis, energy metabolism, locomotion and immune responses.

1.3 Ecdysis behavior and Ecdysis triggering hormone (ETH)

Insects are the dominant terrestrial life form on earth. Their evolutionary success is a consequence of a vast array of unique development and reproductive strategies (Truman and Riddiford, 2002). The transformation from larva to adult, called metamorphosis, enables the same species to minimize intraspecific competition by occupying different ecological niches. This ability of insects is made possible by the process of molting. Apolysis and ecdysis mark the beginning and ending point of molting behavior. Ecdysis triggering hormone (ETH) is one of the peptide hormones that initiates and regulates insect ecdysis behavior, which includes pre-ecdysis, ecdysis and post-ecdysis. ETH was first identified in *Manduca sexta* (Zitnan et al., 1996) and shown to be produced by Inka cells. Identification of the cDNA precursor and gene encoding ETH in *Manduca* revealed two additional peptides produced by Inka cells. One of these is an 11 amino acid, C-terminally amidated peptide. Since this peptide induces only preecdysis behavior in

Manduca, it was named pre-ecdysis triggering hormone or PETH (Zitnan et al., 1999). The third peptide, composed of 47 amino acids with a free carboxyl C-terminus was also synthesized, but has no obvious biological function. It was therefore named ETH-associated peptide (ETH-AP). In the Diptera, three types of peptides are called ETH1, ETH2 and ETH-AP. ETH receptors were first identified in *Drosophila*. The *Drosophila* gene CG5911 encodes two ETH receptor subtypes by alternative splicing: ETHR-A and ETHR-B. These gene products are GPCRs that are highly sensitive and selective for ETH ligands of *Drosophila* (Iverson et al., 2002; Park et al., 2003a).

Preecdysis in moths is initiated by low levels of PETH/ETH induced by corazonin. ETH then acts on its receptor in VM neurons to elicit EH release (Kim et al., 2006a). EH acts back on Inka cells to cause cGMP production and form a positive feedback loop (Ewer et al., 1997). During pre-ecdysis, ETH targets neurons in the CNS that co-express kinins and diuretic hormones (DHs), which induce synchronous pre-ecdysis motor burst patterns (Kim et al., 2006a). The subsequent ecdysis phase induced by elevated ETH levels involves release of crustacean cardioactive peptide CCAP, myoinhibitory peptides (MIP), and bursicon (Kim et al., 2006b; Lahr et al., 2012; Kim et al., 2015).

The persistence of Inka cells into adulthood (Park et al., 2002) suggests that ETH signaling may be important in reproductive functions. Studies in the silkworm *Bombyx mori* (Yamanaka et al., 2008) and yellow fever mosquito *Aedes aegypti* (Areiza et al., 2014) provided evidence that ETH functions as an allatotropin, regulating JH synthesis in adult insects. In recent studies, we reported that ETH promotes JH synthesis in the CA through calcium mobilization (Meiselman et al., 2017; Lee et al., 2017), which likely

induces activity of JH biosynthesis enzymes in *Drosophila*. Interruption of ETH signaling in adult *Drosophila* leads to JH deficiency in both male and female flies. As a consequence, ovary size, egg production, and yolk deposition is reduced significantly in female flies (Meiselman et al., 2017). In male flies, reduction of JH levels by ETHR silencing impairs short-term courtship memory (Lee et al., 2017). Furthermore, we reported that expression of ETH and ETH receptor is dependent on ecdysone. Silencing of the ecdysone receptor in Inka cells reduces fecundity. The endocrine network involving ecdysone-ETH-JH is essential to facilitate female reproduction of adult *Drosophila*. In view the evidence described above, I decided to investigate the function of ETH signaling in regulation of adult *Aedes* reproduction during my Ph.D study.

1.4 Endocrine regulation of female mosquito reproduction

Insects have a significant impact on human life. On the positive side, they are pollinators, scavengers, and soil conditioners. However, on the negative side, some insects are disease vectors and crop pests. Some 75% of all species worldwide are insects. Over a million species of insects are described, but the total number of species is estimated to be between 2.5 and 10 million. One of the most important reasons insects are so successful is that they have such high reproductive potential. During past decades, extensive work has been devoted toward understanding insect female reproductive physiology, which has great potential for targeting future insect control strategies. For hemimetabolous insect orders, it is well established that JH is the major factor controlling reproduction. In holometabolous insects, strategies governing reproduction vary among different orders.

In Coleoptera, JH is the major regulator of reproduction. In Lepidoptera, female reproduction is regulated either by JH or ecdysteroids according to different lineages that evolved independently. In Diptera, the JH is important in conferring competence of fat body to respond to ecdysteroids, which in turn promote vitellogenesis (Raikhel et al., 2005).

In *Aedes aegypti*, the reproductive cycle consists of two phases: previtellogenic and vitellogenic (Hansen et al., 2014). Previtellogenesis starts at eclosion and ends around 72 hr posteclosion with the process of physiological and behavioral preparation of blood feeding. Once this period is complete, competent females enter a state of arrest, which can only be broken by a blood meal. Vitellogenesis starts upon ingestion of the blood meal by inducing expression of the vitellogenein gene (Vg) and synthesis of other yolk protein precursors (YPPs), which is essential for oocyte maturation (Raikhel and Dhadialla, 1992, Raikhel et al., 2005, Raikhel et al., 1983).

JH regulates previtellogenesis by controlling maturation of the mosquito fat body through actions on Met in a time-dependent and concentration-dependent manner by regulating three major gene clusters, named early-posteclosion (EPE) genes, mid-posteclosion (MPE) genes and late-posteclosion genes (LPE). These EPE and MPE genes are active when JH titer is low at eclosion and then suppressed when JH levels increase. EPE and MPE genes are involved in carbohydrate and lipid metabolism. The LPE genes induced by JH can coordinate the protein synthesizing machinery so that the fat body becomes competent a blood meal (Zou et al., 2013). Beside the effect on fat body maturation, JH also regulates maturation and growth of primary follicles, which is

essential for the fate of ovarian follicles (Hagedorn et al., 1977, Gwadz and Spielman 1973).

Hormonal and nutritional cues such as insulin, ecdysone and Target of Rapamycin (TOR) provide nutrient sensors that together regulate vitellogenesis following the blood meal (Roy et al., 2007). During vitellogenesis, large scale synthesis of YPPs occurs in the fat body, an organ analogous to the vertebrate liver with functions in energy storage, lipid metabolism, nutrient sensing and immunity (Zhang Y et al., 2014; Klowden et al., 1987; Hays and Raikhel, 1990). The main YPP genes activated during vitellogenesis are vitellogenins (Vg A, B, C), vitellogenic carboxypeptidase (Vcp), vitellogenic cathepsin B (Vcb) and lipophorin (Lp) (Price et al., 2011; Raikhel et al., 2002). These YPPs are secreted into the hemolymph and accumulate in the oocyte during egg maturation through receptor-mediated endocytosis (Raikhel and Dhadialla, 1992).

The major regulator of vitellogenesis is 20E. Amino acids (AA) and other factors in the blood stimulate the brain, which releases ovarian ecdysterogenic hormone I (OEH I). OEH, belonging to a neuropeptide family called neuroparsins, is synthesized in medial neurosecretory cells during previtellogenesis and stored in the corpora cardiaca (Lea AO, 1972). OEH together with ILPs act on follicle cells in the ovary to secret ecdysone, which is released into hemolymph and transported into the fat body to initiate vitellogenesis together with the active form 20E (Hagedorn et al., 1975; Brown et al., 1998). Except for the master regulator 20E, the insulin signaling pathway and the AA target of TOR signaling pathways also play important roles during vitellogenesis. ILPs not only regulate YPP expression indirectly by inducing ecdysone synthesis, but also regulate YPP gene

expression in fat body directly. *In vitro* fat body culture experiments show that YPP expression is only induced by the combination of 20E and ILP signaling. Neither 20E or insulin has an effect on YPP expression individually (Roy et al., 2007). Components of vertebrate blood are digested by the mosquito gut into free amino acids. They function as building blocks for yolk protein precursor synthesis and also are used for energy production (Marquardt, 2004). The fat body monitors amino acid levels in hemolymph via the TOR signaling pathway, which transmits amino acid signaling to activate vitellogenesis, especially upregulation of YPP gene expression. Vitellogenin cannot respond to 20E stimulation unless with amino acid treatment. Treatment of fat body with the TOR-specific inhibitor rapamycin completely blocks activation of vitellogenin by 20E even in the presence of amino acids (Hansen et al., 2004).

1.5 Thesis objectives and aims

The main goal of my graduate research is to investigate the endocrine cross talk between three hormones: JH, ETH and ecdysteroids and discover their function in mosquito reproductive physiology, which can contribute to current models of mosquito reproduction. In Chapter 2, I identify for the first time the existence of ETH and ETH receptor transcripts, ETH peptide, and Inka cells in adult mosquitoes. In Chapter 3, I show that ecdysteroid regulates expression of ETH and ETH receptor genes in adult mosquitoes. Peak ecdysteroid levels occurring 18 hr post-blood meal induce expression of genes encoding ETH and ETHR. I also find that ETH and ETHR mRNA levels decrease following disruption of the ecdysone synthesis pathway; they can be rescued by

20E treatment. In Chapter 4, I provide evidence that both the first and second post-eclosion JH peaks are regulated by ETH signaling, which in turn regulates follicle size and lipid content during previtellogenesis. Data presented in Chapter 3 and 4 demonstrate that successive hormonal cascades involving ecdysteroids, ETH, and JH are essential for reproductive success of female *Aedes aegypti.* Chapter 5 provides a general summary of my findings and conclusions.

Abbreviations

20E	20-hydroxyecdysone
AA	Amino acids
bHLH-PAS	basic helix-loop-helix Per/Arnt/Sim
CA	corpora allata
CCAP	crustacean cardioactive peptide
DHs	diuretic hormones
E	ecdysone
EcR	ecdysone receptor
EH	eclosion hormone
EPE	early-posteclosion
ETH	Ecdysis triggering hormone
ETH-AP	ETH-associated peptide
FXR	farnesol X receptor
GPCRs	g-coupled protein receptors
ILPs	insulin like peptides
JH	Juvenile Hormone
Lp	lipophorin
LPE	late-posteclosion
Met	Methoprene tolerant
MIP	myoinhibitory peptides
MPE	mid-posteclosion
OEH	ecdysterogenic hormone
PETH	pre-ecdysis triggering hormone
PG	prothoracic gland
PTTH	as prothoracicotropic hormone
RXR	retinoid X receptor
SRC	steroid receptor coactivator
TOR	Target of Rapamycin
USP	ultraspiracle
VBDs	Vector-borne diseases
Vcb	vitellogenic cathepsin B
Vcp	vitellogenic carboxypeptidase
Vg	ellogenein gene
YPPs	yolk protein precursors

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Chapter 2 Ecdysis Triggering Hormone Signaling in the Adult Yellow Fever Mosquito *Aedes aegypti*

Abstract

Ecdysis Triggering Hormone (ETH), a peptide hormone produced by Inka cells in epitracheal glands of insects, is important in scheduling the ecdysis behavioral sequence. Ecdysis is the terminal step of each molt and involves shedding of cuticle surrounding the external surface, the lining of the trachea, and the lining of foregut and hindgut. However, previous work in *Drosophila melanogaster* shows that Inka cells, ETH and ETH receptor transcripts persist through metamorphosis into adulthood, a period during which no molt occurs. In this chapter, I demonstrate the existence of Inka cells, ETH transcript and transcripts encoding ETH receptor isoforms A and B in adult mosquitoes. In female *Aedes*, ETHR transcript levels decrease immediately following pupal-adult eclosion, then subsequently increase post-blood meal. Relative transcript numbers of ETHR-A, ETHR-B and ETH peak at 24 hr post blood meal, which follow the ecdysone peak around 18 hr post-blood meal. This suggests that ETH and ETHRs expression are regulated by ecdysone. In order to map the ETH receptors in the adult females, I created ETHR knock-in lines to locate ETHR-expressing cells, which may give a clue of ETH signaling function in adult mosquitoes.

Introduction

Hormones are bioactive peptides or molecules responsible for regulation of biological process in organisms including insects. Hormonal regulation of insect growth and

development contributes to precisely timed events. One of the most important behaviors is named ecdysis, during which insects shed the exoskeleton in order to grow and undergo morphological changes during metamorphosis. In 1999, Ecdysis triggering hormone produced by Inka cells was first described in larvae of *Manduca sexta* (Zitnan et al., 1996). ETH homologs DrmETH1, DrmETH2 and AeaETH1, AeaETH2 were subsequently identified in *Drosophila melanogaster* and *Aedes aegypti*, respectively (Park et al., 1999; Li and Adams, 2009). ETH receptors were first identified in *Drosophila* as two ETH receptor isoforms (ETHR-A and ETHR-B) processed by alternative splicing (Park et al., 2003a). Two functionally distinct subtypes of ETH receptors (AeaETHR-A, AeaETHR-B) of *A. aegypti* were identified in 2009 (Li and Adams, 2009).

Ecdysis triggering hormones are well known to control ecdysis and molting in all major insect orders during juvenile stages (Zitnan et al., 2003). By contrast, only very recently has ETH signaling and its function been described during the adult stage. One reason for this is that during the adult stage, there are no visible changes of morphology. Recent reports indicate that ETH functions as an obligatory allatotropin in *Drosophila* adults, strongly influencing female reproduction (Meiselman et al., 2017) and male courtship memory (Lee et al., 2017).

In this study, I show that Inka cells, ETH and ETHR transcripts persist in adult female *Aedes aegypti.* Through immunohistochemical visualization of Inka cells, I provide evidence for their existence in female mosquitoes. Post eclosion expression profiles of genes encoding ETH and ETHR suggest that they are regulated by 20E. Thus, in the

following chapters, I describe upstream and downstream of signaling pathways for ETH. I also identify the role of ETH signaling in adult female mosquitoes, elucidating its role in reproduction.

Material and Methods

Mosquito Rearing

The *A. aegypti* wild-type UGAL/Rockfeller strain (CRISPR knock-in injection mosquitoes were derived from *A. aegypti* Liverpool strain.) was reared under laboratory conditions at 28°C and 70% relative humidity with unlimited access to 10% sugar and water. Three to four-day old females were blood-fed on White Leghorn chickens.

Determination of Duration of Pupal Period

Pupae were sexed and collected at 30 minute intervals after the larval to pupal transition. The number of eclosing adults was collected every hour to determine the duration of pupal stage.

Immunohistochemistry

Animals were dissected in cold PBS. The dorsal side of the thorax and abdomen was opened and pinned under saline in a dissection chamber. The muscle in the thorax was removed carefully to expose the main tracheal system. Preparations were incubated in 4% paraformaldehyde overnight at 4°C, washed in PBS (3x5 min) and then washed in PBST (0.5% Triton X-100 in PBS; 3x5 min). Following a block with 5% NGS (normal goat serum) in PBST for one hour at room temperature, samples were incubated with rabbit anti-PETH antiserum (1:1000 dilution in PBST) for two days at 4 °C. After washing in PBST (6x5 min), samples were incubated with goat anti-rabbit Alex Flour 488 (1:500).

For ETHR staining, samples were incubated with rabbit anti-GFP: (1:500 dilution in PBST) and mouse anti-NC82: (1:50 dilution in PBST) for one day at 4°C. After PBST washing (6x5 min), samples were incubated with goat anti-rabbit Alex Flour 488 (1:500) and goat anti-mouse Alex Flour 568 (1:500). After 5x5 min washes with PBST and one wash with PBS, samples were mounted in Aqua Poly/Mount (Polysciences Inc.). Confocal images were acquired with a Zeiss LSM 510 microscope.

Total RNA Extraction and Quantitative RT-PCR (qPCR)

For qPCR of ETHR expression during pupal and adult stage of female and male mosquitoes, 10 animals in each replicate and three replicates were collected. Whole bodies were homogenized and mRNA was extracted using the TRIzol method (Invitrogen) and treated with DNase I (Invitrogen) according to the manufacturer's protocol. For mRNA, cDNA was synthesized from 1µg total RNA ProtoScript II RT Kit (New England Biolabs) and pre-amplified using the SsoAdvanced PreAmp Supermix Kit (Bio-Rad) for unbiased, target-specific pre-amplification of cDNA. qRT-PCR was performed using SYBR green (Cat. #170-8882, Bio-Rad) and by Bio-Rad CFX96 Real Time PCR Detection System. Sequences of primers used for expression analysis are provided below. The specificity of each primer set was validated by construction of a melting curve. S7 ribosomal protein (RPS7) expression was determined as a control housekeeping gene.

RPS7 For: CCCGGAGCCCTACCTATAAACTAT

RPS7 Rev: GCAGCACAAAGATGATTTATGCAC

ETH For: GGCAGACGTAGTGGACTCTT

ETH Rev: GGCTTCATCCAACACATCCC

ETHR-A For: CGTCTGTCTCACCAAAGCCT

ETHR-A Rev: ACTGAGTTCCGGTTTGCTGA

ETHR-B For: CCACACGGCATCGACAAATC

ETHR-B Rev: TGGGACTCCACTTCCACCAC

ETHR common For: AGGTGTGGGTACTGGGAGAA

ETHR common Rev: CACCGTCAGCTCCACGAA

ETHR-A and ETHR-B eGFP Knock-in Plasmid Construction

In order to map ETHR in the adult stage of *Aede*s, ETHR-A and ETHR-B CRISPR knock-in plasmids were designed. I included around 1000 base pairs from the end of ETHR4A exon or ETHR4B exon as homology left arm (HLA) and another 1000 base pairs following 4A exon and 4B exon as homology right arm (HRA). The HLA sequence was followed by a self-cleaving T2A peptide and an eGFP coding sequence to serve as a visual reporter of ETHR-A and ETHR-B expression. I also included a dominant fluorescent marker consisting of 3xP3-dsRed, expressing in the larvae and adult photoreceptors to serve as a transgenesis marker (Fig 2.5).

PCR was used to amplify the homology left arm A (HLAA), homology left arm A (HLAB), homology right arm A (HRAA) and homology right arm B (HRAB) fragments with genomic DNA from the *A. aegypti* Liverpool strain (wt) as template. T2A was ordered from IDT. eGFP was amplified from pBac[3xP3-EGFPafm] and 3XP3-dsRed was amplified from Vector 933C-pBac-HomologyLeft-U6-gRNA-3xp3-tdTomato. ß-lactamase gene conferring resistance to ampicillin (AmpR) and replicon (ori) for the plasmid replication were amplified from pJET1.2/blunt cloning vector. All segments are

aligned in order of HLA-T2A-eGFP-3xp3-dsRed-HRA-ori-AmpR by Gibson Assembly Master Mix (New England Biolabs).

Germ-line Transformation of A. aegypti

A total of 500 G0 wild-type embryos of the Liverpool strain of *Aedes aegypti* was injected with a solution containing the donor plasmid DNA (280 ng/µl), which was purified using the EndoFree Plasmid Maxi Kit (QIAGEN, Valencia, CA), three gRNAs (100 ng/µl) and Cas9 protein (300 ng/µl). For ETHR-A, adults were assigned to 34 male-founder and 27 female-founder pools and outcrossed to wild-type adults of the opposite sex. For ETHR-B, adults were assigned to 45 male-founder and 40 female-founder pools and outcrossed to wild-type adults of the opposite sex. G1 progeny were selected by monitoring the DsRed fluorescent eye marker under a fluorescence microscope (Leica M165FC).

gRNA1: GUCGAAGGUGAUUCUGGCGU

gRNA2: UGCACGGUCGAAGGUGAUUC

gRNA3: GGAAAUCACUGCACGGUCGA

ETHR-B gRNA1: GGGCAGCAGUAGUAGUGAUG

ETHR-B gRNA2: GGAAUGUGAUAUCUAGGCAA

ETHR-B gRNA3: GGCAGCAGUAGUAGUGAUGC

Molecular Analysis

Genomic DNA was extracted from CRISPR knock-in mosquitoes and wild type mosquitoes using DNeasy tissue kit (QIAGEN, Valencia, CA). RT-PCR was performed

using AccuPower PCR premix (Bioneer) and PCR products were sequenced (IIGB core facilities). Primer sequences used for expression analysis are provided below.

ETHR-A F1 CACCTCAACTGCAAGAACAAAG

ETHR-A R1 TGCTTGTCGGCCATGATATAG

ETHR-A F2 GCCCAGTGATGCAGAAGAA

ETHR-A R2 GCTGTCGTCTAATCGACGTATC

ETHR-B F1 ACGCGGAAGTGAGCAAATAG

ETHR-B R1 CTGCTTGTCGGCCATGATATAG

ETHR-B F2 CTGTGACCCAAGATAGCAGTT

ETHR-B R2 AGTCAATCACCACTCGATTTCA

Results

Pupal development duration

In order to show the expression pattern of ETH and ETHR transcripts for pupae and adults, it is crucial to determine the duration of the pupal stage for both females and males under standard rearing conditions. Pupae were collected and sexed at 30 minute intervals. Eclosing animals were scored hourly. Male eclosion began at 41 h (1.44%) after pupation with the majority emerging at 44 h (30.22%), 45 h (38.85%) and a few at 50 h (0.72%). Female eclosion began at 42 h (1.9%). Around 70% of females eclosed at 45 h, 46 h and 47 h post-pupation (Fig 2.1). Thus, in the present study, the duration of the pupal stage for male mosquitoes was ~44.5 hr and female was ~ 46 hr.

Inka cells and ETH signaling persist during adulthood in Aedes.

It was recently reported that the Inka cells persisting in the adult stage of *Drosophila melanogaster* are bilaterally paired and positioned inside the body wall: 6 to 9 pairs of Inka cells are located on the main tracheal tube varying in shape and location (Meiselman et al., 2017; Lee et al. 2017). To visualize Inka cells in adult mosquitoes, I performed immunostaining for ETH in the day 4 adult stage, using the 4th instar larval stage as a control (Fig 2.2). In adult mosquitoes, there are 9 segments in the abdomen. Inka cells are observed at branch points of the tracheal system in the abdominal segments. The shape and size is varied in different locations. However, by traditional dissection, it is very difficult to map their positions.

