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Ecdysis Triggering Hormone and its Role in Juvenile Hormone Synthesis in the Yellow-fever Mosquito, Aedes aegypti

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ECDYSIS TRIGGERING HORMONE AND ITS ROLE IN JUVENILE HORMONE SYNTHESIS IN THE YELLOW-FEVER MOSQUITO, *AEDES AEGYPTI*

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Maria Areiza

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To: Dean Kenneth G. Furton
College of Arts and Sciences

This thesis, written by Maria Areiza, and entitled Ecdysis Triggering Hormone and its Role in Juvenile Hormone Synthesis in the Yellow-Fever Mosquito Aedes aegypti, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

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The thesis of Maria Areiza is approved.

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Florida International University, 2014

DEDICATION

I dedicate this Thesis to my best friend and husband Andrew Barthle. Your unwavering belief in me, even at times when I doubted myself the most, allowed me to complete this journey. Also to my four-legged friends which never let me take myself too seriously and provided much needed stress relief during the writing process. And last but not definitely not least, to every single teacher I have ever had – you have all shaped me and left an indelible mark in my life. Thank you.

"We are like dwarfs on the shoulders of giants, so that we can see more than they, and things at a greater distance, not by virtue of any sharpness of sight on our part, or any physical distinction, but because we are carried high and raised up by their giant size."

"Dicebat Bernardus Carnotensis nos esse quasi nanos, gigantium humeris insidentes, ut possimus plura eis et remotiora videre, non utique proprii visus acumine, aut eminentia corporis, sed quia in altum subvenimur et extollimur magnitudine gigantea."

- Bernard of Chartres

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ABSTRACT OF THE THESIS

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by

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Miami, Florida

Professor Fernando G. Noriega, Major Professor

Ecdysis triggering hormone (ETH) is a neuropeptide known for its role in the orchestration of ecdysis. However, its role in the regulation of Juvenile Hormone (JH) synthesis is unknown. In *Aedes aegypti*, JH is synthesized by the *corpora allata* (CA) and titers are tightly regulated by allatoregulatory factors. In this study I describe the effect of ETH on JH synthesis during the late pupal stage and in the adult female after blood feeding. Analysis of ETH receptor (ETHRs) expression showed that ETHRs are present in both the CA and the *corpora cardiaca* (CC), a neurohemal organ. The data suggest that ETH regulates JH synthesis directly through its receptors in CA. Our results show that in pupa, ETH has a stimulatory effect on JH synthesis while in adult blood fed females, ETH is inhibitory. These findings constitute the first evidence of ETH as a regulatory peptide in mosquito JH synthesis.

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20E	20-hydroxyecdysone
BF	Blood-Fed
bHLH	Basic Helix-Loop-Helix
BR	Brain
CA	Corpora Allata
CC	Corpora Cardiaca
CCAP	Crustacean Cardioactive Peptide
CNS	Central Nervous System
DA	Dopamine
dsRNA	Double-stranded RNA
EH	Eclosion Hormone
ETH	Ecdysis Triggering Hormone
ETHRs	Ecdysis Triggering Hormone Receptors
ETHs	Ecdysis Triggring Hormones
FA	Farnesoic Acid
FAL	Farnesal
FPP	Farnesyl Diphosphate
GPCRs	G-Protein-coupled Receptors
HPLC	High Performance Liquid Chromatography
JH	Juvenile Hormone
JHBPs	Juvenile Hormone Binding Proteins
JHs	Juvenile Hormones

MF	Methyl Farnesoate
MIPs	Myoinhibitory Peptides
MP	Mevalonate Pathway
PGs	Prothoracic Glands
PRXa	PRXamide
PTTH	Prothoracicotropic Hormone
q-PCR	Quantitative Real Time PCR
RNAi	RNA Interference
SF	Sugar-Fed
TF	Transcription Factor
TRH	Thyrotropin Releasing Hormone
TRHR	Thyrotropin Releasing Hormone Receptor
VM	Ventromedial Cells
YFP	Yellow Fluorescent Protein

CHAPTER 1

Summary

Juvenile Hormone (JH) is a master regulatory hormone in insects that controls both metamorphosis in immature insects and reproductive development in adults. In the yellow-fever mosquito, *Aedes aegypti*, JH III is synthesized by a pair of neuroendocrine glands with neural connections to the brain called the *corpora allata* (CA). Hemolymph JH titers are determined by the rate of biosynthetic activity of the CA which is tightly regulated by allatostatic (inhibitory) and allatotropic (stimulatory) factors. Ecdysis triggering hormone (ETH) is a neuropeptide well-established for its role in the orchestration of ecdysis or molts in insects. Ecdysis Triggering Hormone is synthesized and secreted into the hemolymph by endocrine cells called Inka cells. Interestingly, despite the stage specific nature of ecdysis, Inka cells persist in adult insects. However, the role of ETH in adults and/or in JH synthesis is unknown.

In this study I describe the stimulatory effect of ETH on JH synthesis during the maturation process the CA undergoes during the last eight hours of the pupal stage. At this time genes encoding JH biosynthetic enzymes become transcriptionally active and the CA starts synthesizing basal levels of JH. Analysis of the expression of ETH receptors (ETHRs) revealed that they are present in both the *corpora allata* and the *corpora cardiaca* (CC), a neurohemal organ. *In vitro* stimulation of the pupal CA glands with ETH resulted in an increase in JH synthesis. Consistent with this finding, silencing ETHRs by RNA interference (RNAi) in pupa resulted in reduced JH synthesis on 18 h old adults. On the other hand in blood fed (BF) adults, exogenous ETH treatment caused

a reduction of JH synthesis. The decline in synthesis was most striking at 48 h post blood feeding; when JH levels normally begin to increase (Figure. 5). Conversely, silencing of ETHRs resulted in an increase of JH synthesis. Our results suggest that ETH regulates JH synthesis directly through its receptors in the CA. We would like to propose that ETH should be included into the group of regulatory peptides controlling JH synthesis in *Aedes aegypti*.

INTRODUCTION

Aedes aegypti, is a highly anthropophilic mosquito that lives in urban environments. Originating from Africa, it is now distributed worldwide in the tropics and subtropics (Christophers, S. R., 2009). It is found in 23 U.S. states from the Gulf coast to the southeast, Mid-Atlantic and New York. *Aedes aegypti* is the primary arthropod vector of three significant viral diseases: Dengue fever (DF), Yellow-fever, and Chikungunya. Of the three, dengue is the most prevalent mosquito-borne viral disease worldwide. It is estimated that DF causes 50 to 100 million infections per year and 25,000 deaths (Brunkard, J. M *et al*., 2007). Currently there are no vaccines or therapeutics against the dengue virus and vector control is the main option for prevention. An attractive target for control is Juvenile Hormone (JH) a regulatory hormone in insects.

Juvenile Hormones (JHs) are a family of acyclic sesquiterpenoids unique to insects (Riddiford, 2012; Goodman and Cusson, 2012). They are major effector hormones whose primary role in all insects is the regulation of metamorphosis and reproduction (Noriega, 2004; Goodman and Cusson, 2012). In some species, JHs are also associated with the regulation of polyphenisms including color morphs and caste

differentiation of social insects (Goodman and Cusson, 2012). However, it is their universal role in insect metamorphosis and reproduction that has made them an attractive subject of research as possible targets of pest control. Though strides in studies of their chemistry and molecular action have been made since their discovery, much remains to be learned about JH. In particular, much information is still needed to understand the regulatory mechanisms of its synthesis. Not surprisingly, its biosynthesis by the *corpora allata* (CA) is tightly regulated to ensure proper timing of developmental stages and gonadotropic cycles (Goodman and Cusson, 2012). The thrust of my study was to elucidate the role that the peptide hormone Ecdysis Triggering Hormone (ETH) might play in regulation of JH synthesis in the yellow-fever mosquito, *Aedes aegypti*.

Discovery of Juvenile Hormones

The role of JH in regulation of metamorphosis was first discovered in the 1930s by Vincent Wigglesworth (Wigglesworth, 1934). Through a series of surgical manipulations on his model animal *Rhodnius prolixus*, commonly known as the kissing-bug, Wigglesworth was able to conclude that circulating hormones regulate metamorphosis. In particular, he used a technique known as parabiosis in which a developmentally arrested animal was surgically joined with a normally developing one and the circulatory systems joined (Wigglesworth, 1934). Wigglesworth observed that a circulating hormone in young nymphs prevented a fifth (last) stage nymph from progressing to the adult stage. In essence, this hormone with antimetamorphic function promoted the retention of juvenile characteristics in *Rhodnius* and Wigglesworth coined the name Juvenile Hormone (Wigglesworth, 1934). The sole site of synthesis for JH was

found to be a pair of neuroendocrine glands with neural connections to the brain called the *corpora allata* (CA).

Juvenile Hormone Homologues

Since its discovery, several natural occurring JHs have been characterized (Goodman and Cusson, 2012; Riddiford, 2012). Examples of some of the JH homologues characterized to date are: JH-I, JH-II, JH-III JH-0, 4-methyl JH-I, and $JHSB_{3;}$ a common feature of all is their farnesoid structure. Figure 1 shows the structures of the major JHs found in insects (Goodman and Cusson, 2012). Unique to Lepidoptera are JH-I and JH-II, which were the first of the JHs to be discovered during the 1960s. A third homologue, JH-III, is the most ubiquitous and is present in most insects, including in Lepidoptera (Adams, 2009; Riddiford, 2012 Jindra *et al*., 2013). In *A. aegypti*, the CA produces JH-III and its synthesis regulation was the topic of the study. Additional JH homologues have been found in the eggs of the tobacco hornworm, *Manduca sexta*: JH-0 and 4-methyl JH-I (Adams, 2009). Lastly, another JH structure found in higher Diptera is JH bisepoxide ($JHSB_3$) (Riddiford, 2012). Moreover, there are JH analogs which are non-insect derived compounds possessing JH-like biological activity in insects such as: fenoxycarb, pyriproxifen, and methoprene (Figure 1). The latter has exceptional biological activity in insects and has been used commercially as a control agent for insect pests, including mosquitos, since 1975 (Goodman and Cusson, 2012; Riddiford, 2012).

Site of Juvenile Hormone Biosynthesis: Corpora Allata Complex

Juvenile Hormone is synthesized and secreted by the CA, a pair of endocrine glands, which are located posterior to the brain (Figure 2). The CA has neural

connections to brain and is part of the retrocerebal complex along with the *corpora cardiaca* (CC). The paired CC is a neurohemal organ that produces neurohormones and serves as a storage area for peptide hormones produced in the brain (Goodman and Cusson, 2012). Each *corpus cadiacum* is made up of only six neurosecretory cells (Clements, 1992). In the *Aedes* adult female, the CA is approximately 40-50 μm in length (Li *et al*., 2003). In the female, the CA is comprised of approximately 48 cells in the pupal stage and 60 in adult (Clements, 1992). Hence, it is not surprising that handling and dissection of these small glands proves challenging. Juvenile Hormone works in concert with ecdysteroids to modulate both metamorphosis and gonadotropic functions. In larval insects, the neuropeptide prothoracicotropic hormone (PTTH) stimulates the release of α-ecdysone by the prothoracic glands (PGs). However, in the adult these glands degrade and the ovaries become the source of ecdysone (Raikhel, 2005). Once secreted, ecdysone is converted by peripheral tissues to its active ecdysteroid form, 20-hydroxyecdysone (20E) (Riddiford, 2012).

Juvenile Hormone Molecular Basis of Action

Upon secretion from the CA, JH is transported in the hemolymph by high affinity binding proteins to act at distant peripheral sites. Termed hemolymph JH binding proteins (hJHBPs), these proteins also protect JH from degradation by enzymes (Trowell, 1992; Goodman and Cusson, 2012). Only recently was the receptor for JH identified as Methoprene-tolerant (Met). The intracellular hormone receptor is a basic helix-loop-helix (bHLH) protein a member of the Period (Per)-Aryl hydrocarbon nuclear translocator (Arnt) – Singleminded (Sim) (PAS) domain family of transcription factors (Riddiford, 2012; Riddiford, 2013; Jindra *et al*., 2013). Upon binding JH the Met homo-

or heterodimer undergoes a conformational change that allows it to bind to another bHLH-PAS protein known as Ftz-F1 interacting steroid receptor coactivator (FISC) in *A. aegypti* (Jindra *et al*., 2013).

The Role of Juvenile Hormone in Metamorphosis

In holometabolous insects such as *Aedes aegypti*, metamorphosis takes place in two stages: larval-pupal and pupal-adult (Figure 3). After hatching, *Aedes* undergoes four larval instar molts known as the "wrigglers" during which feeding takes place. Hence, JH dictates the nature of the molts which occur in response to 20E (Goodman and Cusson, 2012). For example, in the course of larval development JH ensures that molts are from one larval instar to another larger larval instar. However, in the last larval stadium a rise in 20E coupled with the absence of JH signals a cessation of feeding and onset of pupation. Following is the pupal stage, in which the non-feeding insect undergoes a major developmental restructuring. During this stage a pharate adult, that is, an adult encased in the pupal cuticle, develops until the appropriate time to emerge (eclosion). The pupal stage takes approximately 50 hours.

Role of Juvenile Hormone in Reproductive Maturation

After metamorphosis, JH plays a different role in the adult female mosquito as a regulator of reproductive maturation. In anautogenous mosquitos such as *Aedes*, reproduction is cyclic and a blood meal is required by the female prior to oviposition (Riddiford, 2013). In short, their reproductive cycle has two distinct phases: a previtellogenic stage regulated by JH and a vitellogenic stage regulated by 20E (Raikhel *et al*., 2002). Newly emerged female mosquitos feed on a nectar diet and synthesize low

amounts of JH which, during the first 12 hours post eclosion, increase to 90 fmol/CA/hour. In sugar-fed females JH levels remain high but rapidly decrease after ingestion of a blood meal (Figure 5). Juvenile Hormone levels reach their lowest point 24 h later post blood meal at: < 5 (fmol/CA/hour) (Li *et al*., 2003). Forty-eight hours post blood meal, JH levels begin to rise and return to a relatively high level on the third day (Noriega, 2004). Prior to taking a blood meal the mosquito is in a resting stage in oogenesis is known as the previtellogenic stage. Here, JH exposure leads to competence of the fat body (FB) - the equivalent to the vertebrate liver- to synthesize massive amounts of yolk protein precursors upon later exposure to 20E (Adams, 2005; Riddiford 2012). Another way JH regulates reproductive output is by inducing competence of the ovaries to uptake Vitellogenin (Vg), a major yolk protein, into developing oocytes. Moreover, JH also prepares the mosquito for blood digestion by regulating transcription of the early trypsin gene in the midgut (Noriega *et al.*, 1997). In response to a blood meal, 20E levels rise and the vitellogenic phase of egg development begins. At this time, under the control of 20E, the oocyte completes a process of yolk deposition called vitellogenesis. Three days after blood feeding, 20E levels decrease and JH synthesis begins anew, repeating the preparatory steps necessary for another gonadotrophic cycle (Li *et al*., 2003)

Juvenile Hormone Biosynthesis

The biosynthetic pathway of JH-III involves 13 sequential enzymatic steps and is best divided into early and late steps (Bellés *et al*. 2005). The early steps follow the mevalonate pathway (MP); where two acetyl-CoAs are reductively polymerized into five-carbon (5C) isoprenoid units (Figure 4). These isoprene units are then sequentially

condensed to form farnesyl diphosphate (FPP). Up to the formation of FPP, the MP of insects shares homology with all organisms that use the mevalonate pathway to synthesize cholesterol or other biomolecules. In insects, which do not produce cholesterol, FPP is instead used for production of compounds such as ubiquinone, for protein prenylation, and for JH synthesis (Goodman and Cusson, 2012). In the late steps, FPP is transformed sequentially to: farnesol, farnesal, farnesoic acid (FA), methyl farnesoate (MF), and ultimately JH III.