I next measured relative expression levels of ETH and ETHR-A and ETHR-B transcripts in female adults using q-PCR: just post-pupation animals (Fig 2.3, Fig 2.4). ETH, ETHR-A and ETHR-B transcripts declined in phase following eclosion, then increased in intensity with a peak at 24 hr post blood meal. This pattern of gene expression followed closely the ecdysone peak occurring at 18 hr post-blood meal. The temporal pattern of expression is sexually dimorphic. In male mosquitoes, ETHR (common region) expression level is maintained at a high level post-eclosion (Fig 2.9.B). In general, the expression patterns of ETH, ETHR-A and ETHR-B suggest a common upstream regulator.

Establishment of ETHR eGFP knock-in transgenic lines

The pre-blastoderm embryos of the Liverpool *A. aegypti* strain (wt) were injected with A mixture of plasmid, gRNA and Cas9 protein. For ETHR-A, of 500 injected embryos, a

total of 101 and 89 adult males and females, respectively, survived to adulthood. These G0 mosquitoes were crossed with wildtype animals to produce G1 progeny, which were examined for the presence of dsRED expression in their eyes (Fig 2.6. A). Their progeny was established as homozygous based on the selection for 100% eye-specific dsRED expression of mosquitoes in each line. Results indicated that the transformation efficiency was 0.019%. For ETHR-B, A total of 133 and 118 adult males and females, respectively, survived to the adult stage with transformation efficiency 0.025%.

Genome integration of eGFP into the ETHR-A and ETHR-B loci was confirmed separately by genomic PCR of the ETHR-A knock-in line using two sets of specific primers: homology left arm to eGFP and dsRED to homology right arms (Fig 2.5). Gene amplification analysis confirmed site-specific integration of the donor construct into the ETHR-A locus (Fig 2.6B) and also confirmed integration of the donor construct into the ETHR-B locus (Fig 2.6C).

ETHR mapping by CRISPR eGFP knock in.

In Previous studies, ETHRs were reported expressed in numerous central neurons in the larval stages of *Manduca* (Kim et al., 2006a). ETH initiates ecdysis behaviors through direct actions on the CNS. Expression of ETHRs in the CNS coincides precisely with acquisition of CNS sensitivity and behavioral responses to ETH (Kim et al., 2006a). Here, I tried to identify the ETH receptor (ETHR) in *Aedes* with ETHR-A and ETHR-B CRISPR knock-in lines. According to a previous study, injection of synthetic ETH into 4^{th} instar larval *Aedes* with pupal trumpet rudiments (~9 to 12 hr before ecdysis) induces ecdysis behavior (Li and Adams, 2009). This provided evidence that ETHRs level is high

around 12 hr before ecdysis in 4th instar larvae. In order to map ETHRs in CRISPR knock-in lines, I used 4th instar larvae with pupal trumpet rudiments for immunohistochemistry (Fig 2.10). However, even with high level of ETHRs before ecdysis in 4th instar, there is no obvious staining using an anti-eGFP antibody (Figure 2.7 A). This may be a consequence of 1) low level GPCR expression or 2) an attenuated level of eGFP expression due to its position downstream of T2A (Akbari personal communication).

Discussion

I have shown that Inka cells, ETH genes and ETH peptides persist into the adult stage of mosquitoes. Inka cells, varying in shape and size, labeled by antiserum against PETH, were located in major branches of the tracheal system in adult mosquitoes. Meanwhile, ETH transcripts exist and fluctuate in adult female mosquitoes. ETH gene expression decreases posteclosion and increases again post blood meal, indicating that ETH synthesis is regulated by ecdysone signaling.

I also show the ETHR transcript expression profile in adult mosquitoes. In adult female mosquitoes, ETHR-A and ETHR-B levels increase and peak around 24 hr post blood meal. This pattern also suggests that ecdysone regulates ETHR synthesis in adult female mosquitoes. Previously, it has been reported that ETHR-A-expressing neurons are required for ecdysis throughout development; however, ETHR-B expressing neurons are required during pupal and adult ecdysis, since suppression of ETHR-B-expressing neurons caused only limited lethality during larval stages in *Drosophila* (Diao et al., 2016). In my qPCR studies, I found that in adult female mosquitoes the ETHR-B

expression level is higher than ETHR-A, which has a ct-value higher than 33 cycles. These data suggest that ETHR-A and ETHR-B are differentially required during developmental and mature stages. Furthermore, the expression level of ETHR is sexually dimorphic. Compared with low transcript levels of ETHRs post eclosion in females, ETHR is maintained at a high expression level post eclosion in male mosquitoes. This indicates possible gender-specific functions for ETHR signaling in the reproductive physiology of female and male mosquitoes.

ETHR expression has been reported in the CA of the silkworm, *Bombyx mori*, pupal yellow fever mosquito, *Aedes aegypti* and adult fruit fly *Drosophila melanogaster.* (Areiza et al., 2014; Meiselman et al., 2017; Yamanaka et al., 2008). ETH has been previously shown to stimulate activity of the rate-limiting enzyme in JH biosynthesis, Juvenile Hormone Acid Methyl-Transferase (JHAMT) via calcium release (Areiza et al., 2014, Lee et al., 2017). These findings indicate a vital functional role for ETH as an obligatory allatotropin for maintenance of JH levels. With the development of CRISPR technique in *Aedes aegypti* (Kistler et al., 2015), we would like to map the expression pattern of ETHR-A and ETHR-B in adult mosquitoes by eGFP knock in. Since the regulatory elements of ETHR-A and ETHR-B are not known, it is hard to express a fluorescent marker like eGFP under the control of ETHR-A and ETHR-B promoter. To solve this problem, I tried to incorporate the eGFP coding sequence into the ETHR locus by exploiting the "ribosomal skipping" mechanism of the viral T2A peptide. Viral 2A-like peptides share an Asp-Val/Ile-Glu-X-Asn-Pro-Gly-Pro consensus motif (Donnelly et al. 2001a). During translation, T2A forces the ribosome to skip from Gly to the Pro codon

without forming a peptide bond (Donnelly et al. 2001b). In my CRISPR eGFP knock in, I attempted to express the eGFP sequence at the 3' end of the ETHR sequence, separated by T2A. At the same time, mosquitoes show eye-specific red fluorescent marker (3XP3-DsRED). Even though this was successful, I found the expression level of green fluorescent protein to be too low for microscopic detection in larval mosquitoes, where ETHR-A and ETHR-B expression is at a relatively high level. This may be caused by relatively low expression level of GPCRs. Another possible reason is that eGFP expression level is much lower than ETHRs due to it's location downstream of the T2A cleavage (Akbari, personal communication). I made a second attempt to map ETHRs by CRISPR knock in by modifying the construct in the following ways: 1) exchange the fluorescent marker between ETHR labeling and eye markers. 2). Remove T2A to make an in-frame fusion protein of ETHR and dGFP (Fig 2.8). Results of this experimental approach are pending.

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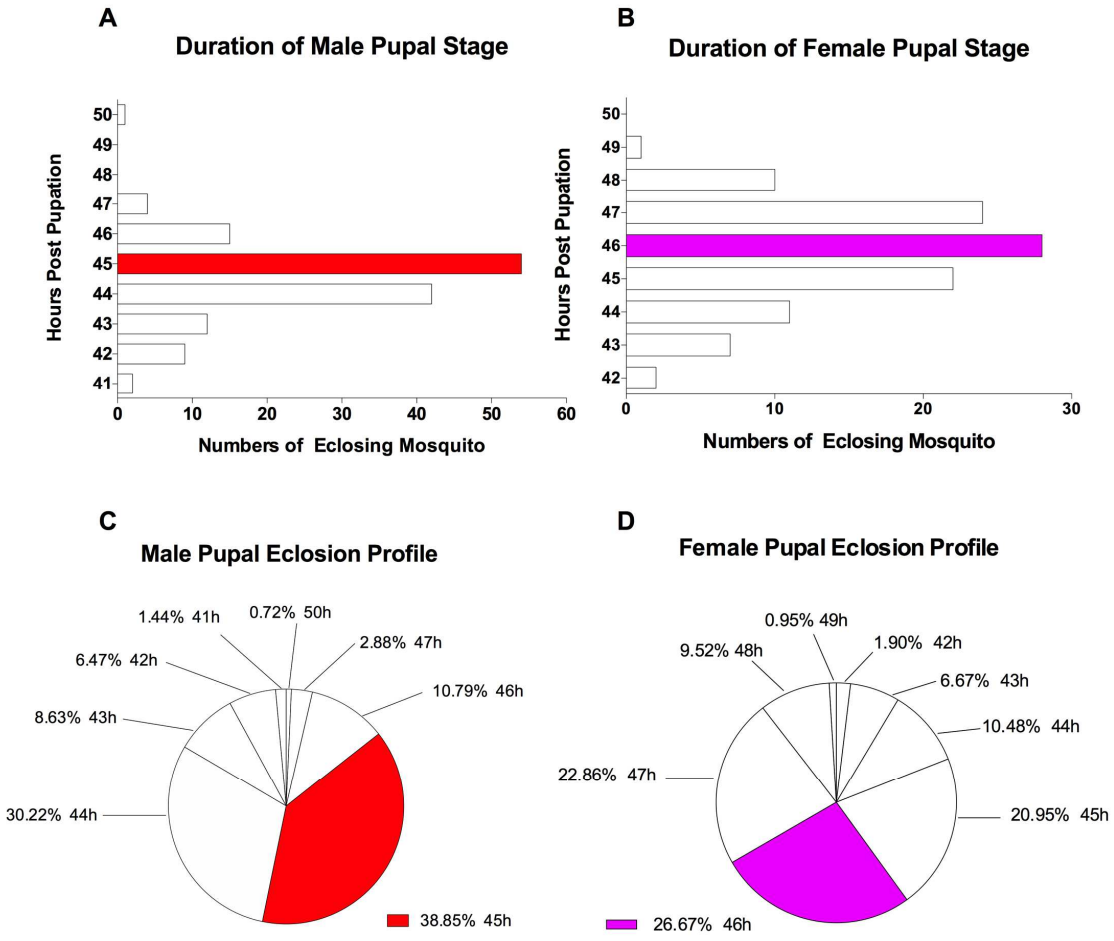


Figure 2.1 Eclosion profiles of male and female *Aedes aegypti*

A. Numbers of eclosed male mosquitoes during hourly intervals after pupation; B. Numbers of eclosed female mosquitoes during hourly intervals after pupation. C. Percentage of eclosed male mosquitoes during hourly intervals. D. Percentage of eclosed female mosquitoes during hourly intervals.

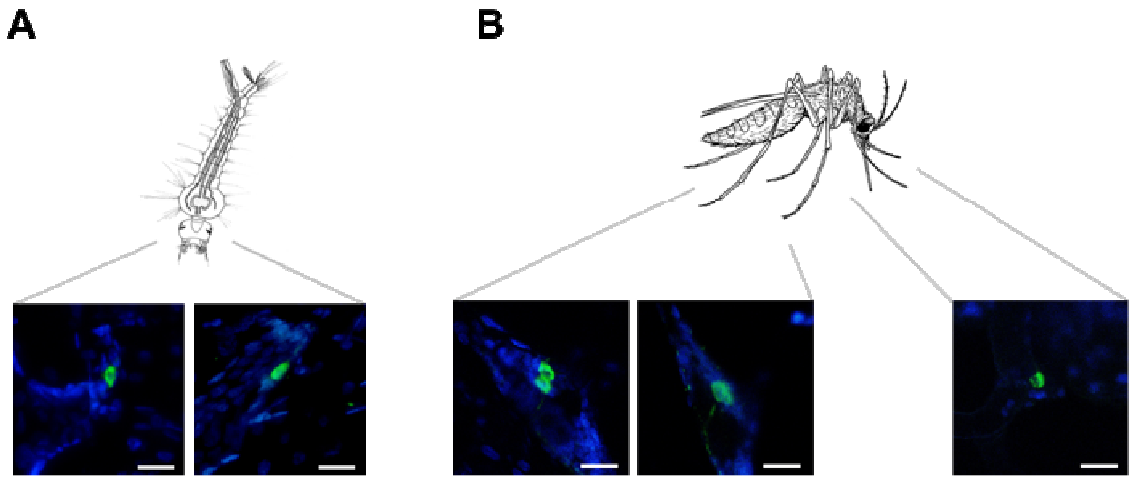


Figure 2.2 Inka cells and ETH peptides persist in adult *Aedes*

Immunohistochemical labeling of Inka cells of *A. aegypti* using an antiserum raised against *M. sexta* PETH. Intense immunoreactivity is observed in Inka cells of larval (A) and day 4 adult (B) mosquitoes in the abdominal segments. Scale bar, 20 μm.

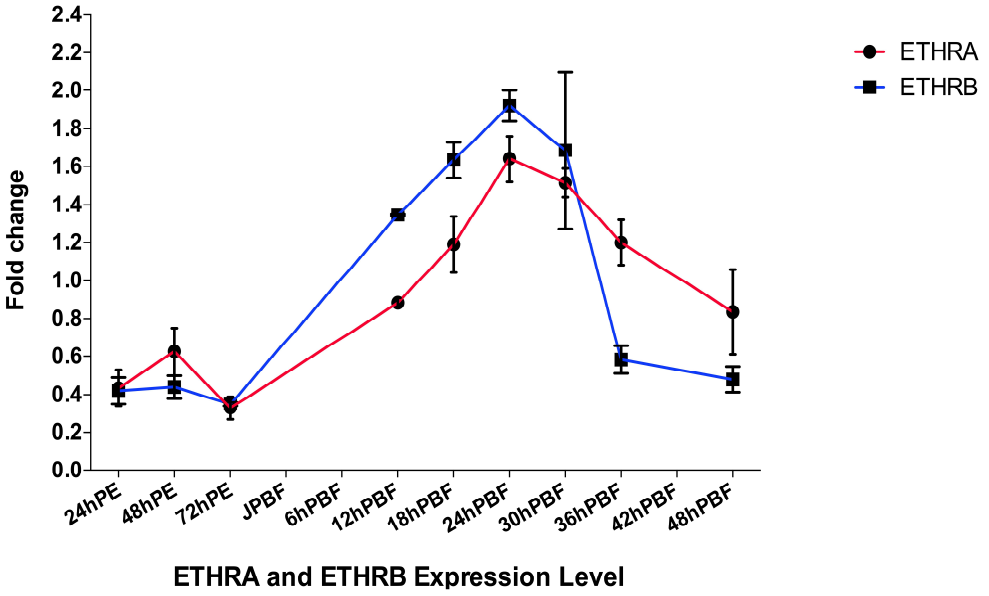


Figure 2.3 Expression pattern of ETHR transcripts in *Aedes aegypti* by q-PCR

Expression patterns of ETHR-A and ETHR-B detected by q-PCR of females during the adult stage. PE: Post-Eclosion; JPBF: just post-blood feeding; PBF: post-blood feeding

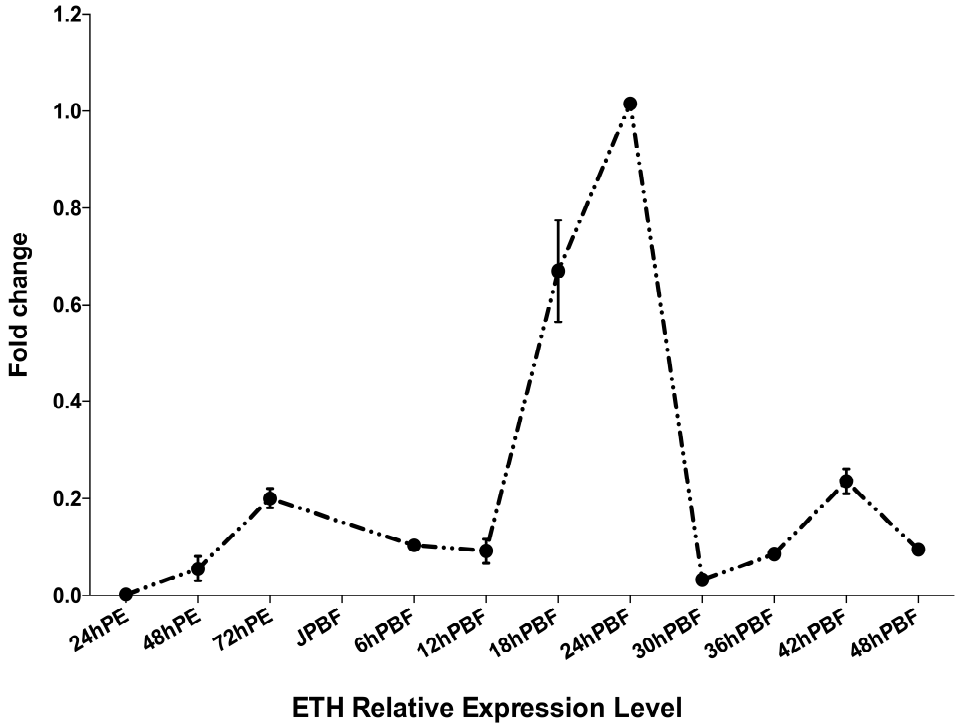
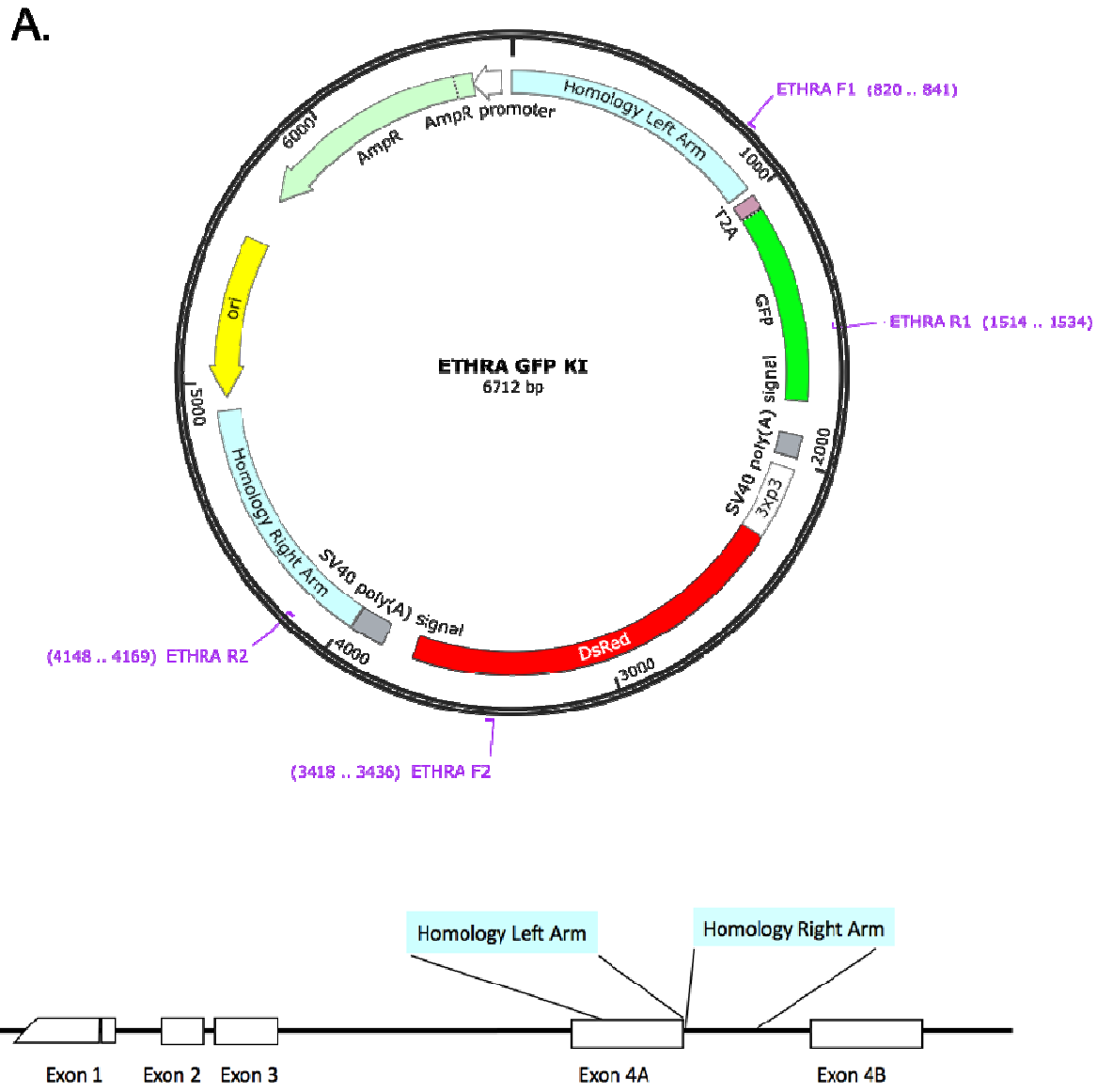


Figure 2.4 Expression pattern of ETH transcripts in adult *Aedes aegypti* by q-PCR

Expression patterns of the ETH gene detected by q-PCR of females during adult stage.PE: Post-Eclosion; JPBF: just post blood feeding; PBF: post blood feeding



Gene structure of ETHR

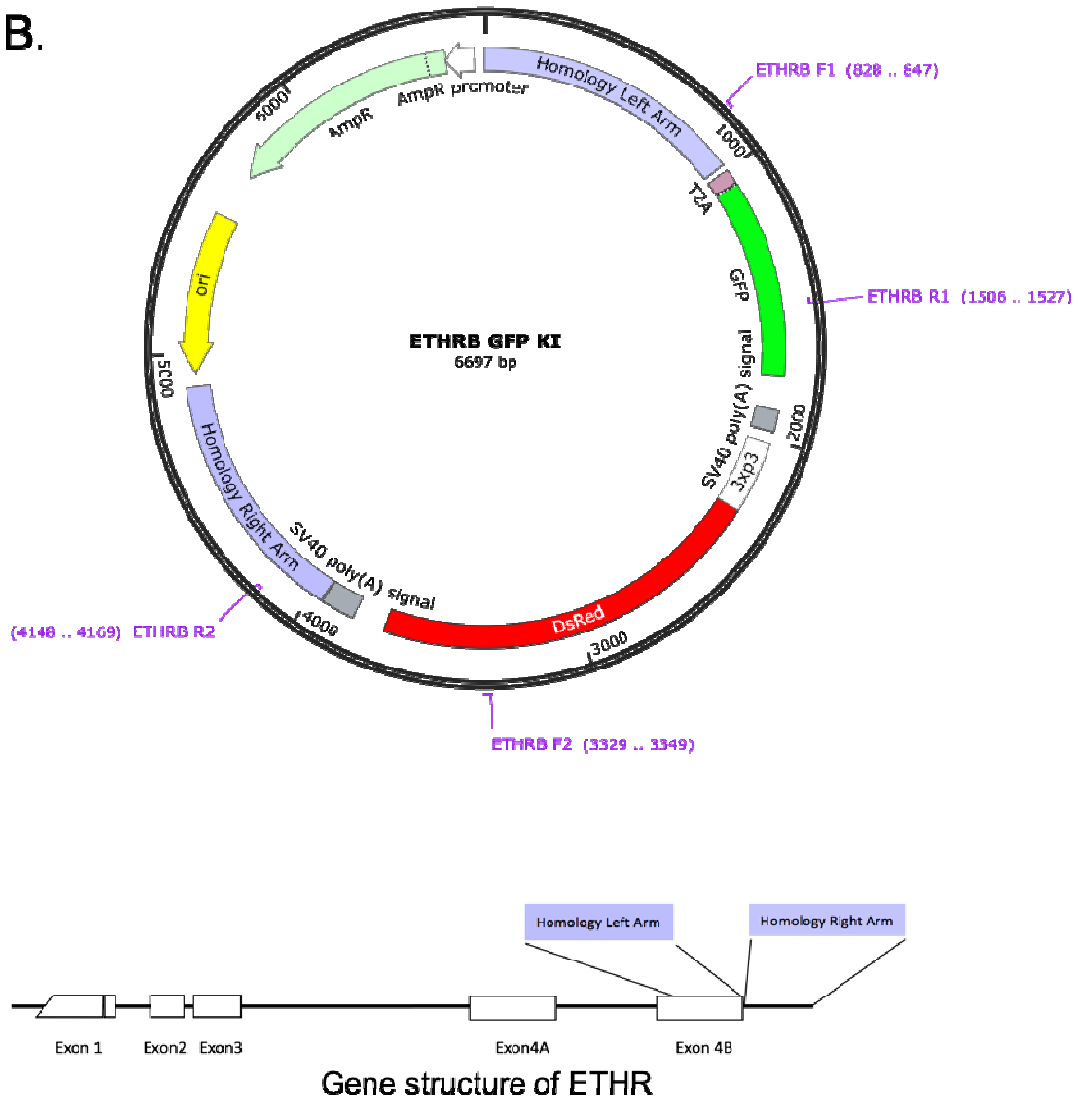


Figure 2.5 Construct of ETHR eGFP knock-in by CRISPR

Schematic representations of the ETHR-A and donor construct (A), and the ETHR-B and donor construct (B). The donor plasmids express red fluorescent eye marker (3xp3-DsRed) and green fluorescent protein is inserted after ETHR-A and ETHR-B separately.