Regulation of Juvenile Hormone Biosynthesis: Allatoregulatory Factors

Considering the important developmental and gonadotropic roles of JH, it is not surprising that biosynthesis of the hormone by the CA is under strict regulation. In essence, the rate of biosynthetic activity of the CA determines the JH titers. To ensure proper titers of JH, the CA is tightly regulated by allatoregulatory factors; such that information on nutritional and developmental status is transduced to the CA by them. These factors are both allatostatic (inhibitory) and allatotropic (stimulatory) in nature. These include neuropeptides, a class of signaling molecules that have been shown to regulate endocrine functions in a vast array of organisms ranging from insects to vertebrates (Woodhead, 1989). Two examples of allatoregulatory neuropeptides are the pleiotropic neuropeptides, allatotropin (AT) and allatostatin (AS). Allatotropins are known to be stimulatory to JH synthesis and have been characterized in *A. aegypti* (Li *et al.*, 2004). Conversely, allatostatins have been demonstrated to have an inhibitory effect in JH synthesis and have also been described in *Aedes* (Li *et al*., 2006). The allatoregulatory potential of these peptides underscores the importance of investigating

the role of other neuropeptide hormones as possible regulators of JH synthesis in the mosquito.

Activation of CA in the pharate adult of *Aedes aegypti*

For female mosquitos, the last six hours of the pupal stage is a period of dramatic developmental changes as the insect prepares itself for its adult life. The CA undergoes a maturation process during which the genes for JH biosynthetic enzymes become transcriptionally active and the gland begins to synthesize basal levels of JH (Nouzova *et al*., 2011). Preliminary data in our lab has shown that several neuropeptide and hormone receptors have a peak of expression in the CA of the late pupa, at the time when transcription of JH biosynthetic enzymes is activated (Figure 6). Among them is the receptor for the neuropeptide, Ecdysis triggering hormone (ETH). Moreover, studies on the tobacco hornworm *Manduca sexta* and the silkworm *Bombyx mori* have also shown that the CA expresses receptors for ETH (Zitnan *et al*., 2007 and Yamanaka *et al*., 2008). Taken together, this leads us to hypothesize that ETH may play a role in the regulation of JH synthesis.

Ecdysis Triggering Hormone

Well conserved among insect orders, ETH is a small C-terminally amidated peptide, known as a major regulator of ecdysis or molts in insects (Adams *et al*., 2006; Zitnan *et al*., 2007). ETH is synthesized and secreted into the hemolymph by specialized endocrine cells called Inka cells that are associated with epitracheal glands (Adams *et al*., 2006; Predel *et al*., 2010). Figure 7 shows the amino acid sequence of several ETHs found in various insects. In insect development, ecdysis allows for shedding of the old

exoskeleton at the end of each life stage. The innate behavioral sequence of ecdysis is stereotypical and comprised of two types of movements: rhythmic contractions and relaxations that loosen the old exoskeleton and peristaltic movements to shed it (Zitnan *et al*., 2003; Margam *et al*., 2006; Adams *et al*., 2006). The preparatory phase of ecdysis is mediated by ecdysteroids such as ecdysone (E) and 20E which are important regulators of gene expression. As previously mentioned, the transition from feeding to molting is triggered by 20E pulses. Rising 20E levels induce gene expression of peptides such as ETH and their cognate receptors (Zitnan et al 1999, Adams *et al.*, 2006; Zitnan and Adams, 2012). The mechanism of steroid action on ETH gene expression appears to be under direct control of 20E. Buttressing this notion is previous work in *M. sexta* which has shown that ETH levels in Inka cells rise in accordance to 20E peaks (Zitnan *et al*., 1999). Furthermore, 20E rises also coincide with expression of the nuclear ecdysone receptor (EcR-B1) in Inka cells. Taken together, this suggests that the steroid-EcR complex may regulate ETH expression by binding to EcR response elements in the promoter of the ETH gene. To date, a putative ecdysone response element (EcRE) (AGGTCA) was identified in the promoter of the ETH gene of *A. aegypti* (Adams, 2009). While high ecdysteroid levels induce expression of the peptide, Inka cells gain secretory competence only when 20E levels decline to <0.1µg/ml (Kingan and Adams, 2000). The mechanism controlling ETHR expression is not yet known. For instance, at this time, it is not known if expression of ETHRs are under the direct control of 20E and its receptor or mediated by other transcription factors. However, it is thought that 20E does induce ETHR expression. Previous studies in *M. sexta* and in *A. aegypti* have shown peak ecdysteroid levels coincident with expression of ETH receptors as well as behavioral

sensitivity (Kim *et al*., 2006; Zitnan *et al*., 2007; Dai and Adams, 2009). Taken together, it appears that expression of both ETH and its receptors are under the regulation of 20E.

Discovery of Ecdysis Triggering Hormone and Inka Cells

The presence of epitracheal glands in insects was first discovered a hundred years ago in the silkworm, *B. mori* (Ikeda, 1913). However, their exocrine and endocrine functions remained unidentified for 76 years until work on histological sections of the waxmoth, *Galleria mellonella* by Dusan Zitnan (Zitnan, 1989). In a search for peptidergenic cells in the waxmoth, Zitnan observed strong immunoreactivity in cells on the surface of tracheal tubes to antiserum for the neuropeptide FMRFamide (Zitnan, 1989). More notably, their peptidergenic nature was identified when staining was lost after ecdysis. This work led to the identification of ETH from Inka cells, which was first accomplished in *M. sexta* (Zitnan, 1996). Thereafter, Inka cells have been described in *Bombyx*, *Drosophila*, and *A. aegypti* to name a few, and appear to occur in all insect orders (Zitnan and Adams, 2012). In *A. aegypti*, Inka cells are located along branch points of lateral epitracheal trunks (Dai and Adams, 2009). The *eth* gene encodes two isoforms of the 17 amino acid peptide, ETH1 (AeaETH1) and ETH2 (AeaETH2) (Park *et al*., 2003). Both of these peptides induce a receptor-mediated signaling cascade in CNS neurons that result in activation of motor programs allowing shedding of the old cuticle (Zitnan and Adams, 2012). Though both ETH peptide isoforms are capable of inducing ecdysis motor programs, work on *Drosophila* and *M. sexta* has noted that ETH1 has a higher binding affinity than ETH2 (Park *et al*., 2003; Dai and Adams, 2009) and therefore was the peptide used throughout the current project. In hemimetabolous insects, Inka cells appear to degrade after adult ecdysis as immunohistochemistry studies have

shown. Remarkably, Inka cells persist in some adult holometabolous insects: beetles, moths, and dipterans (Park *et al.,* 2002; Zitnan *et al*., 2003; Zitnan and Adams, 2012). So far, the physiological roles of Inka cells in adult has not yet been identified but possible roles in modulating JH synthesis have been suggested (Zitnan and Adams, 2012; Hiruma and Kaneko, 2013).

Ecdysis Triggering Hormone Receptors

Like most neuropeptides, ETH acts through G-protein-coupled receptors (GPCRs), the largest family of cell surface proteins. G-protein-coupled receptors are seven trans-membrane proteins that interact with a G protein (comprised of an: α, β, and γ subunits). Upon ligand binding, GCPRs undergo a conformational change altering the intracellular surface of the receptor, thus allowing an activated Gα protein to disassociate from the βγ dimer and interact with target molecules that elicit a signaling cascade in the cell (Caers *et al*., 2012).

The first ETH receptors were identified in *Drosophila* (Iversen *et al*., 2002; Park *et al*., 2002). The expression of ETH receptors has been observed throughout the nervous system of other insects such as *Manduca*, *Bombyx*, and *Aedes*. Figure 8 shows the genomic structure of some of these receptors. In *A. aegypti*, two ETHR isoforms are derived from the same *ethr* gene by alternative splicing of the 3´exon: AeaETHR-A and AeaETHR-B (Dai and Adams, 2009). Additionally, genome surveys have revealed putative ETHRs in various insects including honeybee *Apis mellifera* and *Anopheles gambiae* (malaria mosquito), indicating the conserved nature of ETH-ETHR signaling across the Insecta (Roller *et al*, 2010). To date, all ETH receptors found in insects occur as two subtypes derived from alternative splicing of the 3´exon (ETHR-A and ETHR-B).

These receptors subtypes appear to have differences in ligand sensitivity and specificity (Adams *et al*., 2006). Additionally, there appears not to be much overlapping of receptor subtypes in the same neurons suggesting different functions for the receptors (Adams *et al*., 2006; Zitnan and Adams, 2012). Interestingly enough, ETHRs appear to share monophyletic ancestry with the vertebrate thyrotropin-releasing hormone receptor (TRHR) and the vertebrate receptor for neuromedin U (NMU) (Zitnan and Adams, 2012; Kim *et al*., 2006).

Regulation of Ecdysis Triggering Hormone Release

The proposed mode of action of ETH in inducing ecdysis in insects is thought to take place in two phases of peptide release: early and late (Kim *et al.,* 2004). The initial release of ETH by Inka cells appears to be controlled by a neuropeptide called corazonin which is produced by Ia_1 neurosecretory cells (Fig. 9) (Ewer *et al*, 1996). In *M. sexta* it has been noted that the fall of ecdysteroid titers triggers the release of corazonin into the hemolymph by neurosecretory cells (Kim *et al*., 2004; Zitnan and Adams, 2012). Corazonin then acts on receptors of Inka cells resulting in a low level release of ETH. Then, ETH acts on the ventromedial cells in the brain causing them to release eclosion hormone (EH) into the hemolymph. Eclosion Hormone acting on Inka cells is responsible for the massive ETH release associated with high cGMP levels which ensures the complete depletion of the peptide in the cell (Zitnan and Adams, 2012). The sequential activation of neurons by neuropeptides allows for a controlled progression of the ecdysis sequence in insects.

ETH is an effector of Juvenile Hormone synthesis in vitro: a tale of two stages

In essence, Juvenile Hormone III (JH) is a master regulatory hormone throughout insect life history. In *Aedes aegypti*, JH regulates metamorphosis and previtellogenic ovarian development. As such, understanding the events that control JH synthesis is essential for vector biology and is the subject of the current project. The primary focus of this work was to elucidate the possible role of that the ETH plays in JH synthesis. While its role in inducing stereotypical ecdysis behavioral sequence that results in molts is well characterized; its roles in JH synthesis modulation has not been studied. This is especially striking in *A. aegypti* because expression of ETH receptors is evident in the CA, the JH biosynthetic organ, late in the pupal stage. Furthermore, work on *Manduca sexta* and *Bombyx mori* have also shown that the CA expresses receptors for ETH. Remarkably, despite the stage specific function of ETH, namely as a regulator of ecdysis, Inka cells persist in representative adult holometabolous insects (Zitnan *et al*., 2003; Park *et al.,* 2002). Our results suggest that ETH regulates JH synthesis directly through its receptors in the CA and that it is exerts a biphasic effect 1) stimulatory in the late pupa and 2) inhibitory in the blood-fed female. Although the precise explanation to how ETH modulates JH III synthesis is out of the scope of the study, our studies provides insight into the allatoregulatory roles of ETH in two distinct life stage of *Aedes aegypti*. Both facets of this project provide evidence of ETH as a neuropeptide hormone that should be included into the growing list of regulators of JH biosynthesis.

MATERIALS AND METHODS

Chemicals

Custom made peptide Aea-ETH1 was purchased from Biopetide Co. (San Diego, CA), purified by reverse phase liquid chromatography and assessed to be 98% pure by analytical mass spectrometry (MALDI-TOF MS) and amino acid analysis. Stock of aqueous solutions of *A. aegypti* Aea-ETH1 (DETPGFFIKLSKSVPRI) were prepared at a concentration of 10^{-5} M and stored in aliquots at -80 °C. After two assays, a new aliquot was used.

Insects

A. aegypti of the Rockefeller strain were reared at 28 °C with 80% relative humidity under a photoperiod regime of 16 h light: 8 h dark. Upon emergence adult mosquitos were collected and transferred to a screened container and offered a cotton pad soaked in 3% sucrose solution. Four-day old female mosquitos were fed a blood meal which consists of pig blood equilibrated to 37 °C and ATP was added to a final concentration of 1 mM immediately before use as previously described (Noriega and Wells, 1999).

Determination of Duration of pupal period

Time to eclose for female *Aedes aegypti* mosquitos was determined. Pupae were sexed and collected at 30 minute intervals as they molted from the last larval stadium into the pupal stage. The duration for the pupal stage was determined by scoring at hourly intervals the amount of eclosing adults.

Dissections of corpora allata complexes

Female mosquitos were cold-anesthetized and dissected in *Aedes* physiological saline (APS) ($MgCl_2$ 0.6 mM; KCl 4.0 mM; $NaHCO_3$ 1.8 mM; NaCl 150.0 mM; HEPES 25.0 mM; Sucrose; $CaCl_2$ 1.7 mM). Unless otherwise noted, dissections in this project were of intact *corpora allata-corpora cardiaca* (CA-CC) complex connected to the brain and head capsule and were denoted as BR-CA-CC complex. Dissections were performed on female mosquitos as described by Li *et al*. (2003). Additional preparations of CA complexes for *in vitro* experiments were done using two different surgical separations were performed: (1) of denervated CA-CC complexes in which the CA-CC were separated from the brain; and (2) isolated the CA, in which the CA was isolated from both the brain and the CC.

Quantitative real-time PCR (q-PCR)

Total RNA was isolated using RNA-binding glass powder as previously described (Noriega and Wells, 1993). Contaminating genomic DNA was removed using the DNA-free™ kit (Ambion, Austin, TX). Reverse transcription of RNA was carried out using the Verso cDNA Kit (Thermo Fisher Scientific Inc., Waltham, MA) by an oligo dT priming method according to the manufacturer's recommendations. Relative expression of selected genes was quantified by real-time PCR performed using the 7300 Real-Time PCR System using TaqMan® Gene Expression Assays together with TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Reactions were run in triplicate in a 20 µl volume and normalized to rpL32 mRNA expression for each sample. Standard curves to quantify relative gene copy number were made from serial dilutions of plasmids containing the mosquito genes (300,000; 30,000; 3000; 300; 30 copies of a plasmid per

reaction). Real-time data were collected by the 7300 System SDS Software and analyzed in Microsoft Excel. The primer probes sequences for the house keeping gene 60S ribosomal protein L32 (rpL32, AAEL003396, VectorBase) and Ecdysis Triggering Hormone Receptor (AeaETHR-A/B, VectorBase) are provided in the Appendix.

In vitro ETH Bioassay

The intact *brain-corpora allata-corpora cardiaca* (BR-CA-CC) complex was dissected in *Aedes* physiological saline (APS) ($MgCl_2$ 0.6 mM; KCl 4.0 mM; $NaHCO_3$ 1.8 mM; NaCl 150.0 mM; HEPES 25.0 mM; $CaCl_2$ 1.7 mM). To test the effect of ETH1 on JH III synthesis, samples were treated with different concentrations of ETH; negative controls were run in parallel but not treated with the peptide. The concentration of the peptide was optimized by testing different molar concentrations in pupa (10^{-6} M, 10^{-8} M, and 10^{-9} M) and blood fed adults (10^{-9} M, 10^{-10} M, 10^{-11} M, and 10^{-12} M). Samples were incubated at 32° C for 4 h in (150 µl) tissue culture media M-199 (Lavallette, NJ, USA) containing 2% Ficoll, 25 mM HEPES (pH 6.5) and methionine (50 µM). After incubation, 150 µl of 100 mM sodium sulfide was added and the biological extracts were heated for 30 min at 55 °C. Samples were extracted using 600 µl of hexane, vortexed for 1 min, and centrifuged at 2000 g for 5 min at 4 °C. The supernatants were recovered and analyzed by HPLC-FD using Quantitative Fluorescent Assay as described by Rivera-Perez *et al*., (2012).