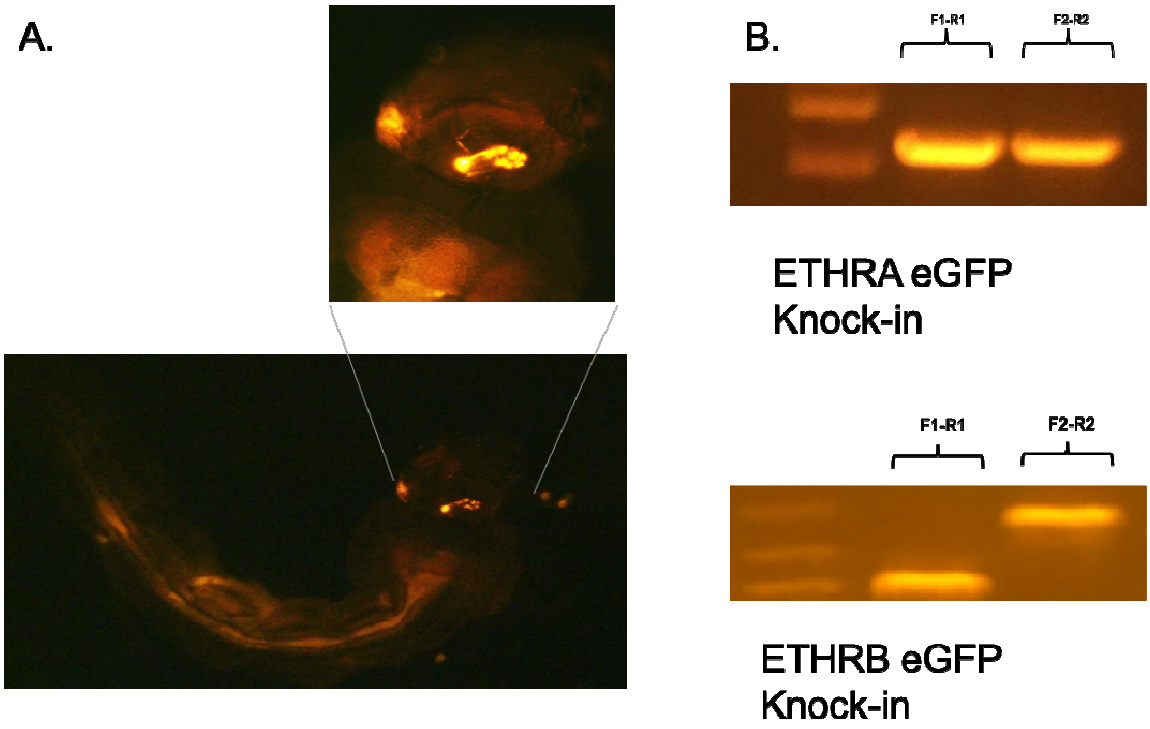


Figure 2.6 Establishment of the *A. aegypti* ETHR-A and ETHR-B eGFP knock-in lines

A. Larvae of knock-in mosquitoes were screened for the presence of eye-specific expression of the selectable markers DsRed. B: Gene amplification analysis confirms site-specific integration of the donor construct into the ETHR-A locus separately using combinations of genomic and plasmid donor-specific primers (ETHR-A F1-R1 expected 715 bp, and ETHR-A F2-R2 expected 752 bp), C: and also confirms the integration of the donor construct into the ETHR-B locus using combinations of genomic and plasmid donor specific primers (ETHR-B F1-R1 expected 700 bp, and ETHR-B F2-R2 expected 841 bp).

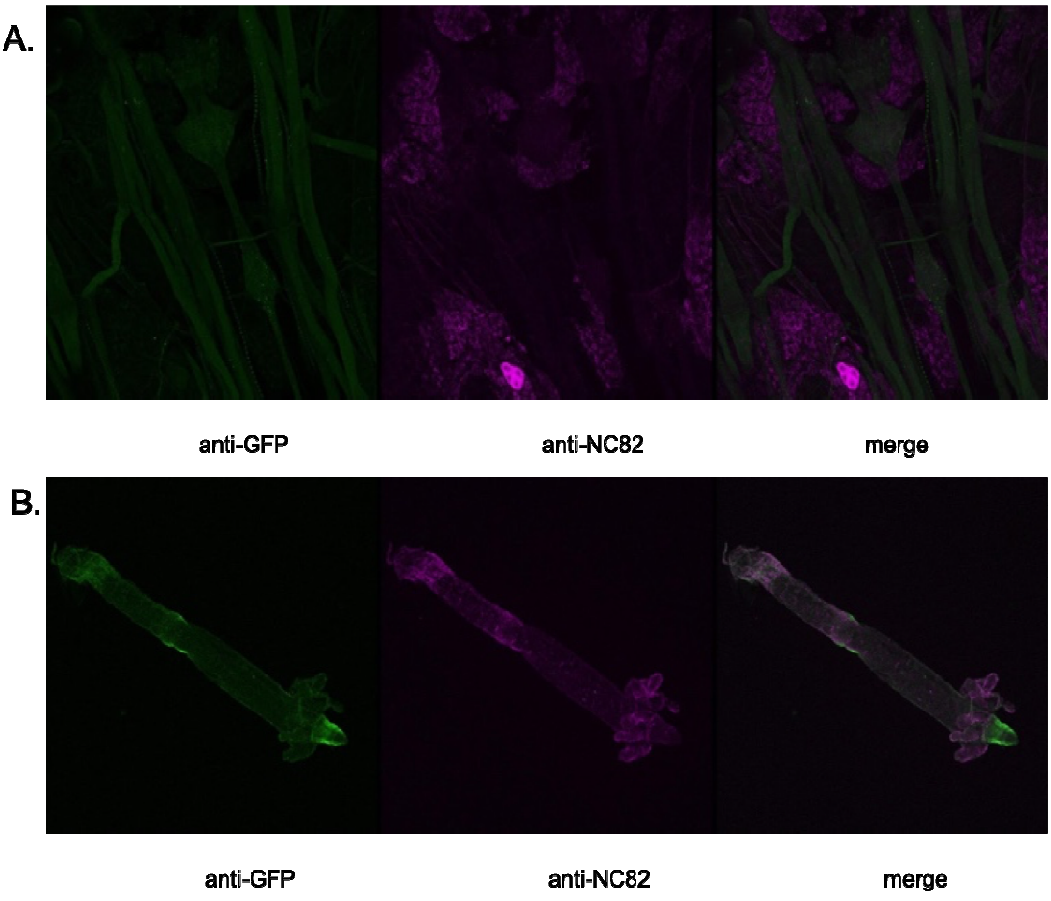


Figure 2.7 Immunohistochemistry of ETHR-A eGFP knock-in

Representative images of ETHR-A eGFP knock in of 4th instar larva central nervous system(A) and gut (B). Larva with pupal trumpet rudiments were dissected for immunohistochemistry with an antiserum raised against eGFP and NC82. Images were obtained using Leica SP5 Inverted confocal microscope.

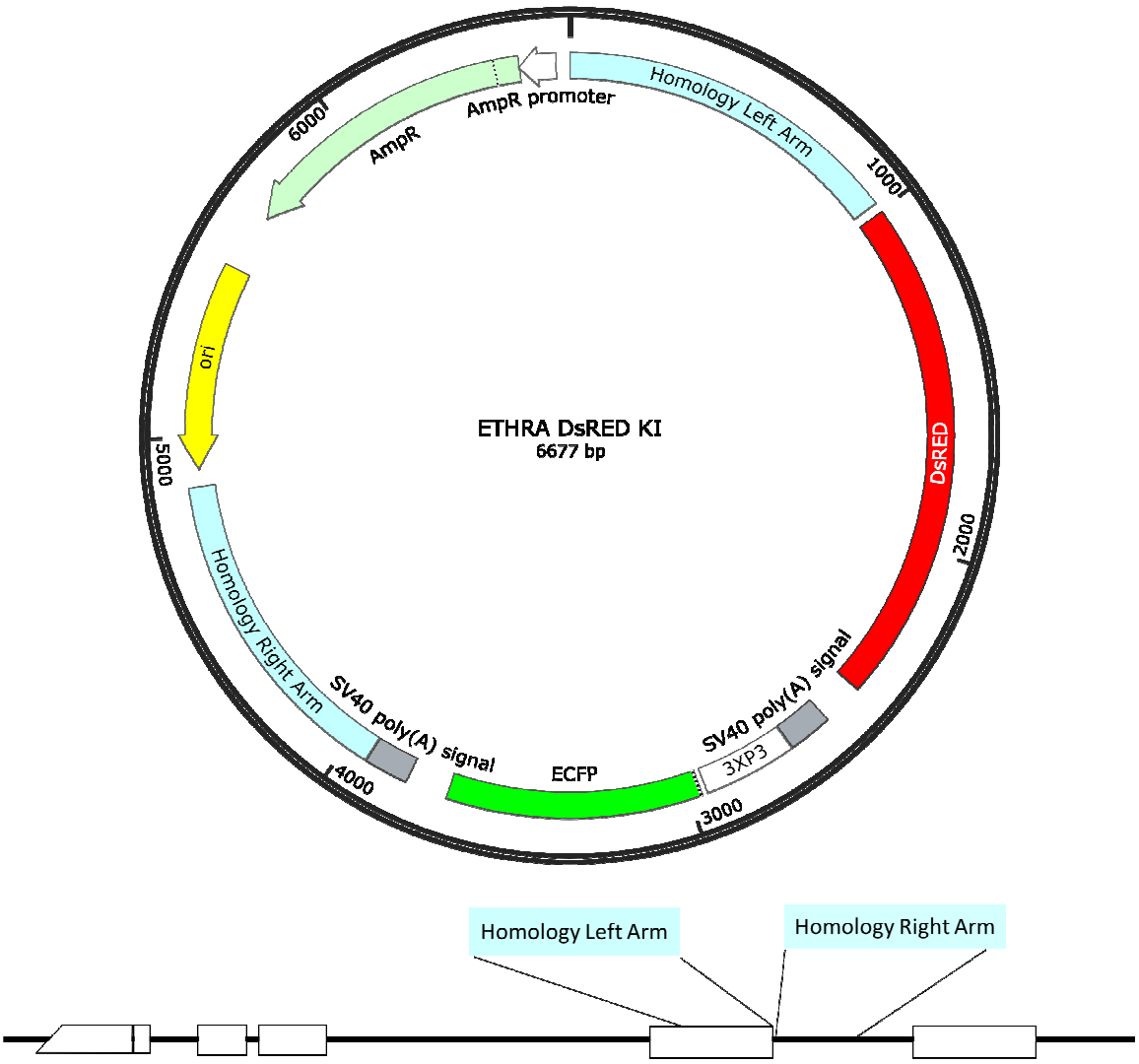


Figure 2.8 . Construct of ETHR-A DsRED knock-in by CRISPR

Schematic representations of the ETHR-A and donor construct. The donor plasmids express green fluorescent eye marker (3xp3-eGFP) and red fluorescent protein (DsRED) is inserted after ETHR-A.

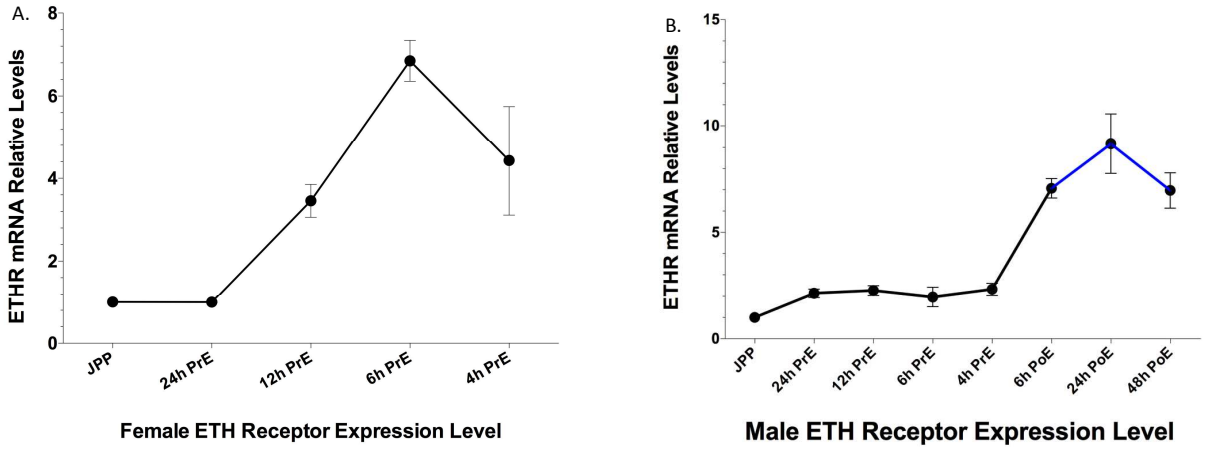


Figure 2.9 Expression pattern of ETHR common region in *Aedes aegypti* by qPCR

(A). Expression patterns of ETHR detected by q-PCR of females during the pupal stage. JPP: just post pupation; PrE: Pre-eclosion. (B). Expression patter of ETHR by q-PCR of males during pupal and adult stage. JPP: just post pupation; PrE: Pre-eclosion; PoE: Post-eclosion

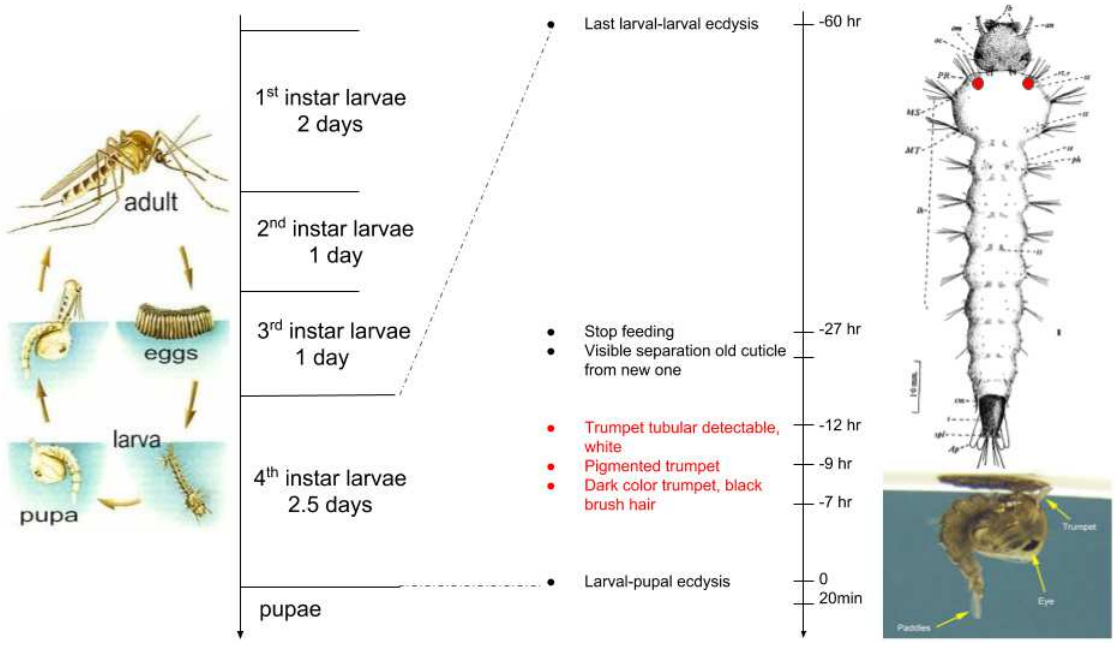


Figure 2.10 Life cycle in *Aedes aegypti* and morphological markers in 4th instar larva

In 4th instar larva, trumpet tubular is detected ~12 hr before ecdysis when mosquitoes are sensitive to ETH. (Pics from US Environmental Protection Agency).

Chapter 3 Ecdysteroids Regulate ETH and ETHR Expression after Blood Feeding

Abstract

Early, early-late and late waves of gene expression initiated by ecdysone are essential for precisely-timed development of insects. Previously it has been shown early and late gene expression events in *Manduca* are essential for precise scheduling of ecdysis. Early events occur around the peak of ecdysteroid levels, which induces ETH and ETHR gene expression during larval stages of moths and mosquitoes. In 2014, STARR-Seq data confirm that 20E induces EcR enhancer activity in promoters of both ETH and ETHR genes. ETH peptide accumulates in Inka cells during early steps gene expression, but release competence of these cells does not occur until ecdysteroid declines to low levels. Here I show ecdysone signaling also regulates ETH and ETHR gene production during adulthood in mosquitoes. After the blood meal, relative levels of ETH and ETHR transcripts closely follow the ecdysone peak. Furthermore, I demonstrate that ETH and ETH receptor gene expression is induced by 20E injection. Furthermore, ETH and ETHRs transcript levels decline dramatically as a consequence of silencing Halloween genes, which encode ecdysteroid synthesis enzymes; this can be rescued by 20E injection. My findings indicate that during mosquito adulthood, ecdysteroids play an important role in regulating genes encoding ETH and ETHR, which could in turn are important regulators of pre-vitellogenesis, as described in Chapter 4.

Introduction

Ecdysteroids are well known molting hormones that, together with juvenile hormones, program the development of insects through larval and pupal stages. However, while the prothoracic glands are the main source of ecdysone during development, they undergo programmed cell death during metamorphosis (Dai and Gilbert, 1997; Gilbert, 1967). In adult female mosquitoes, blood feeding triggers medial neurosecretory cells in the brain to release ovary ecdysteroidogenic hormone (OEH) and several insulin-like peptide (ILP) family members including ILP3 (Brown et al., 1998; Brown et al., 2008; Wu and Brown 2006). Both of these hormones induce the ovaries to produce ecdysteroids (Hagedorn et al., 1975 ; Hoffmann et al., 1980 ; Hagedorn, 1985, 1989).

Although considerable attention has been devoted to ecdysone production, the complete biosynthetic pathway of ecdysteroids is still not fully understood. Similar to the steroid hormone biosynthesis pathway in vertebrates, insects also utilize cholesterol and/or plant sterols as precursors of ecdysteroids (Gilbert et al., 2002). However, it should be noted that insects lack enzymes necessary for the sterol biosynthesis pathways that exist in vertebrates. Thus, most ecdysozoa must acquire cholesterol and/or other sterols from their food for steroid hormone biosynthesis (Kircher, 1982). Cholesterol absorbed from food is transported into the hemolymph by a transport protein, lipophorin (Soulages and Wells,1994). A set of Halloween genes, which encode cytochrome P450 enzymes, have been identified in *Drosophila melanogaster* and *Aedes aegypti* as essential for ecdysteroid biosynthesis, resulting in the synthesis of ecdysone (Niwa and Niwa, 2014;

Sieglaff et al., 2005). In the last step, ecdysone is converted to an active form 20E by 20-hydroxylase (shade/CYP314a1) (Petryk et al., 2003).

Ecdysteroid-induced transcriptional responses that underlie cellular changes leading to molting, metamorphosis, and reproductive maturation are mediated by a heterodimer comprised of the ecdysone receptor (EcR; Koelle et al., 1991) and ultraspiracle (USP). This heterodimer regulates expression of genes by binding to specific promoter sequences called ecdysone response elements (EcREs). Binding of 20E activates the ecdysone receptor, which then binds to EcREs in a number of ecdysone responsive genes, thereby activating transcription of these genes. Genes encoding ecdysis triggering hormone and its receptor are regulated by ecdysone, as demonstrated in larval stage of moths, mosquitoes and adult fruit flies. With initiation of molting, ecdysone levels rise immediately, causing a sharp increase of ETH levels in Inka cells. Injection of 20E or the steroid analog tebufenozide (RH5992) also induces expression of the ETH gene and increased production of peptide hormones and their precursors in Inka cells of larval *Manduca* (Zitnanova et al., 2001). Ecdysone response elements (EcREs) were shown to be present in the promoter of ETH in *Manduca* (Žitňan et al., 1999), *Drosophila* (Park et al., 1999) and *Anopheles* (Zitnan et al., 2003). For *Aedes*, the EcRE (AGCACAtgcaCGAGCT) with four nucleotides separating two imperfect inverted repeats, was identified 300 bps upstream of the open reading frame (ORF) (Dai and Adams, 2009). This evidence suggests that ecdysone regulates ETH gene expression by acting on the ecdysone response element (EcRE) in the promoter region of ETH gene. Ecdysis triggering hormone receptor expression is also regulated by ecdysteroid. Using

qPCR, it was demonstrated that elevation of ecdysone levels induces expression of the ETH receptor in pharate fifth instar larvae of *Manduca* (Kim et al., 2006a). Furthermore, exposure of fifth instar larvae to 20E right after ecdysis induces expression of ETHR-A (Cho and Adams, unpublished). In *Aedes*, elevation of total ETHR transcript number coincides with increased levels of ecdysone, as does behavioral sensitivity to AeaETH injection (Dai and Adams, 2009). Ecdysteroid regulation of ETH and ETHR expression has also been described in the adult stage of fruit flies. 20E injection in adult fruit fly led to significant elevation of ETH precursor and ETHR transcripts expression (Meiselman et al., 2017).

In this study, I demonstrate that ETH and ETHR gene expression is also regulated by ecdysteroids during the adult stage of *Aedes agypti*, especially following the blood meal. ETH and ETH receptor transcripts peak 24 hr post-blood meal, which follows closely the ecdysone peak 18 hr post-blood meal. 20E injection of day 4 adult females leads to significant increases in ETH and ETHR gene expression. Silencing of genes encoding cytochrome P450 ecdysone-synthesis enzymes reduces ecdysone levels post-blood meal, which in turn causes a steep decline of ETH and ETHR transcript levels; this can be rescued by 20E injection. In particular, regulation of ETH and ETH receptor genes by ecdysone provides an essential link to JH signaling, which is essential for reproductive success of female mosquitoes.

Materials and Methods

Mosquito Rearing

The *A. aegypti* wild-type UGAL/Rockfeller strain was reared under laboratory conditions at 28 °C and 70% relative humidity with unlimited access to 10% sugar and water. Three to four-day old female were blood-fed on White Leghorn chickens.

Ecdysteroid Extraction and Enzyme Immunoassay (EIA)

10 mosquitoes were placed into a 1.5 mL Eppendorf tube containing 250 µL methanol and homogenized in methanol (MeOH). Following centrifugation at 16,000 g, the supernatant was transferred to a new tube and dried in a Speed-Vac for 3 h. The MeOH extract was re-suspended in EIA buffer. 20E was reacted with aminooxyacetic acid (AOA), and the derivative was purified as described previously (Kingan, 1989). AOA-20E was coupled to horseradish peroxidase (HRP) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Kingan, 1989), and the conjugate was purified by size-exclusion high-performance liquid chromatography (HPLC) as described by Kingan et al. (1997b). The 20E antiserum (Kingan, 1989) was diluted 1:750 000, and 20E-HRP was diluted 1: 30,000 for use in the EIA.

20E injection

Posteclosion day 4 adult female mosquitoes were injected with 500 pg of 20E in mosquito saline or saline alone. After 1 h, whole bodies were collected. Three biological replicates were performed for each sample, each of which included 10 injected females as technical replicates.

Reverse transcriptase PCR (RT-PCR) and Quantitative RT-PCR (qPCR)

Whole bodies were homogenized and mRNA was extracted using the TRIzol method (Invitrogen) and treated with DNase I (Invitrogen) according to the manufacturer's protocol. For mRNA, cDNAs were synthesized from 1µg total RNA ProtoScript II RT Kit (New England Biolabs) and was pre-amplified using the SsoAdvanced PreAmp Supermix Kit (Bio-Rad) for unbiased, target-specific pre-amplification of cDNA. ETHRs and ETH qRT-PCR was performed using SYBR green (cat # 170-8882 Bio-Rad) and by Bio-Rad CFX96 Real Time PCR Detection System. Primers for expression analysis are provided below. The specificity of each primer set was validated by the construction of a melting curve. S7 ribosomal protein (RPS7) expression was determined as housekeeping gene.

RPS7 For: CCCGGAGCCCTACCTATAAACTAT

RPS7 Rev: GCAGCACAAAGATGATTTATGCAC

ETH For: ATTCATGCGTCCCGCAAAAC

ETH Rev: CATTCTGTTCGTCCCTATCG

ETHR common For: AGGTGTGGGTACTGGGAGAA

ETHR common Rev: CACCGTCAGCTCCACGAA

Mosquito injection and RNA Interference

Double-stranded RNA (dsRNA) was produced as described previously (Roy et al., 2007). In brief, dsRNA was synthesized using the MEGAscript kit (Ambion). Two sets of dsRNA were designed for CYP302A1 and two sets for CYP314. 1 ug (0.23 ul of 4.35 ug/ul) of dsRNA was microinjected into the thorax. RNAi-treated mosquitoes were

maintained on 10% sucrose solution for 4 d and then given a blood meal. Animals were collected 18 hr post blood meal. Transcript abundance was analyzed by means of q-PCR and RT-PCR analysis. RNAi depletion of luciferase (iLuc) served as a control.

dsLuc Forward: CGCCAGGGTTTTCCCAGTCACGAC

dsLuc Reverse: TAATACGACTCACTATAGGGGACACTATAGAATACT

ds302A1.1 Forward: TAATACGACTCACTATAGGGTTCACTGTCCTCCCGACTGA

ds302A1.1 Reverse: TAATACGACTCACTATAGGGTTCCCATGGATCCGGAAGGA

ds302A1.2 Forward: TAATACGACTCACTATAGGGACTGTCGAGCCGTTCTGAAG

ds302A1.2 Reverse: TAATACGACTCACTATAGGGGGCTGGTTGATCAGCTTGGT

ds314A1.1Forward: TAATACGACTCACTATAGGGCCCAAAGCCCCGCAAAATAC

ds314A1.1 Reverse: TAATACGACTCACTATAGGGACTCCACTATTTCCGTCGGC

ds314A1.2 Forward: TAATACGACTCACTATAGGGGCAAGTTACTGCAAGCATTC

ds314A1.2 Reverse: TAATACGACTCACTATAGGGCTTACCGTTTCAATGGCAGC

20-hydroxyecdysone (20E) rescue

Adult female mosquitoes (1 day before blood feeding) were injected with 1 ug dsRNA 302A1.1 and dsLuc. Animals pre-injected with dsRNA 302A1.1 were injected with 500 pg of 20E in 220.8 nL saline or saline alone 18-hr after blood feeding. dsLuc-treated animals were injected with 220.8 nL saline. Mosquitoes were collected 1 hour after all treatments and subjected to RT-PCR and qPCR. RNA extraction, RT-PCR and qPCR procedures were described above.

Results

ETH and ETHRs transcript levels are up-regulated by ecdysteroids.

During juvenile stages, ETH and ETHR gene expression is induced by ecdysteroids (Dai & Adams, 2009; Shlyueva et al., 2014; Žitňan et al., 2007). The goal of this study was to obtain gene expression dynamics of ETH, ETHR and to elucidate the regulatory function of ecdysteroids in governing these gene expression patterns following the blood meal in adult female mosquitoes. To produce a time course expression analysis of ETHRs and ETH in the whole female mosquitoes, I measured mature ETH, ETHR-A and ETHR-B expression levels by quantitative real time PCR (qPCR) analysis. By comparing with just post-pupation animals, I found that expression of mature ETH, ETHR-A and ETHR-B declined post-eclosion; however, expression levels substantially increased following the blood meal (PBF) (Fig. 3.1). I monitored mature ETH, ETHR-A and ETHR-B abundance using nine time points collected over the first reproductive cycle by obtaining total RNA samples from females at 24, 48 and 72 h post eclosion (PE), and 12, 18, 24, 30, 36, 48 h post-blood feeding (PBF). Transcripts for ETH, ETHR-A and ETHR-B were significantly up-regulated post blood meal, reaching a peak by 24 h PBM and declining by 48 h PBM. In order to correlate its correspondence with ETH, ETHRs transcript levels, ecdysteroid levels were determined by EIA (Enzyme Immunoassay). Ecdysteroid levels peak at 18 h post blood meal, followed by ETH, ETHR-A and ETHR-B peaks. These results suggest that the high expression of ETH, ETHR-A and ETHR-B in the female mosquito may be regulated by ecdysteroids and play an important role in tuning events associated with the adult life stage of the female mosquito.

To confirm the function of ecdysteroids in regulating of ETH signaling, I also investigated expression levels of ETH, ETHR-A and ETHR-B following 20E injection into female mosquitoes 4-day post eclosion. As a control, 0.23 ul of mosquito saline was injected. Animals were collected one hour post injection for RNA extraction and cDNA synthesis. Similar to blood-fed mosquitoes above, ETH, ETHR common region, ETHR-A and ETHR-B in the day 4 females increased significantly after injection of 20E, compared with saline injection (Fig 3.2). From these results, it can be concluded that expression levels of ETH and ETHRs are up-regulated by ecdysteroids.

ETH signaling is interrupted by silencing cytochrome P450 enzymes.

In order to silence cytochrome P450 enzymes, two enzymes were chosen for dsRNA synthesis. One is CYP302a1 (disembodied), which catalyzes the conversion of ketotriol to 2-deoxyecdysone. The other is CYP314a1, which catalyzes conversion of ecdysone to 20-hydroxyecdysone. Two dsRNA constructs were designed for each gene. To verify RNAi efficacies of different fragments of CYP302a1 and CYP314a1 sequences, qPCR analyses were performed at 42 h after dsRNA injection (Fig 3.3). All expression levels of target transcripts were normalized using ribosomal protein S7 (RPS7) as a housekeeping gene. The results showed that expression levels of CYP302a1 mRNA decrease dramatically in females injected with ds302A1.1 and ds302A1.2, especially with dsCYP302A1.1, which decreased around 60% compared with the control. CYP314a1 level was significantly reduced following dsCYPA314A1.1 injection. However, CYP314a1 transcript levels did not change significantly following injection with dsCYP314A1.2. In order to test the influence of P450 enzyme silencing on ecdysteroid

levels, I measured levels of E and 20E in dsCYP302A1.1, dsCYP302A1.2 and dsCYP 314A1.1 silenced animals. I found that ecdysteroid levels in dsCYP 302A1.1 and dsCYP302A1.2 injected animals were indeed decreased compared with dsLuc controls. However, no difference of ecdysteroid level was detected in dsCYP314A1.1 injected animals, which may be caused by the equal affinity of the ecdysteroid antibody for ecdysone(E) and 20E (Fig 3.4). In this case, dsCYP 302A1.1 was used for P450 silencing in the following experiment.

Results of CYP RNAi after dsRNA injection indicate that significant reduction in ETH and ETHR transcript levels occurs in day 4 adult females injected with dsCYP302A1.1 (1 μg) at 24 h post injection, and transcript levels of ETHR and ETH decreased by 49 and 62%, respectively, compared with controls (Fig 3.5). These results indicate that synthesis of ETH and ETHR transcript levels is dependent on ecdysone signaling.

To further evaluate ETH and ETHR as the authentic target gene of ecdysone signaling, I conducted rescue experiments by injecting 20E into dsCYP3021.1 treated mosquitoes. Treatment of 20E was expected to alleviate the adverse phenotypes caused by CYP 302a1 silencing, which resulted in decreasing of transcript level of ETH and ETHR. I injected 1 μg of dsCYP301A1.1 1 day before blood meal and then injected 500 pg of 20E into *A. aegypti* mosquitoes at 18 hr post blood meal. ETHR levels in these 20E treated mosquitoes were rescued compared with dsLuc treated and untreated WT mosquitoes. However, ETHR transcript levels of saline-treated animals was still significantly lower than that in dsLuc treated and untreated WT mosquitoes (Fig 3.6A). Similarly, ETH transcript level was partially rescued by 20E treatment but not saline treatment (Fig 3.6B).

According to all data presented above, I demonstrated that genes encoding ETH and ETHRs are targeted by the ecdysteroid pathway, constituting an important step in the reproductive physiology of female mosquitoes.

Discussion

In this study, I show that ecdysteroids play a critical role in regulating the production of ETH and ETHR. Following either the natural elevation of ecdysteroids post blood meal or injection of 20E, expression of ETH and ETHRs genes is strongly induced. Evidence for ecdysteroid regulation of ETH signaling is as follows (i). ETH, ETHR-A and ETHR-B transcript levels increase following the ecdysone peak post blood meal; (ii). 20E injection into day 4 adult females induces ETH and ETHRs synthesis. (iii). Silencing of cytochrome P450 ecdysone synthesis enzymes interrupts production of ETH and ETHRs; this can be rescued by 20E injection. This study defines a significant function for ecdysteroids in regulation of ETH signaling molecules.

Ecdysone mode of action was proposed by Michael Ashburner, which explains the ecdysteroid pathway downstream genes expression (Ashburner et al.,1976; Richards 1997; Thummel, 1996). 20E binding to its receptor actives early gene expression, but represses late gene expression. Proteins produced by early genes repress their own expression and activate late gene expression. The transition from feeding to pharate stage during each stage in insect is initiated by a rise of ecdysteroid levels. Early events include increased production of ETH in Inka cells and onset of CNS sensitivity to ETH.

Previous studies demonstrated that ecdysteroids induce expression of the ecdysone receptor EcR-B1 in Inka cells and that this induces expression of the ETH gene by

interaction with the EcREs that have been identified only in the promoter regions of the *Drosophila, Manduca, Aedes* ETH genes (Park et al., 1999; Zitnan et al., 1999, Li and Adams., 2009) and in the oxytocin gene (Richard and Zingg, 1990; Mohr and Schmitz, 1991). In fifth instar *Manduca* larvae, after each of two successive ecdysone pulses, there is a sharp increase in the rate of PETH and ETH synthesis. Consistent with this, injection of 20E or the steroid analog tebufenozide (RH5992) induced expression of the ETH gene and increased production of peptide hormones and their precursors in Inka cells (Zitnanova et al., 2001). These results demonstrated that ETH synthesis is regulated by ecdysteroids during juvenile stages of *Manduca*. Meanwhile, ecdysteroids also regulate production of ETHRs in *Manduca*. Animals only become sensitive and show specific behavioral responses to PETH and ETH approximately 1–2 days prior to ecdysis, which is the peak of ecdysteroids (Zitnan et al., 1999; Zitnanova et al., 2001). This has been confirmed by injection of 20E, or the ecdysteroid agonist (tebufenozide, RH-5992), which induced ETH sensitivity in freshly ecdysed *Manduca* (Zitnanova et al., 2001).

My study has revealed the regulatory role of ecdysteroid signaling in the expression of ETH and ETHR in the adult mosquitoes. Transcript levels of the ETH and ETHRs, which are early-mid genes started increasing by 12 h post blood meal, reached their maximum 24 h, and then declined drastically to basal levels around 36 h, staying low thereafter. This ecdysteroid-induced increase of ETH and ETHR can be confirmed by 20E injection, which causes a sharp increase of ETH and ETHR transcripts 1 h post injection. Remarkably, I showed for the first time that ecdysone is responsible for ETH and ETHR activation in adult mosquitoes, which is confirmed by dsRNA silencing targeting P450

enzymes essential for ecdysone synthesis. Reduction of ETH and ETHR levels caused by P450 enzyme silencing can be rescued by 20E injection. CYP302a1 and CYP 314a1 were chosen as targets for the following reasons: Transcript abundance of CYP302a1 was greatest in ovaries at 6, 18, and 30h PBM coinciding with the onset and peak in ecdysteroid production (Douglas H et al., 2005), and indicates that this enzyme likely has an important role, as described for *D. melanogaster* CYP302a1 (Chavez et al., 2000; Warren et al., 2002). CYP314A1 is responsible for the final hydroxylation step of ecdysteroidogenesis, which largely occurs in the target tissue. By silencing CYP302, I demonstrated a significant reduction of ecdysteroid level, as determined by EIA. However, CYP314 silencing has no significant effect, which can be attributed to equal affinity of the ecdysteroid antibody for ecdysone (E) and 20E in EIA. These findings are in agreement with previous studies in larval moth, mosquitoes, desert locust and the adult of *Drosophila* (Lenaerts et al., 2017; Li and Adams, 2009; Meiselman et al., 2017; Zitnan et al., 1999; Zitnan et al., 2007). However, a decline in ecdysteroid levels is required for the release of ETH (Zitnan et al., 1999; Kingan and Adams, 2000). The release of ETH is regulated by low ecdysteroid levels, most likely by βFTZ-F1, whose expression is induced only when the ecdysteroid level drops (Rewitz et al., 2010; Zitnan et al., 2007).

This study has provided valuable insight into regulation of ETH signaling by ecdysteroids in adult mosquitoes. Future work should examine ecdysteroid regulation of ETH release. However, characterization of the roles of 20E pathway on ETH and ETHR synthesis represents an essential step in understanding the ETH signaling during mosquito reproduction.

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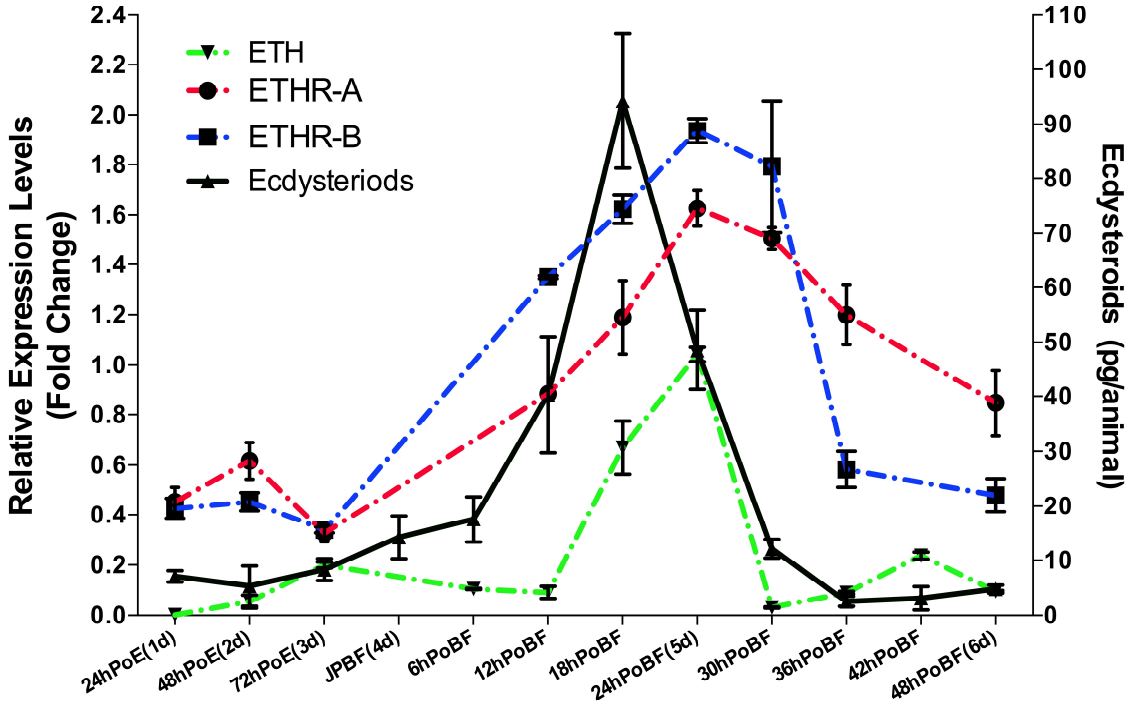

Figure 3.1 The pattern of ETHR and ETH expression follows ecdysteroids levels post-blood feeding

ETHR-A, ETHR-B and ETH transcript levels were determined by q-PCR. Whole body ecdysteroid levels were determined by enzyme immunoassay (EIA). The expression pattern of ETHR follows that of ecdysteroids post blood feeding.