JH Quantitative Fluorescent Assay

To quantify the amount of JH III synthesized by the CA, samples were incubated in tissue culture media M-199 (Lavallette, NJ, USA), with or without ETH1 (10^{-9}M) for 4

h in the dark and rocked at 32 °C. To open the epoxide ring, 150 µl of 100 mM sodium sulfide was added to the vials and they were heated for 30 min at 55 °C. Afterwards, 600 µl of hexane was added and the vials were vortexed for 1 minute. Samples were then centrifuged at 2000 g for 5 min at 4 °C. The recovered organic phase was filtered with a Nalgene filter (0.2 mm nylon membrane, #176) and transferred to new vials. They were then dried under N_2 atmosphere and stored at -20 °C until processing. Derivatization of JH III with a fluorescent tag was performed as described previously (Rivera-Perez *et al*., 2012) and subsequently analyzed by reverse phase high performance liquid chromatography (HPLC) coupled to a fluorescent detector (HPLC-FD). The HPLC-FD analysis was performed using a Dionex Summit System (Dionex, Sunnyvale, CA) equipped with a Ultimate 3000 RS pump, a UltiMate 3000 RS Column compartment oven, and an UltiMate 3000 fluorescence detector connected in series and a Chromeleon software version 6.8 SR10. The tagged compounds were separated on an analytical column Acclaim RSL120 C18 (2.1 x 50 mm ID, particle size 2.2 µm) (Dionex) (Rivera-Perez *et al*., 2012).

RNAi mediated depletion

For RNAi-mediated depletion of ETHR target sequences for double-stranded RNA (dsRNA) were designed against a common region to both splice variants of ETHR (AeaETHR-A and AeaETHR-B). As a control, dsRNA was synthesized encoding the Yellow Fluorescent Protein (YFP). Targeted regions were amplified using the primers included in Supplemental Table 1. dsRNA was synthesized using a MEGAscript RNAi kit (Ambion, Austin, TX) following the manufacturer's instructions.

Injections of dsRNA were performed in cold-anesthetized female mosquitos using a Drummond Nanoject II microinjector and a micromanipulator. For pupal stage experiments, 0.96 μg of dsRNA was injected intrathoracically into newly pupated females within 2 hours past pupation. Injected pupae were maintained in the insectary under normal conditions until eclosion. Silencing was confirmed using mRNA extracted from the abdomens of dsRNA treated 66 h female mosquitos and examined by quantitative - PCR. The effect of dsRNA on JH III synthesis was evaluated using intact BR-CA-CC complexes from ETHR and YFP dsRNA injected mosquitos (18 h after eclosion) and evaluating the endogenous levels of JH by JH Quantitative Fluorescent Assay.

The RNAi mediated knock-down of ETH receptors in adults was accomplished by injecting 1.8 μg of ETHRi or YFPi dsRNA into the thorax of one day old cold-anesthetized female mosquitos. After injections mosquitos were maintained in the insectary under normal rearing conditions and fed 3% sucrose solution for 2 days. On the third day after injections, they were mated and blood fed. Then 48 hours after blood meal the mosquitos were dissected. To assess RNAi-mediated depletion of ETHR transcripts total RNA was isolated from the thoraxes of dsRNA injected mosquitos and used for analysis by quantitative PCR. To evaluate effect of ETHR knockdown on JH biosynthesis the BR-CA-CC complexes of dsRNA treated insects were divided into two experimental groups. In one set only the endogenous JH biosynthesis was evaluated while in the second, complexes were incubated with or without ETH1 (10^{-9} M) and assayed for JH III synthesis by JH Quantitative Fluorescent Assay.

Statistical Analysis

Statistical analysis of the data was performed using a *t*-test in the GraphPad Prism version 3.00 for Windows, GraphPad Software. The results were expressed as mean ± S.E.M. and considered significantly different at $P < 0.05$. The values of percentage stimulation of JH III synthesis by treatment were calculated via the following formula: 100 × (activity with ETH1 – activity without ETH1 / activity without ETH1). Values of percent inhibition of JH biosynthesis were calculated using the following formula: percent inhibition (%) = 100 × (JH biosynthesis activity in the presence of ETH1 - JH biosynthesis activity in the absence of ETH1) / JH biosynthesis activity in the absence of ETH1).

RESULTS

Analysis of length of pupal stage

The first part of this work focused on the role that ETH plays in the female pupa. Consequently, it was crucial to determine the duration of the pupal stage for females under our rearing conditions. To this end, pupae were collected at 30 minute intervals; eclosing females were scored hourly (Figure 10). Eclosion began at 46 h (1%) after pupation with the majority emerging at 50 h (40%) and the last at 52 h (5%). Thus in the present study, duration of the pupal stage for female mosquitos was established to be ~ 50 h. Throughout this work the pupal stage was denoted as starting at -50 h spanning to 0 h, the time of adult ecdysis.

Expression Profile of ETH Receptors in the CA During the Pupal Stage

Real-time q-PCR was used to analyze the stage-specific expression of ETH receptors (ETHR-A and ETHR-B) during different developmental stages of female pupa and adults (Figure 11). In pupa the relative ETHR mRNA levels exhibited two peaks, with the highest shortly after pupation at -50 h and a second at -4 h before eclosion. Transcript levels reach their lowest point at -20 h.

Optimization of Molar Concentrations of ETH1 for JH Bioassays in Pupa

The optimal molar concentration of ETH1 was determined by testing the stimulation effectiveness of the peptide at: 10^{-6} M, 10^{-8} M, and 10^{-9} M (Figure 12). Dissected BR-CA-CC complexes of -2 h pupa were incubated in tissue culture media M-199 containing methionine (see Materials and Methods) in the presence or absence of ETH1. JH III synthesis was evaluated by the JH Quantitative Fluorescent Assay.

Concentrations of ETH1 at: 10^{-6} M and 10^{-8} M were found ineffective in influencing JH synthesis when compared to the control groups. However, JH III biosynthesis significantly increased (56%) with the addition of 10^{-9} M ETH1 when compared to the control. Thus all ETH1 bioassays performed in the pupal stage were done with ETH1 at a concentration of 10^{-9} M.

ETH1 Stimulates the Late Pupa CA in vitro

The effect of ETH1 on CA at different developmental time points of the female pupa was determined. Intact BR-CA-CC complexes were dissected and incubated with or without ETH1 (10^{-9} M) and assayed for JH III synthesis. Dissected CA complexes from newly pupated -50 h females were refractory to ETH1 treatment and no JH biosynthesis was detected, similar to untreated controls. The glands continued to be insensitive to ETH1 treatments up to -12 h prior to eclosion. On the other hand, addition of ETH1 to CA complexes from late pupa ranging from -8 h to -2 h before eclosion showed significantly higher levels of JH biosynthesis in response to ETH1 treatment (> 50%) when compared to controls. For CA complexes of -8 h pupa the rate of JH III biosynthesis increased to (12.23 ± 0.52 fmol/CA/h) in response to ETH1 stimulation compared to control (7.567 ± 0.53 fmol/CA/h). The highest increase in CA biosynthetic activity in the presence of ETH1 took place in -6 h pupa. Here, ETH1 stimulated JH synthesis rate to (13.29 fmol/CA/h ± 1.14) when compared to untreated control rate of (5.07 fmol/CA/h ± 0.50). The stimulatory effect of ETH1 declined as the pupal stage come to a close; this was seen in the period of -2 h to 0 h (Fig. 13). On the whole, these findings show that ETH1 stimulates JH biosynthesis in the late pupal CA.

Effect of ETH1 on isolated CA of the Late Pupa

To assess the direct interaction of ETH1 with the CA, -3 h female pupae were dissected in three different types of preparations: brain connected to the CA and CC (BR-CA-CC), CA-CC, and CA alone (Figure 14). For all three preparations samples were treated with ETH1 (10^{-9} M) while negative controls were run in parallel but not treated with the peptide. Separations of the CA from the CC and brain caused and increase in JH biosynthesis in the untreated samples. In addition application of ETH1 caused significantly increases of JH synthesis in the three types of preparation.

Biosynthesis of JH III significantly increased in all three surgical preparations incubated with ETH1. In untreated denervated preparations, where the brain + head capsule were separated from the CA+CC complex, there was a 2.5-fold increase in JH synthesis. CA-CC complexes in the presence of ETH had a 2-fold stimulation in JH synthesis. Similarly, isolated CA complexes treated with ETH1 showed increases of biosynthesis (<50%) at a rate of (25.64 ± 3.97 fmol/CA/h) when compared to the untreated CA controls (13.99 ± 0.10 fmol/CA/h). Taken together, these findings suggest that ETH interacts directly with the CA and this interaction in turn is responsible for the up-regulation in JH biosynthesis observed in the ETH bioassays of pupa.

Effect of RNAi-Mediated Knockdown of ETH Receptor in Late Pupa

To confirm the role of ETH on JH III synthesis during the pupal stage, -48 h pupae were injected with 1) dsRNA targeting the isoforms of ETH receptors (ETHR-A and ETHR-B), or 2) a control dsRNA for yellow-fluorescent protein (YFP). Animals were evaluated ~18 h after adult emergence. To verify the efficiency of silencing, abdomens were used to assess RNAi-mediated depletion of ETHR transcripts. The ETHR

relative transcript levels were quantified by q-PCR and the RNAi-mediated suppression of mRNA levels was found to be 48%. A representative sample of this experiment is shown in Figure 15A. In animals injected with ETHr dsRNA there was a 56.9% reduction of endogenous JH III synthesis when compared with control YFP injected insects. This reduction indicated that RNAi suppression of ETHR expression resulted in a decrease in JH III biosynthesis (Figure 15 B).

Optimization of Molar Concentrations of ETH1 for JH Bioassays in the Blood-Fed Adult

The second facet of this study was to evaluate the role that ETH plays in the adult blood-fed female. To this end, the optimal molar concentration of ETH1 was determined by testing the effectiveness of the peptide at: 10^{-8} M, 10^{-9} M, 10^{-10} M, 10^{-11}M and 10^{-12} M (Fig. 16). Dissected BR-CA-CC complexes of 48 h blood-fed (BF) females were incubated in tissue culture media M-199 containing methionine in the presence or absence of ETH1. JH III synthesis was evaluated by the JH Quantitative Fluorescent Assay. Statistically significant effects on JH III synthesis were seen at the concentrations of 10^{-8} M, 10^{-9} M, and 10^{-10} M when compared to controls. Interestingly, treatments of exogenous ETH1 down regulated JH III synthesis (~ two-fold decrease) in these test groups. The effect of the peptide on JH synthesis at concentrations of 10^{-11} and 10^{-12} M were not significant when compared to the controls. The biosynthetic rates amongst the assays performed at 10^{-8} M, 10^{-9} M, and 10^{-10}M were not significantly different. Therefore, for consistency with the pupal experiments, all ETH bioassays in adult mosquitos were performed with ETH1 at a concentration of 10^{-9} M.

ETH1 Down-Regulates in vitro JH Synthesis in the Blood-Fed Adult

Since ecdysteroids rise after a blood meal in the adult female, the effect of ETH1 on JH synthesis was evaluated at different hours post blood feeding (BF). Intact BR-CA-CC complexes were dissected and incubated in tissue culture media M-199 containing methionine with or without ETH1 (10^{-9} M) and JH synthesis was assessed using the JH Quantitative Fluorescent Assay. Exogenous ETH1 treatment on dissected CA complexes from four day sugar fed (SF) females did not significantly affect JH III biosynthesis; the measured rate of JH synthesis was not statistically different to levels seen in untreated controls. Surprisingly, the addition of ETH1 had an overall inhibitory effect on JH synthesis in blood-fed females ranging from 24 h to 96 h after blood feeding (Figure 17). Statistically significant reductions in JH synthesis (>50%) were measured in ETH treated CA complexes of 24 h, 48 h, and 96 h blood-fed females. The biggest reduction in JH synthesis (71.7%) took place in CA complexes of 24 h blood-fed females treated with ETH1. Here, CA complexes incubated with ETH1 had a reduced JH synthetic rate of (3.131 ± 0.86 fmol/CA/h) when compared to the control rate of (10.86 ± 1.381fmol/CA/h).

Effect of ETH1 on CA Separations of the Blood-Fed Adult

The direct interaction of ETH1 with the CA was determined in 48 h blood-fed (BF) female mosquitos. The ETH bioassays were performed *in vitro* on CA dissected in three different types of preparations: brain connected to the CA and CC (BR-CA-CC), CA-CC, and CA alone (Figure 18). Samples for the three types of preparations were incubated in presence or absence of ETH1 (10^{-9} M) and JH III synthesis was evaluated. Overall, treatment with ETH1 significantly decreased JH biosynthesis in blood-fed CA

complexes. In BR-CA-CC preparations there was a 39% decrease in JH synthesis when compared with controls. Declining rates of JH synthesis were most evident in CA-CC separations incubated with ETH1; JH synthesis was reduced by 67%. Similarly ETH1 inhibited JH synthesis on isolated CA complexes as demonstrated by an inhibition of 38% when compared to the untreated CA controls. These results, when taken together, indicate that ETH interacts directly with the CA in the BF female and mediates the observed inhibition of JH III biosynthesis.

Effect of RNAi-Mediated Knockdown of ETH Receptor in the Blood-Fed Adult

The effect of ETHR depletion on JH synthesis was examined by performing *in vitro* ETH bioassays on BR-CA-CC complexes dissected five days post dsRNA injections (Figure 19). Dissected BR-CA-CC were incubated with or without ETH1 (10^{-9} M) and assayed for JH III synthesis. To assess the efficiency of RNAi-mediated depletion of ETHR transcripts, total RNA was isolated from the thoraxes of dsRNA injected mosquitos and analyzed by q-PCR. The ETHR knockdown efficiency was found to be 67- 78% in dsRNA injected insects when compared to controls. A representative sample of this experiment is shown in Figure 19A. To assess the role of ETH in the observed inhibition of JH synthesis in BF adults, endogenous JH levels were quantified from dsRNA injected insects. As expected, ETHR knockdown resulted in higher endogenous levels of JH synthesis in dsRNA ETHR injected insects (25.04 ± 1.46 fmol/CA/h) when compared to YFP controls (15.59 ± 1.92 fmol/CA/h). In contrast, addition of ETH1 to YFP treated complexes decreased JH synthesis 3-fold when compared to dsRNA ETHR injected samples (Figure 19B).

DISCUSSION

ETH stimulates in vitro JH synthesis in the maturing CA of the late pupa

Previous work on neuropeptide GCPRs of *Bombyx mori* has shown that the CA of 4th and 5th instar larva has a high expression of ETH receptors (Yamanaka *et al*., 2008). The expression analysis coupled with results of *in situ* hybridization has suggested a putative role of ETH in the observed rises of JH titers during ecdysis (Baker *et al*., 1987). Moreover, preliminary data in our lab has corroborated the presence of both ETH receptor isoforms in the CA-CC complexes of the late pupa of *Aedes aegypti* (Figure 6). The primary focus of this work was to study the possible role of the neuropeptide ETH in JH synthesis. To this end, the effect of ETH on JH synthesis was evaluated in the context of two distinct developmental stages of the mosquito: the pupal stage and the adult. These two stages represent two different phases of the CA, the activation of the CA before adult emergence, and the cyclic rise in JH associated with the second gonadotropic after a blood-meal.

The first facet of the current work focused on the female pupa. The rationale behind the approach is that the CA undergoes a maturation process in the late pupal stage which is demonstrated in the coordinated expression of all JH biosynthetic enzymes (Nouzova *et al*., 2011; Rivera-Perez *et al*., 2013; Nyati *et al*., 2013). The working hypothesis was that, in the pupa, ETH could act through its receptors in the CA to regulate this process. We posited that if ETH played a role in the immature pupal CA, then *in vitro* stimulation with the peptide would induce premature JH synthesis. Testing of ETH1 in an *in vitro* assay on the BR-CA-CC complex revealed that in the late pupa, a period ranging from -8 h to -2 h prior to emergence, ETH has a stimulatory effect on JH biosynthesis. Notably,

this stimulatory effect coincides with the previously observed coordinated expression of JH biosynthetic enzymes like methyl farnesoate epoxidase (the last enzyme in the JH biosynthetic pathway) (Figure 4). In contrast, ETH bioassays showed that the CA was refractory to ETH stimulation in early pupa: newly pupated females -50 h extending to -12 h pupa and immediately after emergence at 0 h (Figure 13).