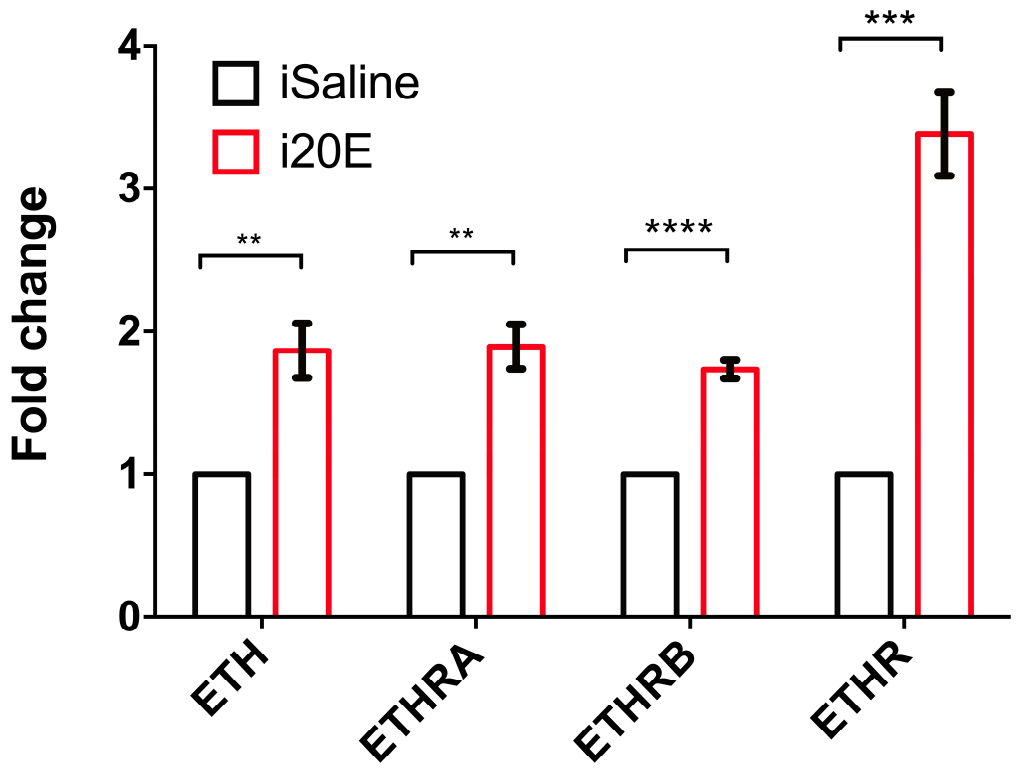

Figure 3.2 ETH and ETHR expression level increase after 20E injection

500 pg of 20-hydroxyecdysone was injected in each adult female mosquito. Animals were collected 1 hr post-injection. ETH, ETHR common region, ETHR-A and ETHR-B expression levels were detected by q-PCR.

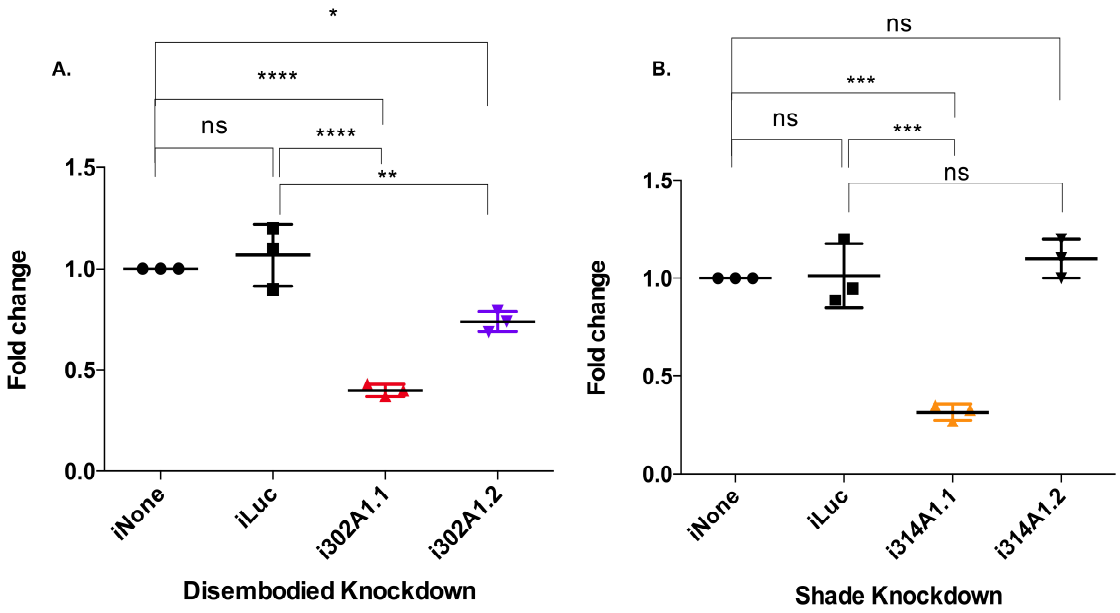

Figure 3.3 Knock down efficiency of 302A1 and 314A1 dsRNA injection

Two sets of dsRNA were designed for CYP302A1 (A) and two sets for CYP314 (B). dsRNAs were injected 1 day before blood feeding. Animals were collected 18 hr post-blood meal. Transcript abundance was analyzed by q-PCR; dsRNA targeting luciferase (iLuc) was used as control.

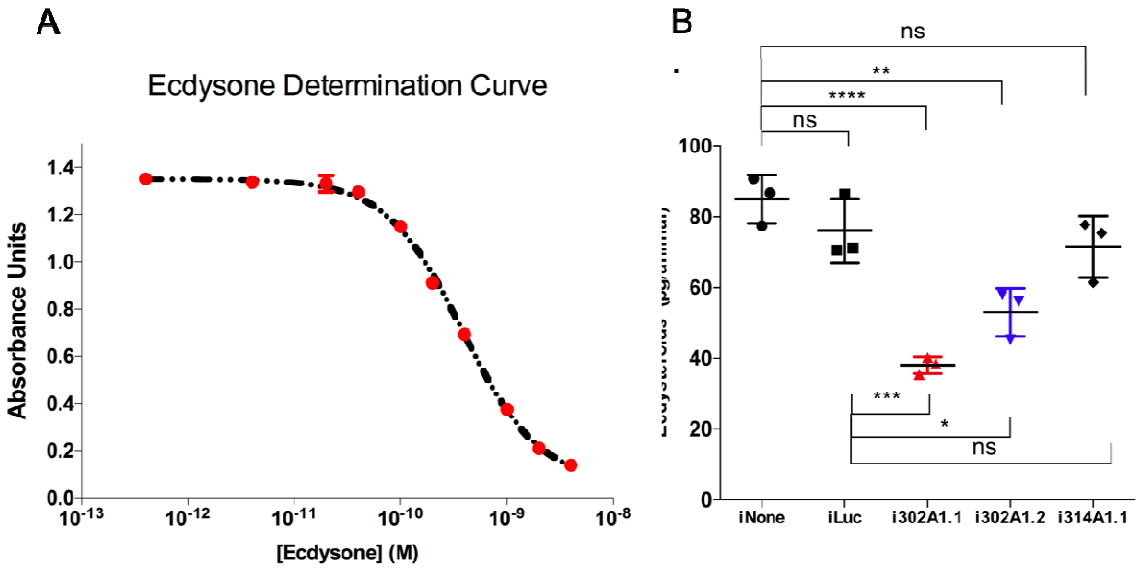

Figure 3.4 Ecdysteroid levels decrease after P450 silencing

dsRNAs were injected 1 day before blood feeding. Animals were collected 18 hr post-blood meal. Ecdysteroid levels were quantified by EIA.

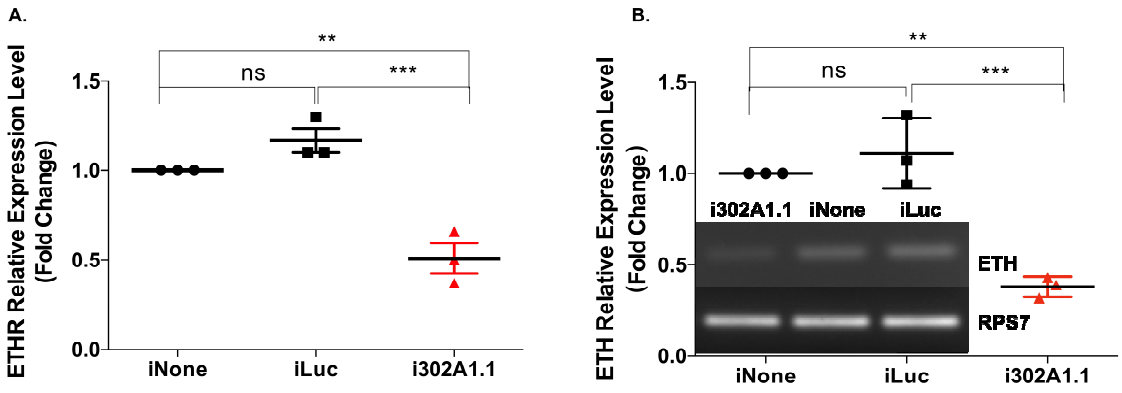

Figure 3.5 ETH and ETHR transcript levels decrease after P450 silencing

dsRNAs were injected 1 day before blood feeding. Animals were collected 24 hr post-blood meal. ETH levels were determined by RT-PCR and qPCR. ETHR common region transcript level was determined by qPCR.

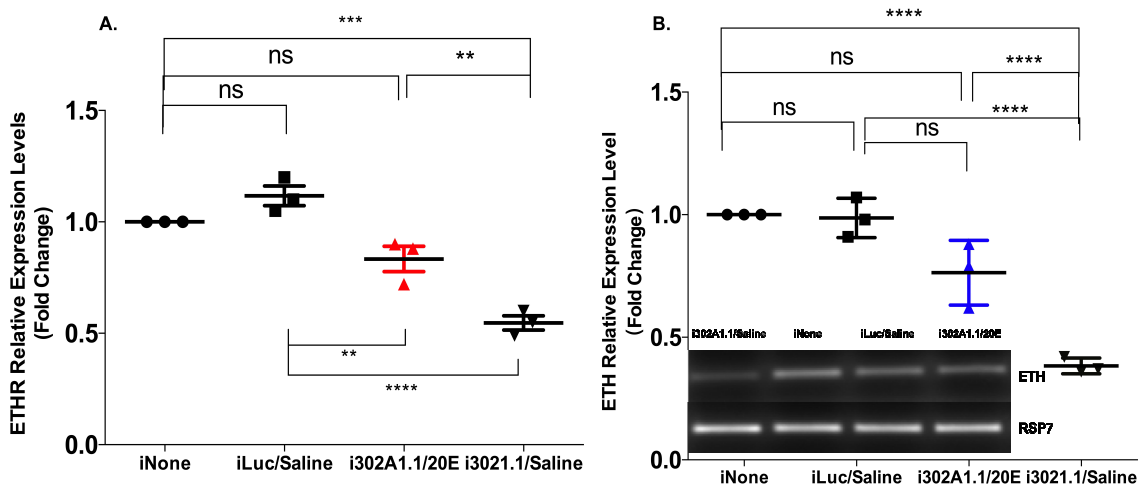

Figure 3.6 ETH and ETHR transcript levels are rescued by 20E

1 µg of 20-hydroxyecdysone was injected into dsRNA treated mosquitoes 18 hour post-blood meal. Animals were collected 1 hour after injection. ETH expression levels were detected by RT-PCR and q-PCR. ETHR levels were determined by q-PCR.

Chapter 4 ETH Signaling Regulates Previtellogenesis Through JH Signaling

Abstract

In *Aedes*, egg development can be divided into two periods: previtellogenesis and vitellogenesis. During the previtellogenesis phase, mosquitoes acquire competence to produce eggs in response to the blood meal. Juvenile hormone (JH) plays an important role during previtellogenesis, by increasing metabolic machinery and protein synthesis capacity. After acquisition of competence, the vitellogenic phase is initiated by the blood meal and consequent elevation of ecdysone levels, leading to yolk protein production. Here I show that ETH is an important allatotropin, promoting the first peak of JH production following eclosion and the second JH peak following the decline of ecdysteroids after the first blood meal. ETH signaling is essential for egg development during previtellogenesis by regulating JH levels following the first blood meal cycle and second blood meal cycle. Reduction of JH by ETHR silencing reduces follicle size and lipid content, which can be rescued by methoprene. Finally, I find that ETHR knockdown causes decreased egg numbers laid by female after the first blood meal and second blood meals; egg viability is not affected. Our findings indicate that the hormonal cross-talk between ecdysone, ETH and JH is essential for reproduction in female mosquitoes.

Introduction

Precise hormonal regulation of development and reproduction is critical to survival of insects. Tissues are organized and hormones are purposed for roles in a stage-specific way. Previous studies showed that ecdysis triggering hormones (ETHs) and ETH

receptors (ETHR) regulate insect ecdysis behavior by targeting central peptidergic neurons (Kim et al., 2015; Kim et al., 2006; Park et al., 2002b; Park et al., 2003; Park et al., 1999; Zitnan et al., 1996; Zitnan et al., 2003). However, ETH and ETHR transcripts and Inka cells persist into adulthood, where has no ecdysis occurs, suggesting possible reproductive functions of ETH signaling (Catalan et al., 2012; Graveley et al., 2011; Park et al., 2002a).

Presence of ETHR transcripts in the corpora allata of the silkworm*, Bombyx mori* was the first reported occurrence of ETHR expression outside the CNS, which indicated a possible role of ETH in regulation of JH production (Yamanaka et al., 2008). JH is a sesquiterpenoid hormone with well-known morphogenetic and gonadotropic functions. Release of JH from the corpora allata maintains the juvenile body plan, which has been extensively studied in a broad range of insect. During adulthood, JH is re-purposed as a gonadotropic hormone, coordinating vitellogenesis, ovary maturation, pheromone synthesis of females, and mating behaviors of both males and females (Argue et al., 2013; Bilen et al., 2013; Lin et al., 2016; Ringo et al., 1991; Sroka and Gilbert, 1974; Teal et al., 2016; Wilson et al., 2003).

According to evidence described above, ETH functions as an obligatory allatoropin to promote JH production in *Drosophila* (Meiselman et al., 2017). ETHR silencing in the corpora allata (CA) and ETH deficits result in marked JH deficiency in both male and female *Drosophila*, resulting in reduction of ovary size, egg production and yolk deposition in mature oocytes (Meiselman et al., 2017; Lee et al., 2017).

Precisely timed hormonal signaling is important for basic understanding of cyclic physiological processes and enhances our efforts to develop and implement strategies for human health and disease vector control. In *Aedes,* JH acts through its receptor, Methoprene-tolerant (Met) during previtellogenesis from eclosion to pre-blood meal to regulate genes involved in preparation and acquisition of competence for vitellogenesis (Zou Z et al., 2013). In this study, I hypothesized that ETH signaling regulates the previtellogenesis phase of mosquito reproduction by acting as an allatotropin, leading to JH production during first JH peak postelosion as well as the second JH peak following the first blood meal. I also investigated physiological responses of JH deficient mosquitoes by silencing ETHR. Interruption of ETH signaling reduces follicle size, lipid content and egg numbers by creating JH deficiency. I confirmed that persistence of ETH signaling throughout adulthood functions by regulating reproductive physiology of female mosquitoes through maintenance of JH levels.

Materials and Methods

Mosquito Rearing

The *A. aegypti* wild-type UGAL/Rockfeller strain was reared under laboratory conditions at 27 °C and 80% relative humidity with unlimited access to 10% sugar and water. Three to four-day old females were blood-fed on White Leghorn chickens.

Mosquito injection and RNA Interference

dsRNA was synthesized using the MEGAscript kit (Ambion). dsRNA was designed to target regions of the ETHR sequence common to ETHR-A and ETHR-B. dsRNA was microinjected into the thorax. RNAi-treated mosquitoes were maintained on a 10%

sucrose solution. Transcript abundance was analyzed by means of q-PCR analysis. RNAi depletion of luciferase (iLuc) served as a control.

Hemolymph Extraction

Hemolymph of adult mosquitoes was obtained by perfusion (Hernandez et al., 1999). Fine needles were made from 100-µl micro-glass capillary tubes using a pipette puller P-30 (Sutter Instrument, Novato, CA) and mounted in a pipette pump (Drummond, Broomall, PA). Needles were inserted manually through the thoracic intersegmentary membrane into the thoracic cavity, and insects were perfused with 20 µl of a "bleeding solution" of phosphate-buffered saline (PBS) (100 mM NaCl, 25 mM NaHCO3, pH 7.2). The hemolymph was obtained from a small tear made laterally on the intersegmentary membrane of the last abdominal segment. The first drop of perfused hemolymph was collected directly on a glass silanized tube (Thermo Scientific) placed on ice. For each data point, three independent samples of hemolymph were collected from pools of five insects each.

Determination of JH levels

Certified standard solutions for JH III and its deuterated analog (JH III-D3) were obtained from Toronto Research Chemicals (Toronto, Canada). After hemolymph extraction, 10 µL of 6.25 ppb of JH III-D3 in acetonitrile were added to each sample, followed by 600 µL of hexane. Samples were vortexed for 1 minute, and spun for 5 minutes at 4ºC and 2000 g. The organic phase was transferred to a new silanized vial, dried under nitrogen flow, and stored at -20ºC. Dried extracts were re-suspended in 50 µl of acetonitrile, vortexed 1 minute, transferred to a new silanized vial with a fused 250 µL

insert. JH quantifications by high performance liquid chromatography coupled to electrospray tandem mass spectrometry (HPLC-MS/MS) were done as previously described by Ramirez et al. (2016) Briefly, we employed a HPLC-MS/MS workflow based on multiple reaction monitoring (MRM) using the two most abundant fragmentation transitions: 267->235 (primary) and 267->147 (secondary). In order to accurately quantify the amount of JH III present in the hemolymph, the heavy deuterated JH III analog (JH III-D3) was utilized as an internal standard to normalize recoveries during the sample preparation, extraction and analysis steps. An extraction recovery of near 55% was routinely observed regardless of the analyte concentration (Ramirez et al, 2016).

Follicle size measurement

Each female has paired, symmetrically arranged ovaries. Each ovary consists of variable numbers of tubular epithelial ovarioles, which contain a germarium and a maturing, presumptive follicle. Each follicle includes groups of nurse cells and one oocyte. The follicle size was measured using a Zeiss LSM510 confocal microscope. RNAi depletion of luciferase (iLuc) served as a control.

Methoprene rescue

For rescue of JH deficiency phenotypes, (S)-methoprene was applied topically in acetone to the ventral side of the thorax in females following cold anesthesia with a Nanoject II (Drummond) applicator. Vehicle treatment was performed with acetone only.

Ovarian lipid staining

Ovaries from mosquitoes were dissected in APS before fixation in 4% paraformaldehyde (PFA) for 1 hour. The PFA was replaced with Oil red-O working solution (100 μL) for 1 hr. Oil red-O specifically stains triglycerides and cholesteryl oleate (Ramirez-Zacarias et al., 1992). Ovaries were removed from the stain and rinsed thoroughly in PBS before being placed under a coverslip and photographed.

Determination of TAG Levels

Ovary lipids were detected using a Triglyceride Colorimetric Assay (Cayman). In this kit, triglycerides are hydrolyzed into glycerol and free fatty acids. Glycerol is then oxidized to generate a product which reacts with a colorimetric probe. The absorbance of the colorimetric probe can then be read at OD 530-550 nm. For each experimental treatment, 20 pairs of ovaries were dissected in phosphate buffered saline (PBS). Ovaries were thoroughly cleaned of any contaminating fat body tissue and placed in 50 μL of NP40 substitute assay reagent, then homogenized and centrifuge at 10,000xg for 10 minutes at 4^{o}C. Supernatants (10 μL) were incubated with Enzyme Mixture Solution (Cayman) and absorbance was measured at 530-550 nm with SpectraMax 190 Absorbance plate reader.

Results

ETH is an obligatory regulator of JH levels in female Aedes aegypti during the first and second reproductive cycles.

In order to assess functions for ETH signaling in regulation of JH synthesis in the adult female mosquito, I employed dsRNA knockdown of the ETHR targeting the common region of ETHR-A and ETHR-B. dsRNA targeting luciferase was designed as a control. I

interrupted ETH signaling during the first and second reproductive cycle separately: 1) an RNAi construct targeting the ETHR common region was microinjected 2 hr post pupation; this treatment was intended to influence the JH peak during the first reproductive cycle and 2) the same RNAi construct was microinjected into Day 3 old females with the objective of influencing the JH peak during second reproductive cycle. To evaluate efficiency of dsRNA injection, I monitored ETHR levels by qPCR at ~40 hr post injection for the first cycle and ~48 hr post injection for the second cycle. Injection of dsRNA at doses of 0.5, 0.7, and 1 μg during the pupal stage caused significant reduction (~40%, ~50% and ~60%, respectively) of ETHR transcript number compared with the luciferase control (iLuc) (Fig 4.1). For adult injections, treatment with 1 and 1.2 μg caused ~25% and ~45% reduction of ETHR transcript level, respectively (Fig 4.8). The mortality associated with pupal injection was ~20% and for adult, it was ~1% (Fig 4.15).

A recent report (Areiza et al., 2014) showed that ETHR knockdown during the pupal stage results in a 56.9% reduction of endogenous JH III synthesis compared with controls. To investigate further the role of ETH signaling in JH synthesis, I show that adult ETHR dsRNA injection causes 49% reduction of JH III level in the hemolymph compared with dsLuc injection animals at 48 h posteclosion during the first reproductive cycle (Fig 4.2). Similarly, dsETHR injection causes 30% reduction of JH III level compared with dsLuc injection post oviposition during the second reproductive cycle (Fig 4.9). These data demonstrate that ETH signaling is essential for normal JH synthesis during both the first and second reproductive cycles. ETH therefore plays an obligatory role as an allatotropin

in adult female *Aedes*. This reduction has significant consequences for female reproduction.

Disruption of ETH signaling impairs the development of follicle during the first reproduction cycle and second reproduction cycle.

Follicles develop during the previtellogenesis phase, which regulated by JH (Zou Z et al., 2013). In order to determine whether ETH signaling influences development of follicles, I evaluated the state of ovarian development in mosquitoes during the first and second previtellogenesis phases. Each female has one pair of ovaries, each composed of ~75 ovarioles. Each ovariole contains a primary follicle, an undifferentiated secondary follicle, a germarium, and a mass of germ and somatic stem cells. During the first gonadotrophic cycle, only primary follicles develop into mature eggs, while secondary follicles grow during the second cycle (Clements, 2000). Within the primary follicle lies a single oocyte and a cluster of several nurse cells providing nourishment for the oocytes during the early stages of their growth (Bryant B et al., 2000). Ovaries of wild type UGAL mosquitoes were dissected at 24, 48, 72 hr post eclosion for follicle size measurement. The follicle is ~60 μm 24 hr post eclosion, ~90 μm 48 hr post eclosion and ~110 μm 72 hr post eclosion (Fig 4.3.B).