To further evaluate the results from ETH bioassays, the relative expression levels for ETH receptors were analyzed in the pupal stage. Previous work on *Manduca sexta* and *Bombyx mori* has shown that the transcriptional activation of both the ETH peptide and its cognate receptors rises in accordance to 20E levels (Zitnan *et al*., 1999; Zitnan and Adams, 2012; Adams *et al.*, 2006). Results from our ETHR expression survey of the pupal stage confirmed these previous findings (Figure 11). Moreover, ETHR expression levels follow the previously reported 20E levels in pupal stage of *Aedes aegypti* (Margam *et al*., 2006). In this context, the ETH bioassay results in early pupa indicate that despite the high expression of ETH receptor transcripts, JH stimulation by ETH is not feasible as there is no transcriptional activation of the JH biosynthetic enzymes (Nouzova *et al*., 2011).

Notwithstanding, there is a clear phenotype of increased JH III synthesis derived from exogenous treatments with ETH1 in the late pupa. To discern if the observed up-regulation in JH synthesis is a result from the interaction of ETH ligand with receptors in the CA, *in vitro* ETH1 assays were performed on surgical separations of the BR-CA-CC complex. All separations treated with ETH1 had a stimulatory effect on JH synthesis. But more importantly, it showed that the CA was directly responsive to ETH stimulation as the stimulatory effect was observed in isolated CA complexes (Figure 14). RNAi

mediated silencing of ETH receptors in pupa further corroborated the stimulatory effect of ETH on JH biosynthesis. Silencing of both receptor isoforms resulted in a reversal of the physiological effect of ETH on JH synthesis; in short there was a decrease of JH synthesis in all ETHRi samples (Figure 15). Hence as expected the interruption of ETH signaling by RNAi knockdown resulted in a reduction of JH synthesis. Knock-down efficiency of ETH transcripts was verified by real-time q-PCR to be ~ 48 %. The moderate knock-down efficiency can be attributed to the time constraint presented by the duration of pupal stage (50 hours) in our study. While the norm for RNAi experiments is to wait for 4 days post injection to verify RNAi effect (Perez-Hedo *et al*., 2013; Nyati *et al*., 2013), we had to assay RNAi insect ~ 3 days post injection (Blandin *et al*., 2002). Nonetheless, the silencing did result in a phenotype that had a reduction in JH III synthesis.

The release of ETH from Inka cells is predicated by a decrease in 20E titers (Kingan and Adams, 2000). With this in mind, it is expected that in the early pupa high titers of 20E would not have allowed Inka cells to gain competence to secrete ETH. Therefore for ETH mediated stimulation of JH synthesis in the pupa it appears two caveats must be met: 20E titers must be low and the transcriptional activation of JH biosynthetic enzymes should be in place. A plausible notion is that ETH may be an effector that indirectly modulates the flux of the biosynthetic pathway but may not necessarily be directly involved in transcriptional activation of the enzymes. For instance, the ETH signaling mechanism in itself may be regulating JH synthesis by increasing Ca^{2+} levels involved in signal transduction within the CA. It has been shown that ETH signaling occurs via ETHR (GPCRs) which activate the Gαq pathway to release intracellular Ca^{2+} stores (Kim

et al., 2004). Furthermore, work in *M. sexta* and the cockroach *Diploptera punctata* have shown that rises of intracellular Ca^{2+} correlate with increases in JH synthesis (Kikukawa *et al*., 1987; Granger *et al*., 1992; Rachinsky and Tobe, 1996).

The current study is the first body of work to focus on the role of ETH in pupal stage of an insect; even in *Aedes*, previous studies have focused on the larval stage (Dai and Adams, 2009). While the precise mechanism on how ETH stimulates JH synthesis is not yet available, this study provides valuable insight into a possible allatoregulatory role of ETH in the late pupa.

In the blood-fed adult ETH down-regulates in vitro JH synthesis

Spurring the notion that ETH may be more than just a regulator of ecdysis is the persistence of Inka cells in the adult stage of insects. Immunohistochemistry work on Inka cells has demonstrated that immune-reactive cells do not disappear in the adult stages of representative holometabolous insects such as: *Bombyx mori*, *Drosophila melanogaster*, and *Aedes aegypti* that suggests the likely role of ETH signaling in adults (Park *et al.,* 2002; Zitnan *et al*., 2003; Zitnan and Adams, 2012). This coupled with the known regulation by rising ecdysteroid titers of ETH and ETHR expression prompted the following question: Given the rise of 20E titers after blood-feeding, does ETH play a role in the subsequent modulation of JH synthesis post-blood feeding? To answer this question three approaches were taken: 1) performed *in vitro* ETH1 bioassays on BR-CA-CC complexes of blood-fed females, 2) determined the effect of ETH on isolated components of the CA complex of 48 h blood-fed females, and 3) RNAi mediated knock-down of ETH receptors. Surprisingly, results from ETH1 *in vitro* BR-CA-CC assays

showed that the peptide had inhibitory effect on JH III synthesis (Figure 17). The down-regulation of JH biosynthesis was evident in adult females at: 24 h, 48 h, and 96 h after blood-feeding. To further assess if this effect was a result of a direct interaction of ETH and the CA, surgical separations were performed as in the experiments done in pupa. Once again, ETH bioassays showed that the CA was indeed directly responsive to the effect of ETH (Figure 18). Additionally, measured JH levels were lower for complexes still connected the CC both in the presence and absence of ETH, indicating that the CC may also play an inhibitory role on synthesis. The effect of ETH on the CC was not unexpected as we analyzed the expression of ETH receptors of the CA-CC complex and found transcripts in both endocrine organs (data not shown). When these results are taken together they show that the allatostatic effect of ETH is mediated by a direct interaction of ETH with the CA. Silencing of ETH receptors by RNAi further corroborated the inhibitory effect of ETH on JH synthesis in blood-fed females. As expected, disruption of ETH signaling by RNAi mediated knock-down of receptors resulted in higher basal levels of JH synthesis in dsRNA ETHR injected samples when compared to the control YFP (Figure 19b). Thus, indicating that silencing of ETH receptors resulted in the reversal of the decrease in JH biosynthetic rates seen in YFP animals. Since silencing of ETHR by RNAi was systemic it was important to verify if the allatostatic role of ETH on synthesis was due to a direct modulation of CA activity or by an indirect effect, to this end *in vitro* ETH1 bioassays were performed on dsRNA injected females (Figure 19b). Here, addition of ETH1 resulted in down-regulation of JH biosynthesis in both YFP and ETHR complexes; however a marked three-fold decrease in synthesis was seen in dsRNA YFP injected insects when compared to dsRNA ETHR samples. To verify that results

from silencing experiments were due to effective ETHR knock-down, suppression of ETHR mRNA levels was confirmed by real-time PCR of thoraces of dsRNA injected females and found to be 67-78% (Figure 19a). It is important to note, that despite robust ETHR silencing there are still ETH receptors present that are able to mediate the reduction in JH biosynthesis seen in both control YFP and the ETHR samples (Figure 19a). However, the knock-down of the receptors significantly disrupts the inhibitory role of ETH in ETHRi samples exposed to ETH1 and as consequence JH biosynthetic rates do not drop as do the YFPi controls. Overall, the observed down-regulation of JH synthesis in dsRNA YFP injected animals underscores the inhibitory role of ETH in JH biosynthesis in the blood-fed female.