For the first reproductive cycle, dsRNA was injected 2 hr post pupation and follicles were removed 72 hr post eclosion. Following injection of 0.5 μg or 0.7 μg dsRNA, follicle size was not significantly different compared with control. However, ovarian follicle growth was drastically inhibited following 1 μg dsETHR injection; average primary follicle length was reduced to ~75 μm compared to controls measuring. The

degree to which females treated with the dsRNA displayed this phenotype varied considerably across individuals. Ovaries from dsLuc females were similar to those in non-injected female mosquitoes, with primary follicles reaching 95 – 100 μm in length on average (Fig 4.3.B). During the second reproductive cycle, 1.2 μg ETHR dsRNA was injected 1 day before the blood meal. Females laid eggs 3 days post blood meal and follicles were removed 72 hr post oviposition. Similar to the first reproductive cycle, dsETHR injected females during second cycle displayed significantly reduced ovarian development. Follicle size from the dsLuc injected females was similar to that of wild type female mosquitoes at 72 hr post first oviposition with primary follicles reaching 94.96 μm in length on average (Fig. 4.10). Ovarian follicle growth was considerably reduced in the dsETHR injected animals at 72 hr post first oviposition compared to control mosquitoes, with an average primary follicle size of 81.84 μm in length (Fig 4.10).

Disruption of ETH signaling impairs lipid content in the follicles during the first reproduction cycle and second reproduction cycle.

JH and its receptor play critical roles in controlling lipid metabolism during female mosquito reproduction. Indeed, RNAi of Met resulted in a reversal of the lipid metabolism phenotype in female mosquitoes during previtellogenesis phase, causing dramatic increase of lipid metabolism gene transcripts, β-oxidation enzymes, and metabolites and the decrease in levels of lipid stores and FFAs (Wang X, et al., 2017). In order to evaluate the role of ETH signaling in follicle development, I investigated the apparent differences in ovarian and follicle morphology more thoroughly. A neutral lipid specific stain (Oil red-O) was used to stain the ovaries and visualize accumulation of

lipids in the oocytes. Lipid content of untreated mosquito oocytes or those treated with iLuc dsRNA treatment appear to contain massive lipid content compared to those observed from ETHR-knockdown mosquitoes in both the first (Fig 4.5.A) and second reproductive cycles (Fig 4.12.A). This result has been validated by triglyceride quantification. During the first reproductive cycle, the lipid content of ovaries from ETHR knockdown animals is around 300 pmol/female, which is significantly lower than luciferase dsRNA-treated animals, which is around 500 pmol/female (Fig 4.5.B). For the second reproductive cycle, injection of dsETHR into female mosquitoes led to sustained decrease of lipid content, with an average of 582.4 pmol/female compared with dsLuc controls with an average of 786.3 pmol/female (Fig 4.12.B). This result suggests that ovarian lipids may be an important energy source for transcriptional, translational and endocytotic activities during vitellogenesis, which is integral to the coordination of fecundity of mosquitoes.

ETH regulates development and lipid content of follicles through downstream JH signaling.

After showing that ETH signaling is necessary for regulating the size and lipid content in follicles, I next tested the hypothesis ETH-knockdown phenotypes occur through creation of JH deficiency. I conducted rescue experiments through JH analog methoprene treatment of dsRNA injected female mosquitoes. This approach has been successfully used in rescuing ETH knockdown phenotypes in *Drosophila* (Meiselman et al., 2017; Lee et al., 2017). It was expected that the JH analog treatment would alleviate the adverse

phenotypes caused by ETHR silencing if ETH signaling influence follicle development through downstream JH signaling.

First, I attempted to determine the maximum dose of methoprene that causes no effect on pre-vitellogenesis on wild type females. During the first reproductive cycle, I found that the follicle size of methoprene-treated females (107 fmol/female) is not significantly altered compared to that of acetone-treated animals (Fig 4.4.A). Using the same method, I found that the comparable dose during the second reproductive cycle is 1067 fmol/female (Fig 4.11.A).

Topical application of methoprene (107 fmol) immediately after eclosion rescued follicle size reduction in dsETHR knockdown animals. Inhibition of ovary development and lipid deficient phenotypes were not observed in dsETHR/methoprene treated mosquitoes at 72h post eclosion, while females treated with the dsETHR and acetone control displayed reduced ovary development and reduced egg deposition characteristic of dsETHR-treated mosquitoes. The follicle size of dsETHR and acetone treated animals was ~19% smaller compared to rescued animals (Fig 4.4 B). Additionally, ovary development phenotypes were not observed in the dsLuc/methoprene-treated and dsLuc/acetone control mosquitoes at 72 h post eclosion (Fig. 4.4B).

Meanwhile, methoprene rescue was performed to visualize lipid content by Oil Red. Similarly, methoprene-treated dsETHR treated mosquitoes exhibited partial rescue of lipid deficiency phenotypes observed in dsETHR treated females (Fig 4.5.B). For the second cycle, methoprene treated females (1067 fmol/animal) exhibited recovery of follicle size compared to ETHR-knockdown animals. The follicle size of acetone treated

ETHR silenced animals is ~23% smaller than the rescued animals (Fig 4.11.B). Deficiency of lipid content during second cycle was also rescued by methoprene treatment (Fig 4.12.B). Hence, methoprene dramatically restores proper follicle development and lipid content in dsETHR knockdown females, suggesting that ETHR signaling regulates reproduction through JH signaling.

The fate of follicles after a blood meal is affected by previtellogenic development.

Posteclosion physiological functions for JH have been associated with changes in the fat body that prepare it for vitellogenesis (Flanagan and Hagedorn, 1977; Raikhel and Lea, 1990; Hagedorn, 1994). These changes make the fat body competent to respond to the steroid hormone 20-hydroxyecdysone (20E), leading to synthesis of massive amounts of yolk protein required for egg maturation (Raikhel and Lea, 1983; Dittman et al.,1989). However, functions of JH are not only restricted to the fat body. A study by Clifton and Noriega showed that JH levels determine the fate of individual ovarian follicles in *Aedes* by determining the final egg numbers that are produced in a gonotropic cycle (Clifton and Noriega, 2012). In order to confirm the reproductive phenotype associated with ETHR silenced females, I counted eggs laid by the ETHR silenced animals, dsLuc injected animals and wild type controls. dsETHR-treated females displayed reduce fecundity, laying a significantly lower number of eggs per mosquito as compared to controls, with an average of 60.7 eggs per mosquito (Fig 4.6). dsLuc treated females laid a similar number of eggs as non-injected female mosquitoes, with 91.2 and 96.35 eggs per female on average, respectively (Fig 4.6). On the average, ETHR silencing resulted in a ~34% decrease in egg production compared with dsLuc injection. Next, I checked the viability

of eggs laid by dsRNA-injected animals and found that ETHR silencing does not cause a significant change in viability (Fig 4.7). For the second reproductive cycle, dsRNA was injected one day before the first blood meal, followed by oviposition three days post-first blood meal. The second blood meal was taken three days post-oviposition. Egg numbers following the second blood meal were not significantly different from controls (Fig 4.13.A). Another 1.2 μg dsRNA was delivered via a second injection 12 hr after the blood meal to enhance the silencing phenotype. Double dsETHR-injected females laid a significantly reduced number of eggs per mosquito as compared to controls, with an average of 71.2 eggs laid per mosquito (Fig 4.13.B). Overall, double dsRNA injection caused ~20% difference of egg numbers between dsLuc and dsETHR treatment following the second blood meal (Fig 4.13.B). Hatching rates 3 days post-oviposition, revealed no difference in egg viability between dsETHR-treated and controls (Fig 4.14). Together, these results indicate that ETH signaling in the female mosquito, in large measure, has a crucial influence on fecundity following both the first and second blood meals.

Discussion

Key findings reported in this study are that ETH is an obligatory allatotropin for maintenance of JH levels required for normal previtellogenesis in the female mosquitoes. My findings demonstrate RNAi knockdown of ETHR in female adult mosquitoes results in defects linked to previtellogenic ovarian development and egg production. The basic findings are summarized as follows. First, interruption of ETHR expression leads to a ~49% drop of JH levels during the first reproductive cycle and a ~30% drop during the

second reproductive cycle. Second, JH deficiency resulting from by interference with ETH signaling leads to reducing of lipid content and follicle size for both first and second reproductive cycle. This phenotype is rescuable by treatment with methoprene. Third, the fate of follicles after a blood meal is dependent on JH levels during previtellogenesis phase, which are regulated by ETH. Fourth, even though interruption of ETH signaling results in significantly reduced egg production, viability is not significantly changed. This study defines a significant regulatory function ETH signaling in maintenance of JH synthesis, which is essential for reproductive success in mosquito disease vectors. Future studies taking advantage of the CRISPR knockout technique may reveal that an evenmore profound influence on JH signaling as well as mosquito reproductive physiology.

JH is a major endocrine regulator in insects. Increased levels of JH in early posteclosion female mosquitoes initiates the reproductive process. The rate of JH synthesis depends on the activity of corpora allata (CA), which is controlled by allatoregulatory factors that play allatostatic (inhibitory) or allatotropic (stimulatory) roles. Brain factors exerting such modulatory roles on the CA have been described in mosquitoes (Li et al., 2004). In *A. aegypti*, allatostatin-C (AST-C) and allatotropin (AT) are present in the brain of mosquitoes; both modulate JH synthesis *in vitro* (Li, Y et al., 2003; Li, Y et al., 2006; Nouzova, M et al., 2014). Starvation decreases JH synthesis via reduction in insulin signaling in the CA (Perez-Hedo et al., 2013). In addition, exposure of CA to ETH leads to significant increases in JH methyltransferase activity (JHAMT) and JH synthesis (Areiza et al., 2014). In *Drosophila*, we recently reported on peptidergic regulation of JH synthesis by ETH (Meiselman et al.,2017). Here, I provide evidence that ETH signaling

is critical for JH synthesis not only during the first reproductive cycle, where ETH signaling peaks during eclosion. ETH signaling, regulated by ecdysone, also functions as an obligatory allatotropin for JH synthesis during the second reproductive cycle, which implies the likely role of ETH signaling during all subsequent reproductive cycles.

Primary ovarian follicles of *A. aegypti* are undifferentiated after adult eclosion. During the following 60 hr of previtellogenesis, they reach maximum size. This phase of ovarian development is regulated by JH (Hagedorn, 1994; Klowden, 1997). In newly emerged animals, JH acts on ovaries, fat body and midgut to make them competent for the blood meal and the subsequent vitellogenic phase (Klowden, 1997). During my studies, I also found that JH regulates previtellogenesis in female mosquitoes, by showing that JH-deficiency caused by ETHR knockdown leads to low lipid content and small follicle size. These phenotypes can be rescued by methoprene application. My results confirm the role of JH as a downstream target of ETH signaling. In *Drosophila,* we also showed that RNAi knock down of ETHR by the CA driver JHAMT-GAL4 causes JH deficiency leading to reduced fecundity and decreased ovary size (Meiselman et al., 2017). The function of previtellogenic lipid in the oocytes is unknown, but current evidence in vertebrates suggests that oocyte lipids are essential for energy production (Sturmey and Leese, 2003). Since endocytosis of yolk components and protein synthesis post-bloodmeal are energy-dependent processes (Raikhel and Dhadialla, 1992), it is reasonable to hypothesize that previtellogenic oocyte lipids are essential for energy needed at the beginning of vitellogenesis.

In this study, I found that the egg production of ETHR silenced animals is significantly lower than controls during both first and second reproductive cycles. JH controls yolk accumulation in oocytes of many insects and lack of this hormone is associated with oosorption; i.e. resorption of oocytes (Chapman, 1998). In 2001, Noriega and colleagues reported that reduction of JH levels induces follicle resorption in *Aedes* (Clifton and Noriega, 2011). By resorbing reproductive tissues, insects redirect resources away from reproduction (Osawa, 2005). I therefore hypothesize that JH deficiency caused by ETH signaling contributes to oocyte resorption in mosquitoes as reported recently for *Drosophila* (Meiselman et al., 2017).

In conclusion, my work establishes a fundamental role for ETH in regulating JH productionin female *Aedes aegypti*. My ETHR knockdown experiments have revealed an intriguing role for the ETH signaling pathway in female mosquito reproduction by regulation of the follicle development and lipid content. This investigation has opened a new avenue towards understanding signaling pathways that are important for mosquito reproductive physiology.

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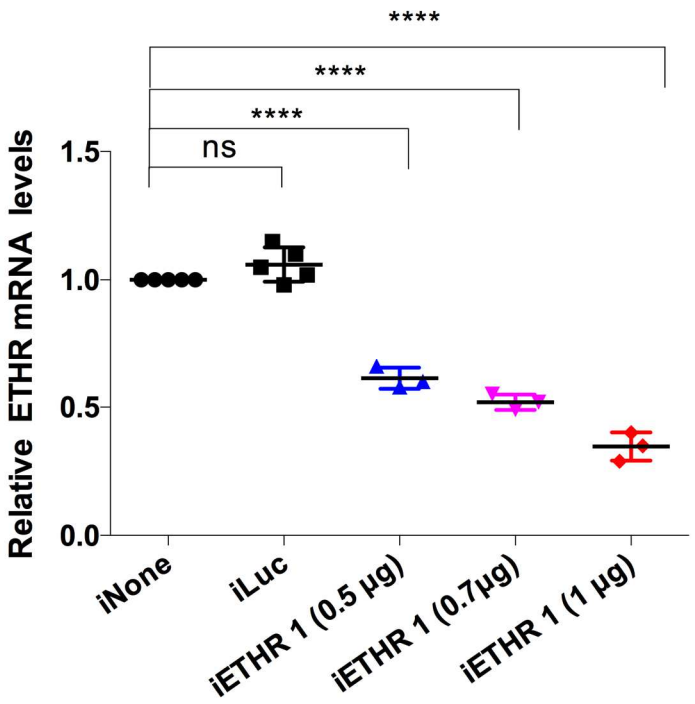

Figure 4.1 ETHR silencing efficiency during the first reproductive cycle

Different doses of dsRNA were injected 2 hr post pupation. Animals were collected 6 hr before eclosion for qPCR.

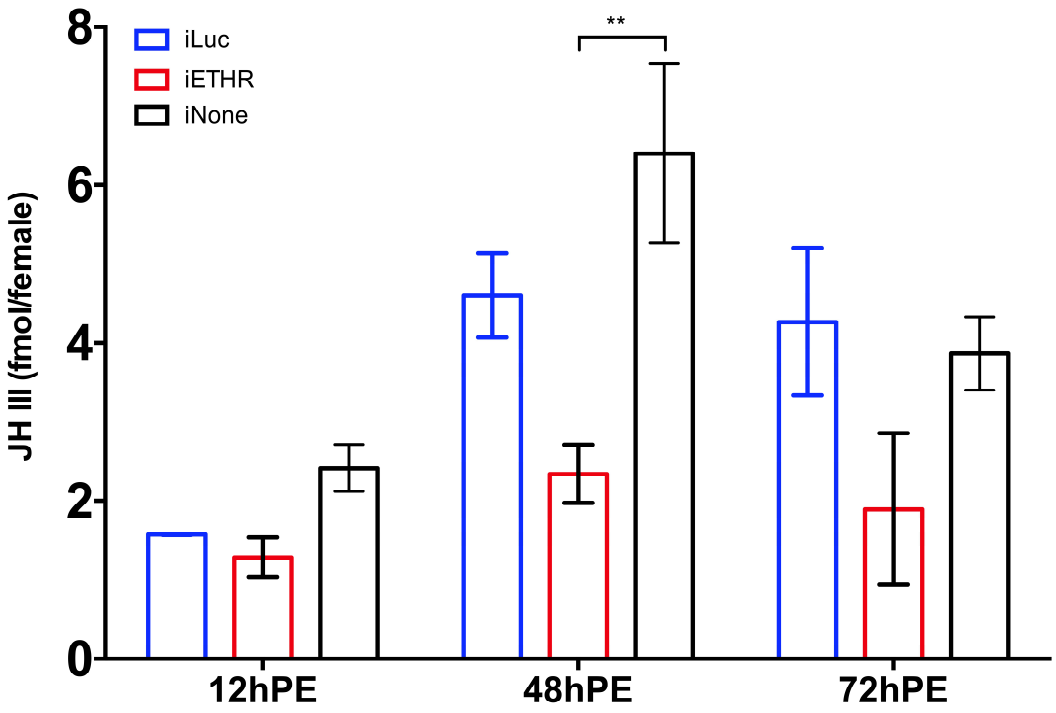

Figure 4.2 First JH peak is reduced after ETHR dsRNA silencing

1 µg of dsRNA was injected 2 hr post pupation and animals emerged ~44 hr after injection. Hemolymph were collected from dsETHR, dsLuc and non-injected post eclosion for JH measurement. 12hPE: 12 hr post eclosion; 48hPE: 48 hr post eclosion; 72hPE: 72hr post eclosion (JH level was determined by Dr. Dr. Noriega Fernando and Dr. Francisco Fernandez-Lima).

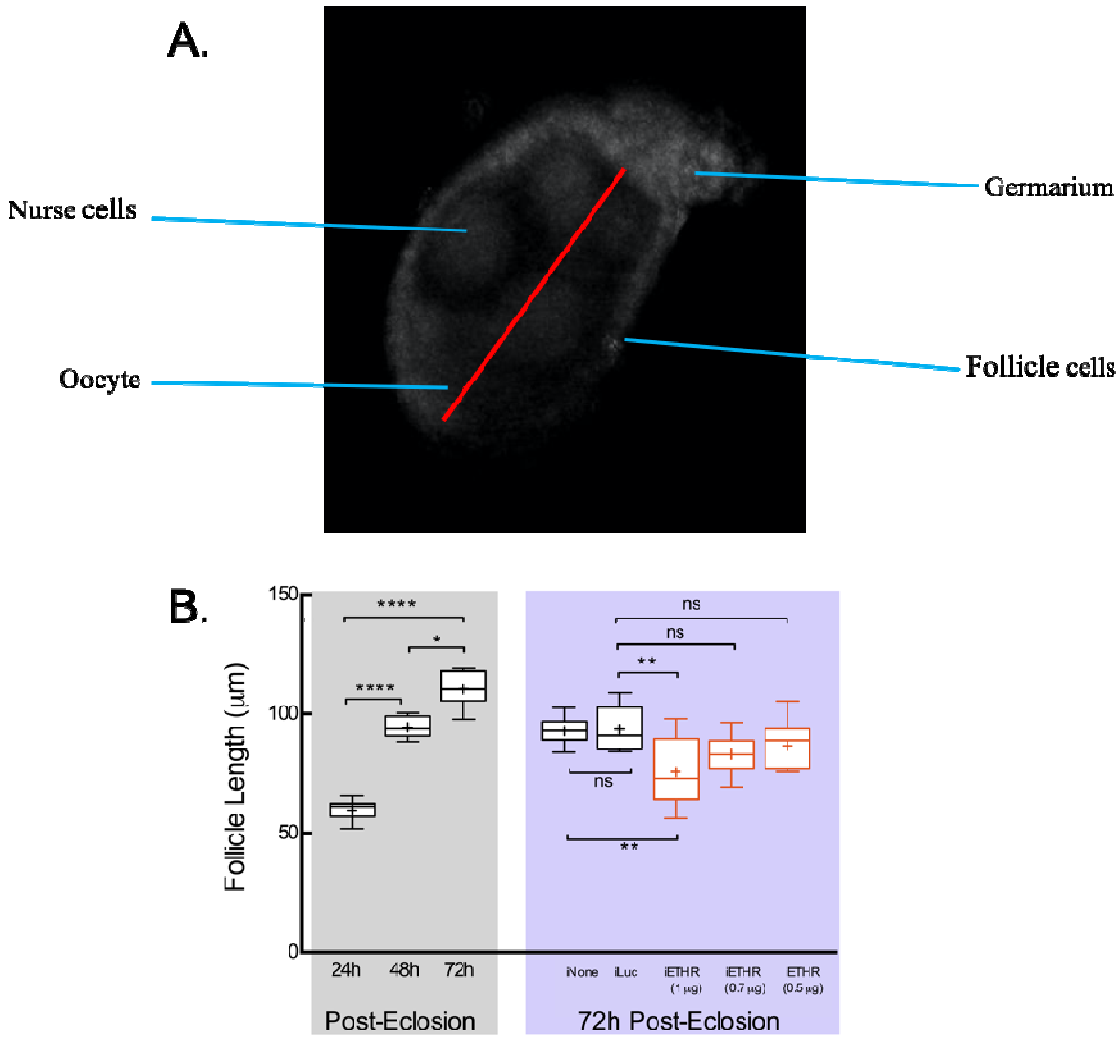

Figure 4.3 ETHR knock down reduces follicle length in the first reproductive cycle

dsRNA was injected 2 hr post pupation and animals emerged ~44 hr after injection. Follicles were collected 72 hr post eclosion for measurement (B). Follicle size was measured as indicated by the red line with Zeiss LSM510 confocal microscope (A).

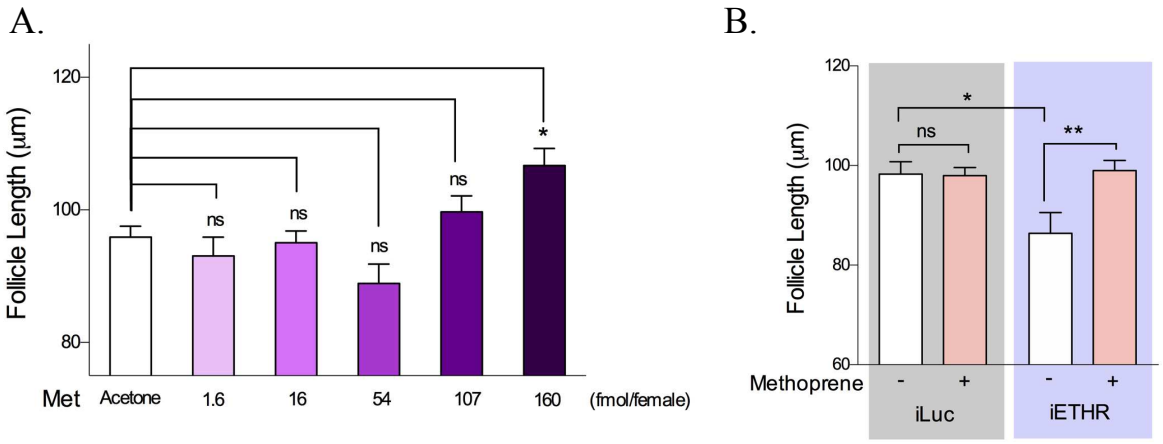

Figure 4.4 Follicle size is partially rescued by methoprene treatment

Different doses of methoprene were topically applied on the dorsal thorax after eclosion. Follicles were collected 72 hr post eclosion for length measurement(A). For rescue experiments, dsRNA was injected 2 hr post pupation; methoprene (107 fmol) was applied to posteclosion animals ~44 hr after injection. Follicles were collected 72 hr post eclosion for measurement. Follicle size could be rescued by methoprene treatment (B).

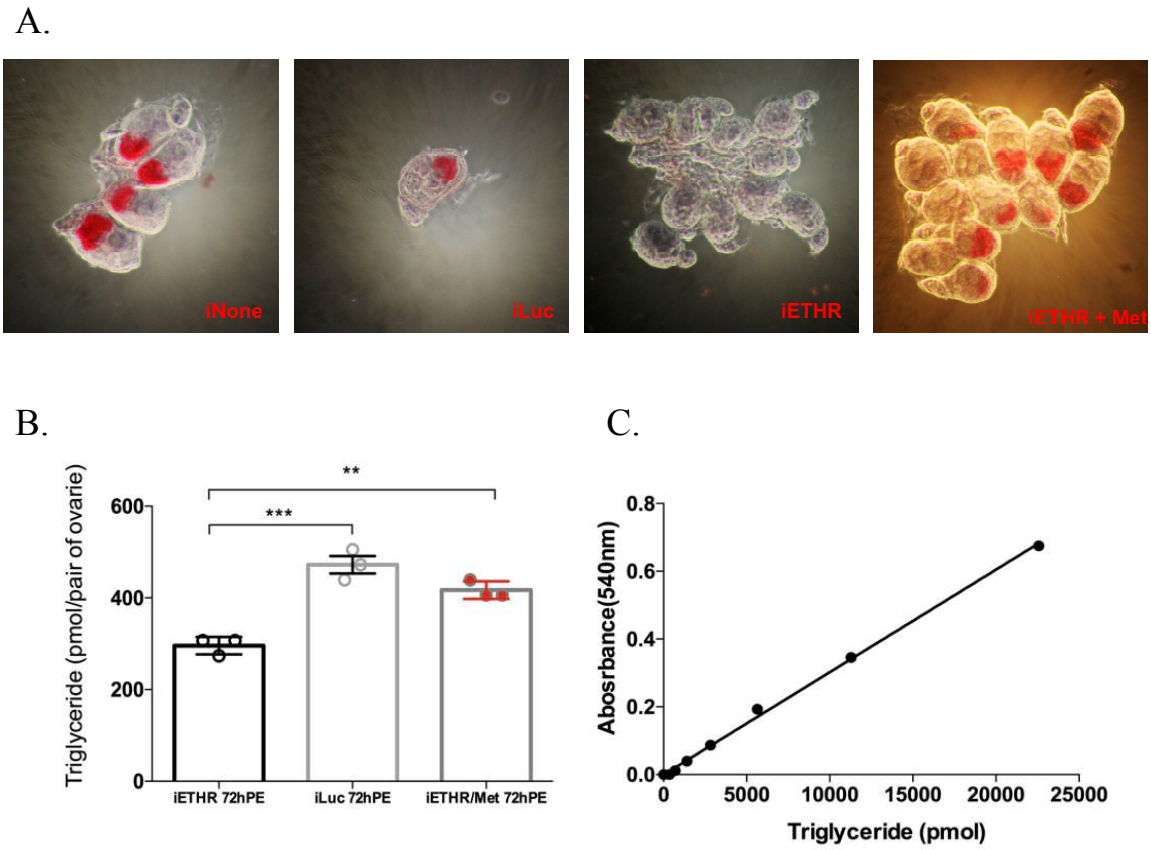

Figure 4.5 ETHR knockdown reduces oocyte lipid content in the first reproductive cycle

Hemocoel injections were performed 2 hr post-pupation; animals emerged ~44 hr after injection. Follicles were collected ~72 hr post eclosion. Lipids accumulate predominantly in the oocyte and proximal nurse cells as indicated by oil red-o staining of neutral lipids. Lipid content can be rescued by methoprene treatment. (iNone: non injection; iLuc: Luciferase dsRNA injection; iETHR: ETHR dsRNA injection; iETHR+Met; ETHR dsRNA injection with methoprene rescue).

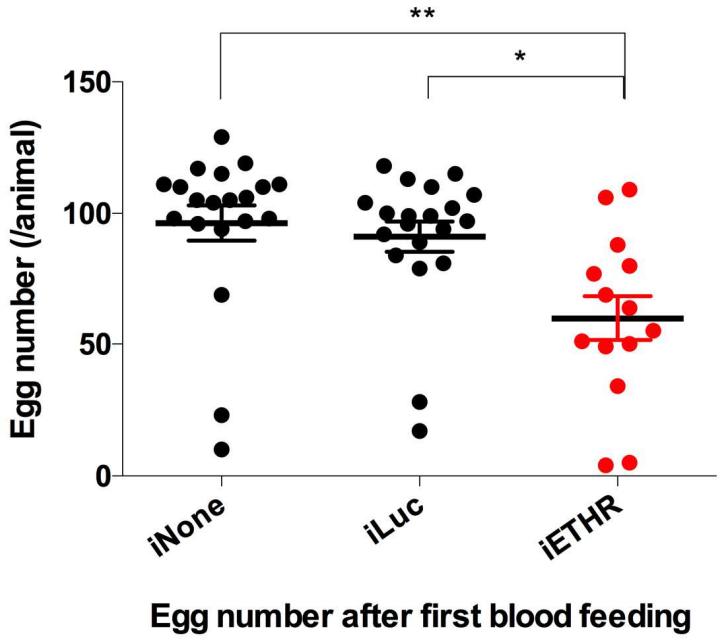

Figure 4.6 Egg number of ETHR knock down mosquitoes decreases following the first blood meal

dsRNA was injected 2 hr post-pupation; animals emerged ~44 hr post-injection. Animals were blood fed 4 days post-eclosion and eggs were collected 3 days after blood feeding.

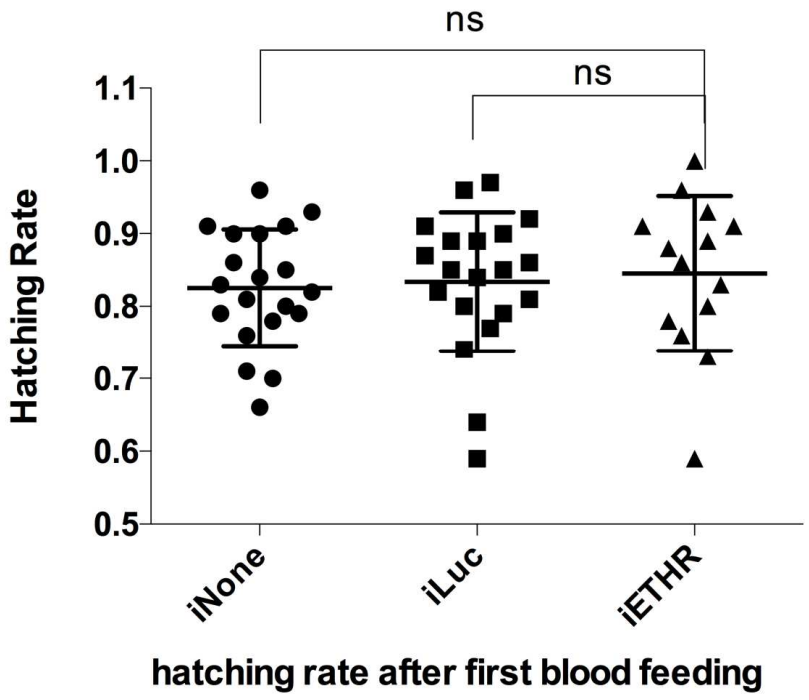

Figure 4.7 ETHR knockdown has no effect on mosquito egg viability following the first blood meal

dsRNA was injected 2 hr post-pupation; animals emerged ~44 hr post-injection and were blood fed 4 days post-eclosion. Eggs were collected 3 days after blood feeding and hatching occurred 3 days after collection.

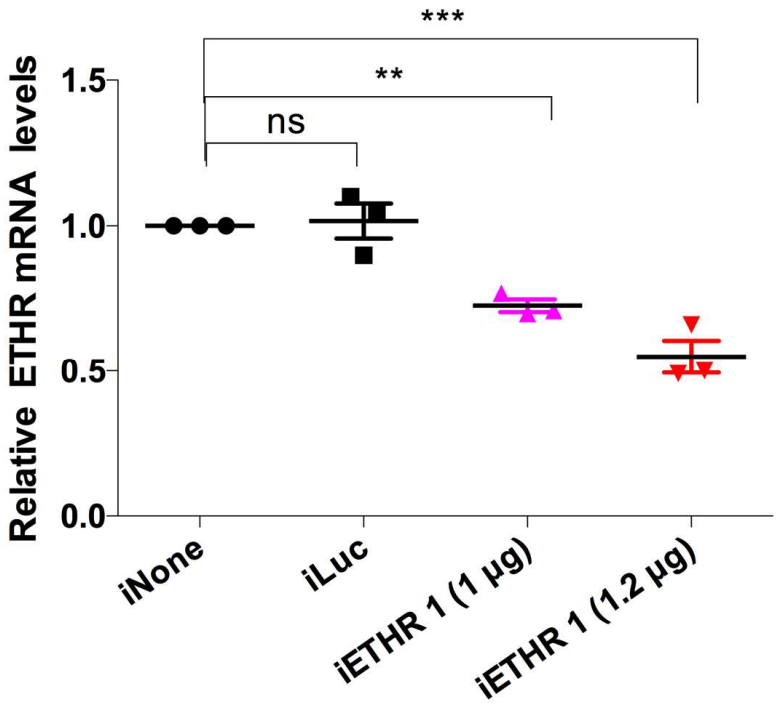

Figure 4.8 ETHR dsRNA silencing efficiency during the second reproductive cycle

Different doses of dsRNA were injected 1 day before blood meal. Animals were collected 24 hr post blood meal for qPCR.

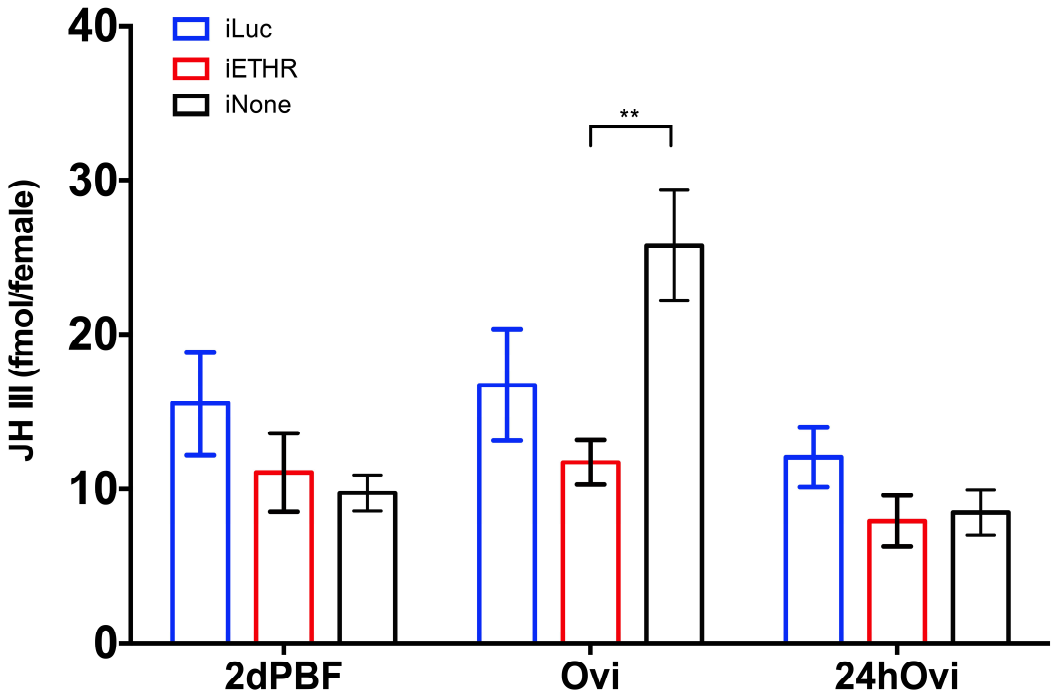

Figure 4.9 Second JH titer peak is reduced after ETHR dsRNA silencing

1.2 µg of dsRNA was injected 1 day before the blood meal. Hemolymph was collected from dsETHR, dsLuc and non-injected mosquitoes for JH measurement. 2dPBF: 2 day post blood feeding; Ovi: Oviposition: 24hOvi:24 hr post oviposition. (JH levels were determined by Dr. Dr. Noriega Fernando and Dr. Francisco Fernandez-Lima).

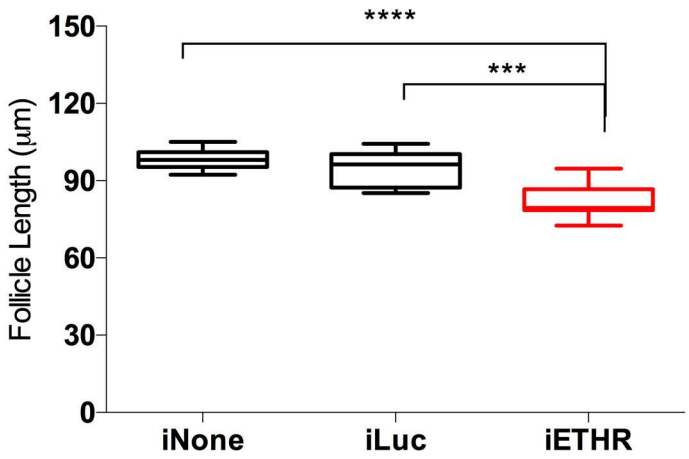

Figure 4.10 ETHR knock down reduces follicle length during the second reproductive cycle

dsRNA (1.2 ug) was injected 1 day before blood feeding. Follicle sizes were registered 72 hr post egg laying. Follicle size was measured with Zeiss LSM510 confocal microscope.

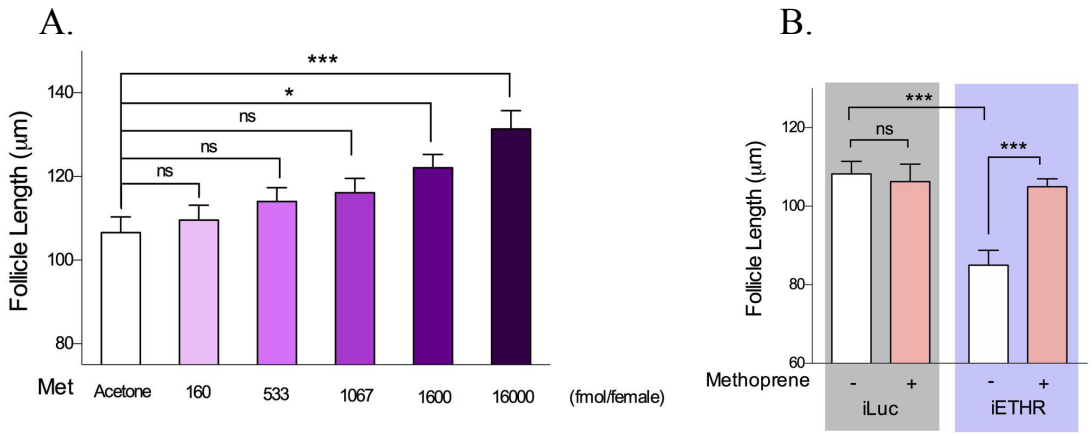

Figure 4.11 Follicle size is partially rescued by methoprene treatment

Different doses of methoprene were topically applied on the dorsal thorax 2 days after blood meal. Follicles were collected 72 hr post egg laying for length measurement(A). For rescue experiments, dsRNA was injected 1 day before blood meal; methoprene (1067 fmol) was applied 2 days post blood meal. Follicles were collected 72 hr post egg laying for measurement. Follicle size could be rescued by methoprene treatment (B).

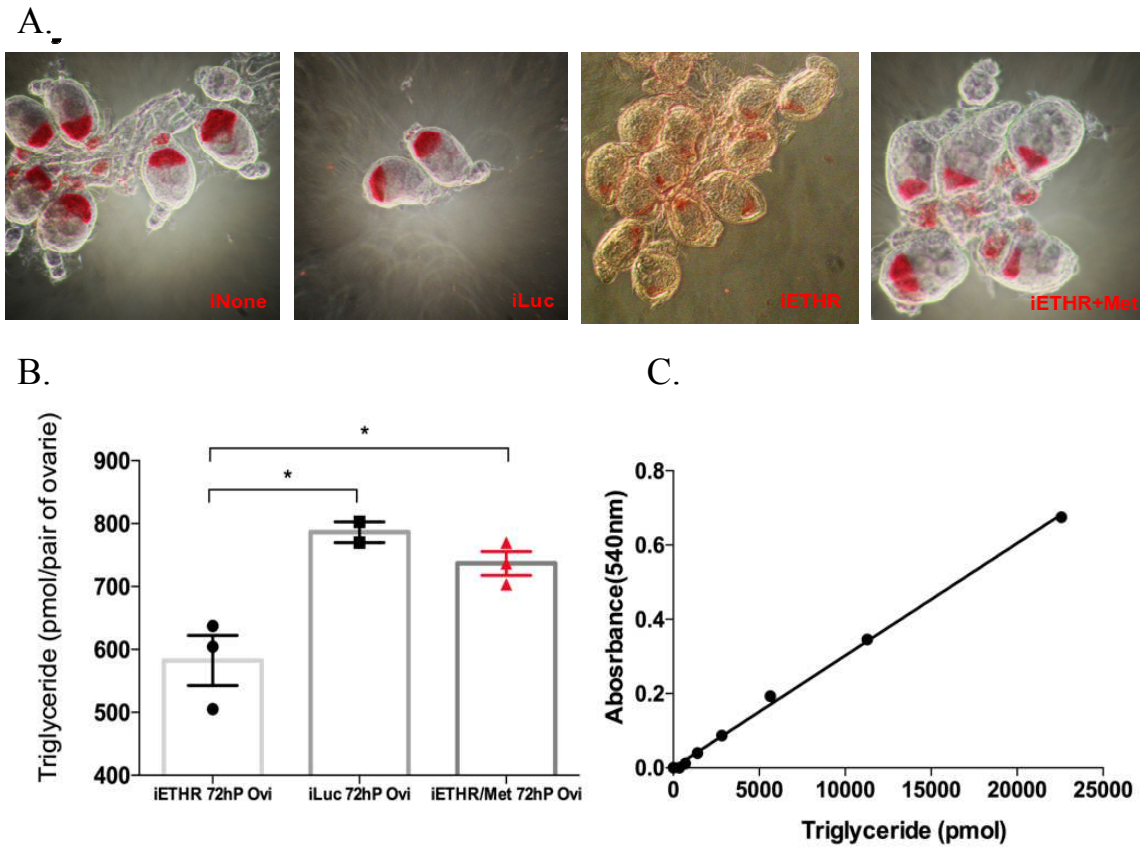

Figure 4.12 ETHR knockdown reduces lipid content of oocytes in the second reproductive cycle

dsRNA was injected 1 day before the blood meal. Different doses of methoprene were topically applied 2 days after the blood meal. Lipids accumulate predominantly in the oocyte and proximal nurse cells as indicated by oil red-o staining of neutral lipids. Lipid content can be rescued by methoprene treatment. (iNone: non injection; iLuc: Luciferase dsRNA injection; iETHR: ETHR dsRNA injection; iETHR+Met; ETHR dsRNA injection with methoprene rescue)

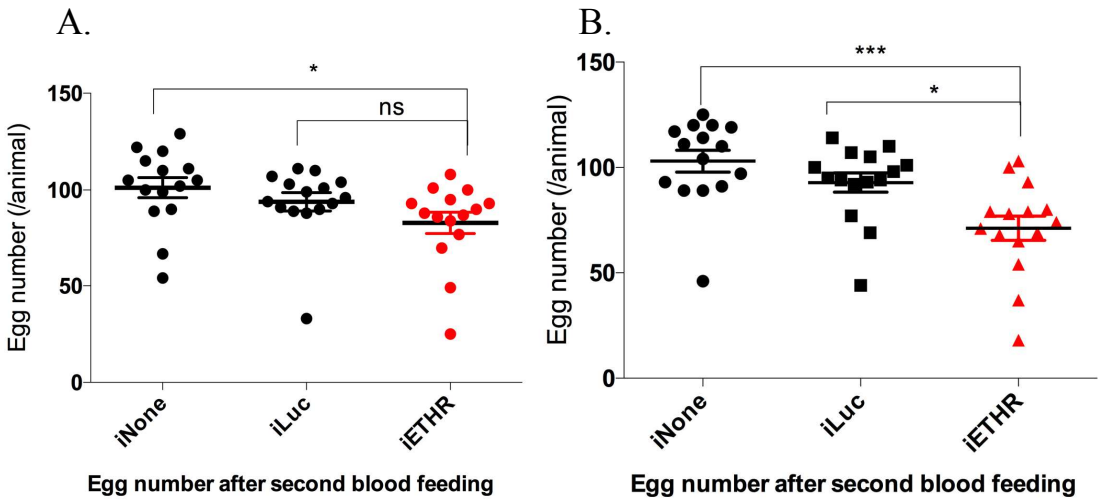

Figure 4.13 Egg number of ETHR knockdown mosquitoes following the second blood meal

(A) dsRNA was injected 24 hr before blood feeding. Eggs were collected 3 days after blood feeding. (B) First dsRNA injection was 24 hour before the bloodmeal; second dsRNA injection was 12 hr post-bloodmeal. Eggs were collected 3 days after the bloodmeal. ETHR knockdown decreases the egg number during the second reproductive cycle.

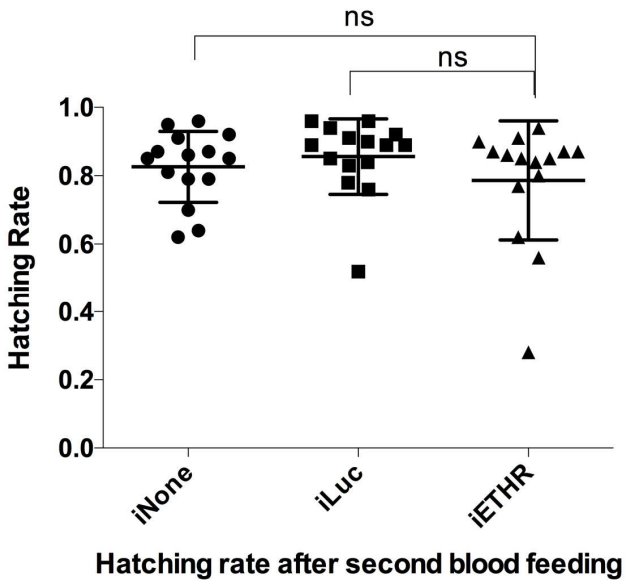

Figure 4.14 ETHR knockdown has no effect on egg viability of mosquitoes following the second blood meal

dsRNA was first injected 24 hr before the bloodmeal; the dsRNA injection was 12 hr post-bloodmeal. Eggs were collected 3 days after blood feeding and hatched 3 days after collection.

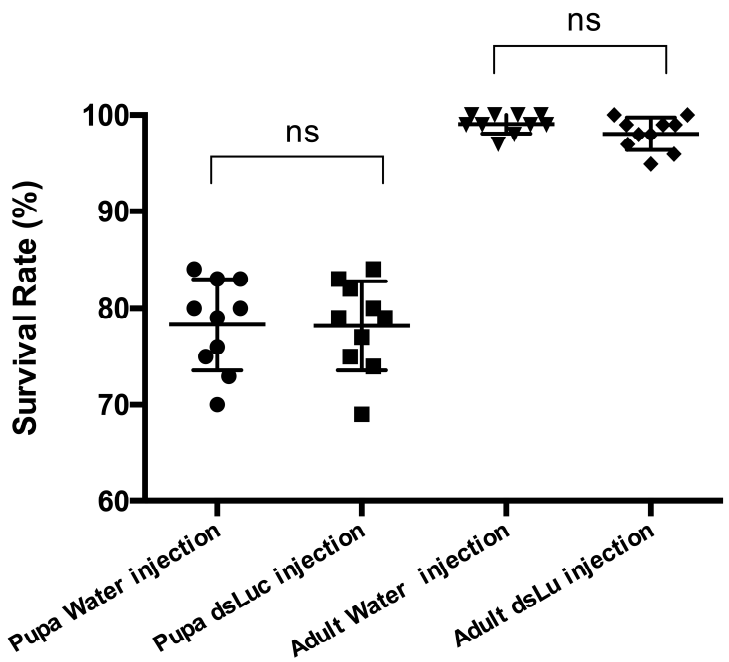

Figure 4.15 Survival rate three days post-injection

dsLuc or water (0.23 µl) was injected into pupa or adults separately. Survival rate was registered 3 days post-injection.

Chapter 5 Conclusions of the Dissertation

5.1 Summary

During development, insects perform ecdysis behaviors required for shedding of old cuticle at the end of each molt. These behaviors are orchestrated by circulating peptides known as ecdysis triggering hormones (ETHs) (Zitnan et al., 1996; Zitnan et al., 1999; Park et al., 1999). Secreted by endocrine Inka cells, ETH acts on target neurons in the CNS via two GPCR isoforms (ETHR-A and ETHR-B) (Park et al., 2003) to recruit downstream peptide signaling cascades. Recent work shows that Inka cells and transcripts encoding ETH and ETHR receptors persist through metamorphosis into adulthood, a period during which no ecdysis occurs (Graveley et al., 2011; Meiselman et al., 2017; Park et al., 2002). This suggests possible regulation of reproductive functions by ETH.

The yellow fever mosquito *Aedes aegypti*, a major public health threat throughout the world, has been established as an excellent model organism for study of vitellogenesis and reproductive physiology. In *Aedes* species, egg development proceeds through two developmental phases: previtellogenic and vitellogenic. The previtellogenic phase is controlled by JH, necessary to prepare the mosquitoes to be competent to the blood meal and subsequent vitellogenesis. During vitellogenesis, the fat body produces the yolk protein precursor vitellogenin. At the same time, oocytes accumulate vitellogenin to form protein yolk, leading to egg maturation (Hansen et al., 2014).

This dissertation examines the role of ecdysis triggering hormone signaling in regulation of reproductive physiology in female mosquitoes. Chapter 2 provides the evidence of existence of Inka cells, ETH and ETH signaling in the adult stage of mosquitoes. Chapter 3 focus on discovery upstream of ETH signaling that ecdysone signal pathway regulates ETH and ETHR synthesis. Chapter 4 investigates JH as downstream signaling of ETH regulating previtellogenesis during both first and second reproductive cycle. These studies signify the function of ETH in adult mosquitoes, especially important regulating mosquito reproduction, which depict signaling cross talk among members of the 20E-ETH-JH endocrine network.

5.2 Ecdysis triggering hormone signaling in the Adult yellow fever mosquito *Aedes aegypti*

In Chapter 2, I described the existence of ETH signaling in adult stage of mosquitoes in the following aspects: (1) In insects, there are 8-9 pairs of epitracheal glands occur near the spiracles, each with a single, prominent Inka cell associate with 3-4 other cells. (Zitnan et al., 2002a, 2003). In this study, I demonstrated presence of ETH and Inka cells in adult mosquitoes by immunohistochemical labelling with PETH antibody. (2). I provided expression profiles of ETH, ETHR-A and ETHR-B after eclosion by comparing with animals just post pupation. ETH, ETHR-A and ETHR-B transcripts decreased post eclosion, then increased post-bloodmeal, peaking at 24 hr post bloodmeal. (3). In order to map ETHR-expressing cells in adult mosquitoes, I made two mosquito lines with ETHR-A and ETHR-B GFP in-frame fusion knock-in separately by CRISPR. However, although qPCR data verified success of the CRISPR knock-in, GFP fluorescence was not

sufficient for microscopic visualization. This may be attributable to 1) relatively low expression of GPCRs and 2) gene products 5' to the T2A ribosomal skipping sequence may be expressed at very low levels (Akbari Unpublished).

5.3 Ecdysteroids regulate ETH and ETHR expression after blood feeding

It was reported previously that in the larval stages of moth, mosquitoes, desert locust and the adult of *Drosophila*, ecdysteroids are important regulators of ETH synthesis and release as well and expression of ETHR (Lenaerts et al., 2017; Li and Adams, 2009; Meiselman et al., 2017; Zitnan et al., 1999; Zitnan et al., 2007). In Chapter 3, I demonstrated that ecdysone signaling regulates ETH signaling in the adult stage of mosquitoes. ETH and ETHR exhibits high levels of expression in the female mosquito post blood meal (PBM), suggesting that ecdysteroids might have a role in regulation of ETH signaling in female mosquito adult stages. I set out to functionally characterize ecdysteroids in the female mosquito using both gene silencing and rescue methods. Using RNA silencing, I injected dsRNA targeting P450 enzymes involved in ecdysteroid synthesis. RNA silencing indicated that ecdysteroids function in regulation of ETH and ETHR synthesis in adult mosquitoes. Furthermore, using 20E injection for rescue, along with P450 enzyme silencing, I confirmed that 20E promotes ETH signaling, providing evidence that ecdysone is a critical regulator of ETH signaling of the female mosquitoes. This investigation has opened a new avenue towards understanding ETH signaling in adult signaling, which implies a potential function of ETH signaling in mediating mosquito reproduction.

5.4 Acting as an allatotropin, ETH regulates previtellogenesis in *Aedes aegypti*

Together, Chapter 2 and Chapter 3 described the existence and regulation of ETH signaling in adult mosquitoes. In Chapter 4, I investigated downstream actions of ETH on mosquito reproduction through JH signaling. Suppression of ETH signaling by impairing the ETH-JH cascade resulted in reduction of lipid content, follicle size during previtellogenesis phase during the first and second reproductive cycles decreased fecundity post-bloodmeal. To confirm that ETH signaling regulates reproduction through the JH pathway, I also showed ETHR silencing animals leads to JH deficiency. In the results, I show a peak of JH around 24 hr post eclosion, which declines to baseline levels within 48 hr. A fluctuation was observed during the previtellogenic phase of the second reproductive cycle. Further validating JH as a ETH signaling target, ETHR knock down in methoprene-treated animals resulted in normal follicle size and lipid content during previtellogenesis. Therefore, ETH-JH signaling not only regulates previtellogenesis, it also determines the fate of follicles after the bloodmeal during both first and second reproductive cycles. However, ETH-JH deficiency does not lead to altered egg viability.

5.5 Concluding remarks

In this dissertation, I have established ETH as a critical regulator of reproductive physiology in adult female *Aedes.* The reproductive cycle is controlled by alternating peaks of two important insect hormones: juvenile hormone (JH) and 20-hydroxyecdysone (20E). Previously it was shown in the mosquito that JH levels are high during the preparatory posteclosion period, dropping sharply immediately following a blood meal (Zhao et al., 2016). However, levels of 20E show an opposite trend, rising after a blood

meal to a maximum ~18 hr post-bloodmeal before declining. At the termination of vitellogenesis, 20E levels have dropped to baseline, and JH levels surge again, setting the stage for another cycle. According to my studies described here, ETH signaling exists in the adult stage and plays a crucial role in regulation of reproductive cycles. During the first reproductive cycle, ETH and ETHR production is regulated by ecdysone signaling prior adult molting. However, when ecdysone titer is high, ETH cannot release from Inka cells (Cho et al, 2013). Only after ecdysone levels decrease does ETH release occur, which in turn regulates JH synthesis, leading to the first posteclosion JH peak (Fig 5.1 A). For the second reproductive cycle, ecdysone peaks post-first bloodmeal, causing increased expression of ETH and ETHR (Fig 5.1. B). Ecdysone levels decline to baseline around 30 hr post blood meal (Fig 5.1. A), allowing release of ETH and consequent induction of the second JH peak (Fig 5.1A).

I propose a model for hormonal regulation of mosquito reproduction. Ecdysone in the pupal stage regulates ETH and ETHR synthesis. ETH release is regulated most likely by βFTZ-F1 post eclosion after ecdysone levels decrease (Rewitz et al., 2010; Cho et al, 2013). ETH acts on the corpora allata (CA) and regulates JH synthesis posteclosion. In the meantime, the TOR nutritional signaling pathway also contributes to JH biosynthesis (Perez-Hedo et al., 2013). JH promotes previtellogenesis in the following ways: regulation of resource allocation to the ovaries, leading to growth and maturation of primary follicles (Clifton and Noriega, 2011; Clifton and Noriega, 2012; Gwadz and Spielman, 1973). JH also regulates development of fat body and midgut in preparation for the bloodmeal (Raikhel et al, 2005). JH acts on the JH receptor complex (Met and

Taiman) to induce gene expression by binding the promoters of Kr-h1, Hairy, and regulator of ribosomal synthesis 1 (RRS1). RRS1 regulates translation of JH-controlled ribosomal protein genes such as RpL32, leading to massive ribosomal biogenesis in the fatbody post eclosion (Wang et al., 2017). Met downstream factors Hairy and Kr-h1 play promote posteclosion maturation of the mosquito ovary (Roy et al., 2018). The blood meal taken by female mosquitoes triggers release ILPs and ovary ecdysteroidegenic hormone from the brain (OEH). AAs, OEH, ILP3 and ILP4 stimulate synthesis and release of ecdysone from ovaries (Dhara et al., 2013). 20E binds the heterodimeric receptor complex of EcR and USP. Receptor-bound 20E activates transcription of the early genes, BrC, E74, and E75 (Chen et al., 2004). Early proteins are involved in the transcriptional regulation of genes encoding YPPs in the fat body (Raikhel, 2005). The TOR pathway also promotes nutrition-dependent activation of Vg synthesis. Secreted YPPs are subsequently taken up by oocytes during maturation (Fig 5.2) (Hansen et al., 2005).

Although great progress has been made in our understanding of mosquito reproduction, many unknowns remain. Development of novel molecular techniques, especially CRISPR as well as bioinformatics knowledge, more and more study will be added to understand the complex endocrinal networks of mosquito reproductive physiology.

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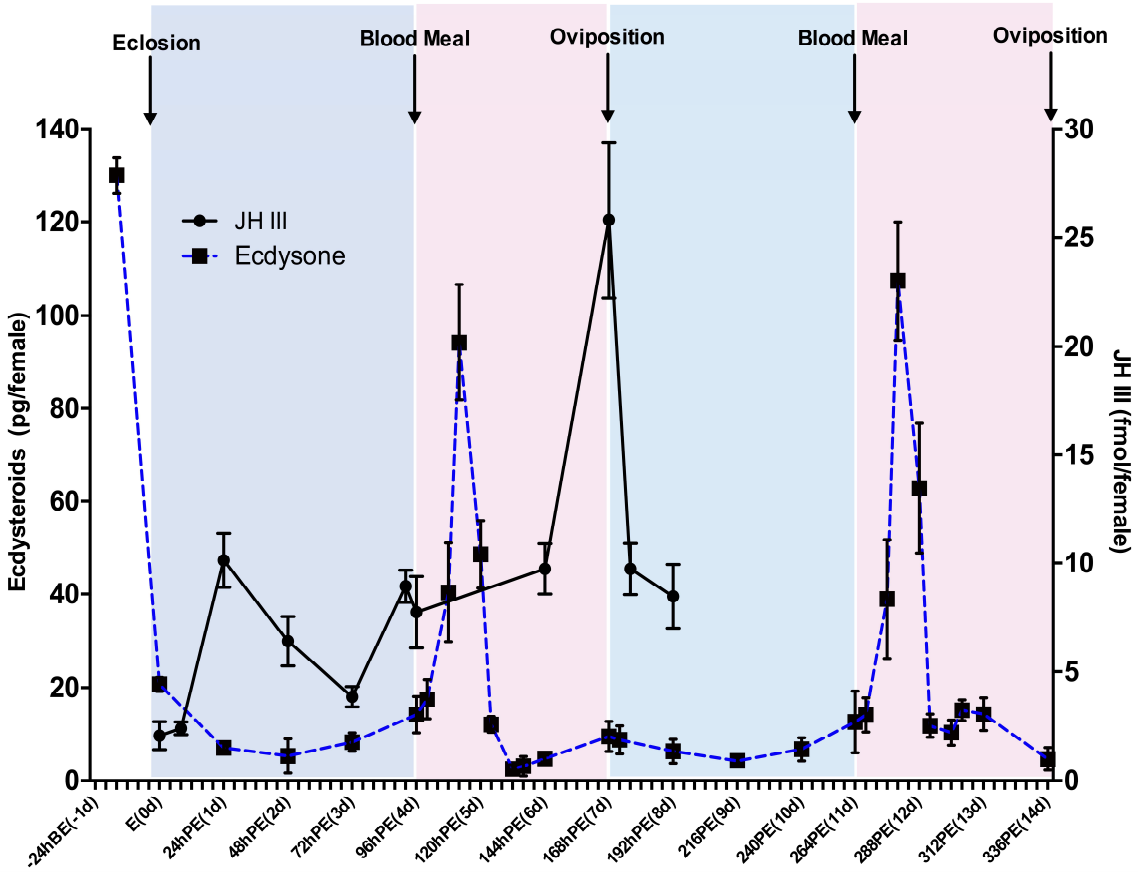
A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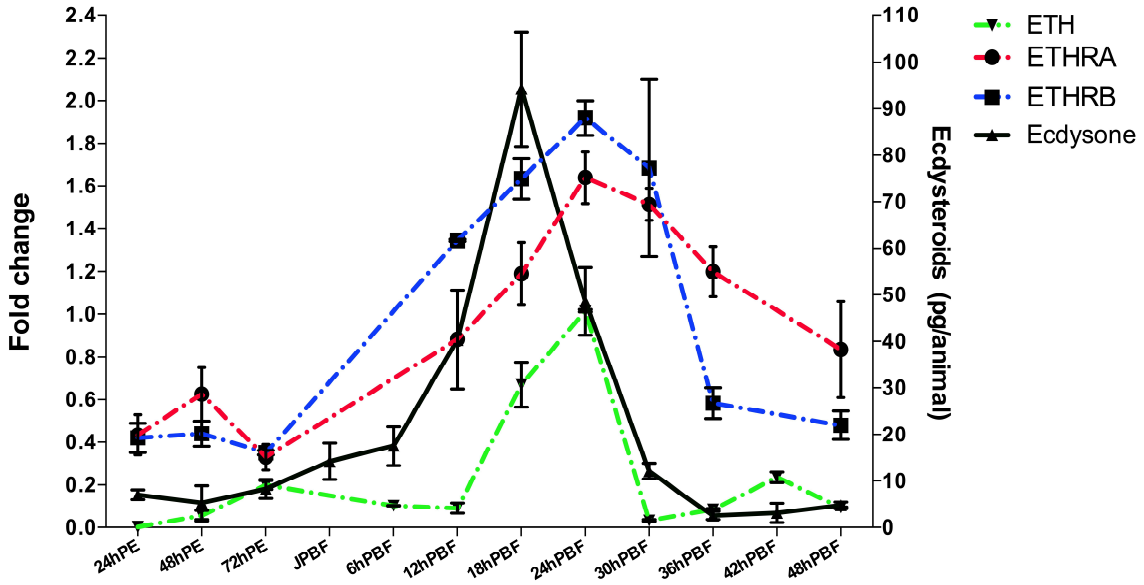

Figure 5.1 Fluctuations of ETH, ETHRs transcript levels (B) and their correlation with changes in JH and ecdysone levels (A) in adult female mosquitoes.

(A). Ecdysteroid levels were determined by EIA and juvenile hormone levels were determined by HPLC-MS/MS. BE: Before- Eclosion; E: Eclosion; PE: Post-Eclosion; (B). Ecdysteroid levels were determined by EIA. ETH, ETHR-A, ETHR-B transcript levels were determined by q-PCR. PE: Post-Eclosion; JPBF: just post-blood feeding; PBF: post-blood feeding

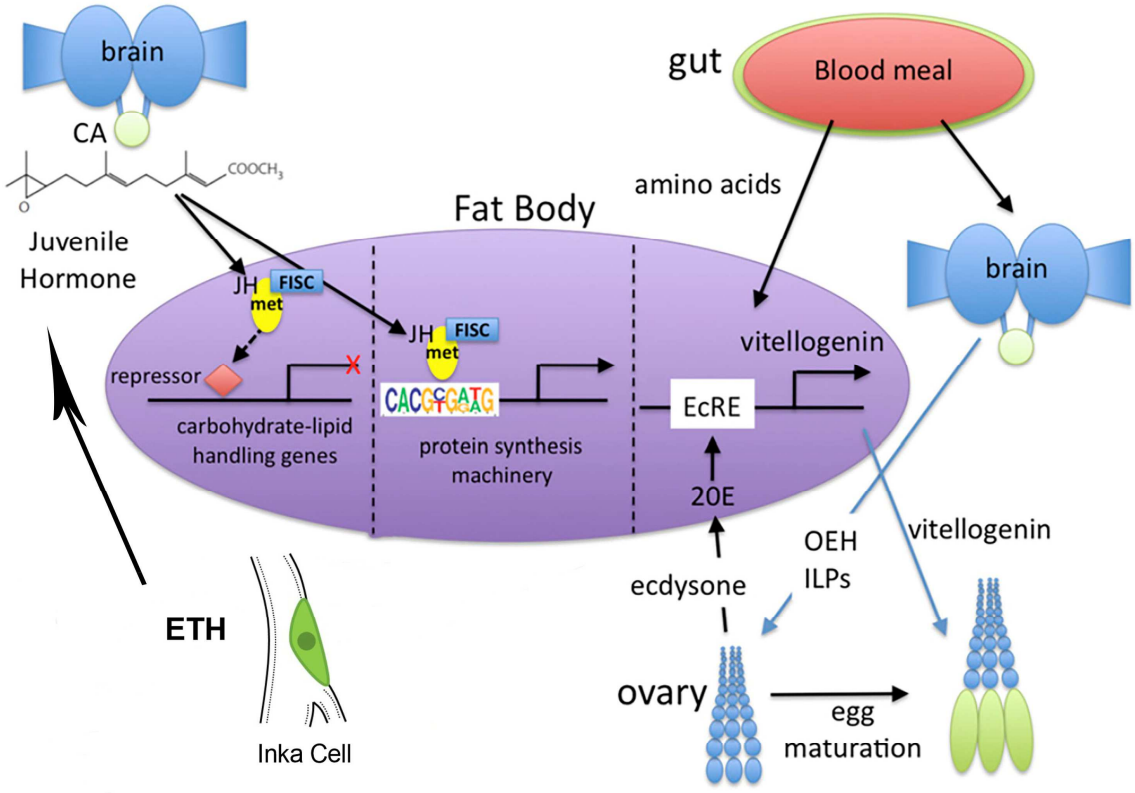

Figure 5.2 Model of hormonal network consisting of 20E, ETH, and JH in *Aedes aegypti* adults

corpora allata (CA); 20-hydroxyecdysone (20E); ecdysone response element (EcRE); neuropeptides ovarian ecdysteroidogenic hormone (OEH); insulin-like peptides (ILPs); (Modified from Riddiford 2013)