At first glance the allatostatic role of ETH in the blood-fed females appears counter intuitive. How does a neurohormone have opposite actions on JH biosynthesis throughout development, from a putative allato-stimulatory factor in the late pupa to an inhibitory factor in the blood-fed female mosquito? However, the biogenic amine dopamine (DA) is an example of allatoregulatory factor that has been established to have stage-dependent differences in the modulation of JH synthesis in the insects: *B. germanica, D. melanogaster*, and *M. sexta* (Pastor *et al*., 1991; Granger *et al*., 1996; Gruntenko and Rauschenbach, 2008). For instance, in *M. sexta*, DA has been demonstrated to stimulate the biosynthesis of the CA in the first two days of the fifth larval stage, but it is inhibitory at the prepupal stage (Granger *et al*., 1996). For both *M. sexta* and *Drosophila* the ontogenic differences in DA action on JH synthesis has been suggested to be mediated by two types of D1-like and D2-like GPCRs (Granger *et al*., 1996; Gruntenko *et al*., 2012). So it is a possible that different ETH receptor isoforms may be mediating the stimulatory

and inhibitory roles that ETH plays in the female mosquito. Perhaps, during development the differential expression of ETHR-A versus ETHR-B in the CA may dictate the mode of action of ETH. It is widely known that there is not much overlap of ETHR-A and ETHR-B in neurons; suggesting that two ETHR isoforms may mediate different roles in the ecdysis sequence (Kim *et al*., 2006a). Still, not much is known of the signaling of ETHR-B neurons, but our work shows that in the late pupa both isoforms are present in the CA with ETHR-A being more highly expressed (Figure 6). Experiments in the present project did not discriminate both ETHR isoforms, so additional experiments would need to be done to determine their specific roles. Another plausible way in which ETH maybe inhibiting JH biosynthesis is by an ETH induced rise in cytosolic Ca^{2+} from intracellular stores which might disrupt an optimal Ca^{2+} homeostasis within the CA. For instance, work on adrenal glands of rats, has suggested that intracellular Ca^{2+} levels are finely tuned to an optimal concentration such that rise or fall below this threshold due to action of Ca^{2+} ionophores results in activation or deactivation of corticosterone production (Lymangrover and Keku, 1984). Moreover, work on the CA of fifth stadium larva of *Manduca sexta* showed that there were stage specific differences to how Ca^{2+} affected JH synthesis; this was further supported using both caffeine and ionophores which induce the release of Ca^{2+} from intracellular stores (Allen and Granger, 1992b). Likewise, the cockroach allatostatin peptide Dippu-allatostatin 7 (AST) is another example of an allatoregulatory peptide which has been shown to have ontogenic differences in the control of sesquiterpenoid synthesis; stimulating synthesis in early embryos while inhibiting it in mid to late embryos (Stay *et al*., 2002). At present, the precise mechanistic mode in which ETH inhibits JH III synthesis in the blood-fed female

is unknown; this is the first body of work to study the role of ETH in the adult stage of *Aedes aegypti*.

Lastly, besides the direct effect of ETH on the CA, one cannot discount the possibility that the effect of ETH on JH synthesis may be due to the down-stream signaling process of ETHR responsive neurons. So that activated neurons may be mediating the inhibition of JH synthesis through release of allatostatic peptides. Indeed, ETHR-A responsive neurons release various neuropeptides including: crustacean cardioactive peptide (CCAP), eclosion hormone (EH), kinins, and myoinhibitory peptides (MIPs) (Kim *et al*., 2006a; Kim *et al*., 2006b). For example, *in situ* hybridization work in the larva of *M. sexta* has shown that ETHR-A responsive neurons of the abdominal ganglia produce short neuropeptide F (sNPF) and allatostatins (Type A) upon activation (Kim *et al*., 2006). Additionally, in *B. mori*, sNPF is expressed in the CC-CA complex and *in vitro* CA assay revealed an inhibitory effect on JH synthesis (Yamanaka *et al*., 2008).

Evolutionary perspective

For a neurohormone like ETH to have more than one role in the life history of an insect, from a major regulator of ecdysis to a putative regulatory peptide of JH synthesis is not uncommon. Juvenile Hormone and 20E are excellent examples of the versatility of hormones, with both having pleiotropic functions in the life history of insects. In the immature insect both hormones are known as regulators of molting and metamorphosis, whereas in the adult they play a role in insect reproduction (Hartfelder, 2000; Flatt *et al*., 2005). In fact, the original function on JH has been suggested to have been regulation of reproduction, with its metamorphic role being acquired secondarily in evolution (Sehnal

et al., 1996; Tobe and Bendena, 1999). Underscoring the pleiotropy of JH are the other known roles it has in insect physiology such as facultative polymorphism and caste differentiation which have allowed insect flexibility in life history strategies (Hartfelder, 2000). Furthering our understanding of JH biosynthesis not only has practical purposes such as in vector control but also in compounding our understanding of the evolution of hormones.

Most modern animals belong to either of two evolutionary lineages, Protostomia (of which most invertebrates including insects belong) or Deutorostomia (to which vertebrates belong and some invertebrates like echinoderms) (Grimmelikhuijzen and Hauser, 2012). The split of the two lineages from a common bilaterian ancestor is believed to have taken place between 700 - 600 million years ago (Peterson et al., 2008). There is debate as to whether the urbilaterian ancestor had a centralized nervous system prior to the phylogenetic split. This idea has been suggested by work on the mushroom bodies of annelids and the vertebrate pallium (cortex) of mice which indicates that a common ancestor possessed a precursor structure to both brain centers and that complexity was acquired divergently (Tomer *et al*., 2010). Conversely, it is commonly regarded that nervous system and peptide signaling evolved from cnidarian ancestors independently in the two lineages (Grimmelikhuijzen and Hauser, 2012; Wirmer et al., 2012).

Nonetheless, many peptide hormone families, endocrine cells, and endocrine organs appear to share homology in both protostomes and deuterostomes (Park *et al*., 2002; Loof *et al*, 2012; Wirmer *et al*., 2012). An example of a peptide hormone family

that has been evolutionarily conserved is the vertebrate peptide neuromedin U (NMU) and the insect peptides which have a C-terminal –PRXamide (PRXa) motif (Claeys *et al*., 2005). Insect PRXa peptides bind GPCRs and among them are members of the pyrokinins (-FXPRXa), cardioactive CAP2b-like peptides (-FPRXa), and ecdysis triggering hormones (-PRXa) (Park *et al*., 2002; Claeys *et al*., 2005). In fact, work in *Drosophila* has shown that ETHR is related to both NMUR and the vertebrate thyrotropin-releasing hormone receptor (TRHR) (Park *et al.*, 2003; Kim *et al.*, 2006). Lending weight to this hypothesis is work on *Drosophila melanogaster* which showed that TRH was able to elicit ETH receptor activation at 10^{-5} M (Park *et al.*, 2003). The same effect has also been documented in *Manduca sexta* in which TRH induced weak but significant activation of ETHR-A at 10^{-5} M (Kim *et al.*, 2006).

Thyrotropin-releasing hormone is a releasing hormone in vertebrates which controls the release of thyroid-stimulating hormone (TSH) from the pituitary (Loof *et al*., 2012). Vertebrate releasing hormones are synthesized in the hypothalamus and then transported by the portal blood vessel system to the pituitary (De Loof *et al*., 2012). In contrast, insects which do not have a portal blood vessel system instead, have neurosecretory cells that directly innervate neurohemal organs such as the CA-CC complex (Virant-Doberlet *et al*., 1994). Interestingly, in insects there are also neurosecretory cells (NCS) such as ventral medial cells (VM) which are located in the pars lateralis of the brain (Ewer *et al*., 1996). Remarkably, Inka cells which secrete ETH are non-neuronal cells that are located on the surface of the lateral tracheal trunks. Likewise in vertebrates there are examples of hormones that are also peripherally produced: ghrelin and leptin (Loof *et al*., 2012). Moreover, structurally and functionally

there are similarities between the hypothalamus-pituitary complex of vertebrates and the pars intercebralis (PI)/ pars lateralis / (PL) - corpora cardiaca- corpora allata complex (Loof *et al*., 2012; Wirmer *et al*., 2012). For instance, the CC are paired glands like the pituitary in vertebrates and they are considered homologs of the neurohypophysis (Hartenstein, 2006). Located proximal to the neurohypophysis is the adenohypophysis which has been suggested to also be homologous to the CA of insects (Wirmer *et al*., 2012). Lastly, JH controls development and vitellogenesis in insects which parallel some of the products released by the adenohypophysis such as: gonadotropins and thyrotropin (Wirmer *et al*., 2012).

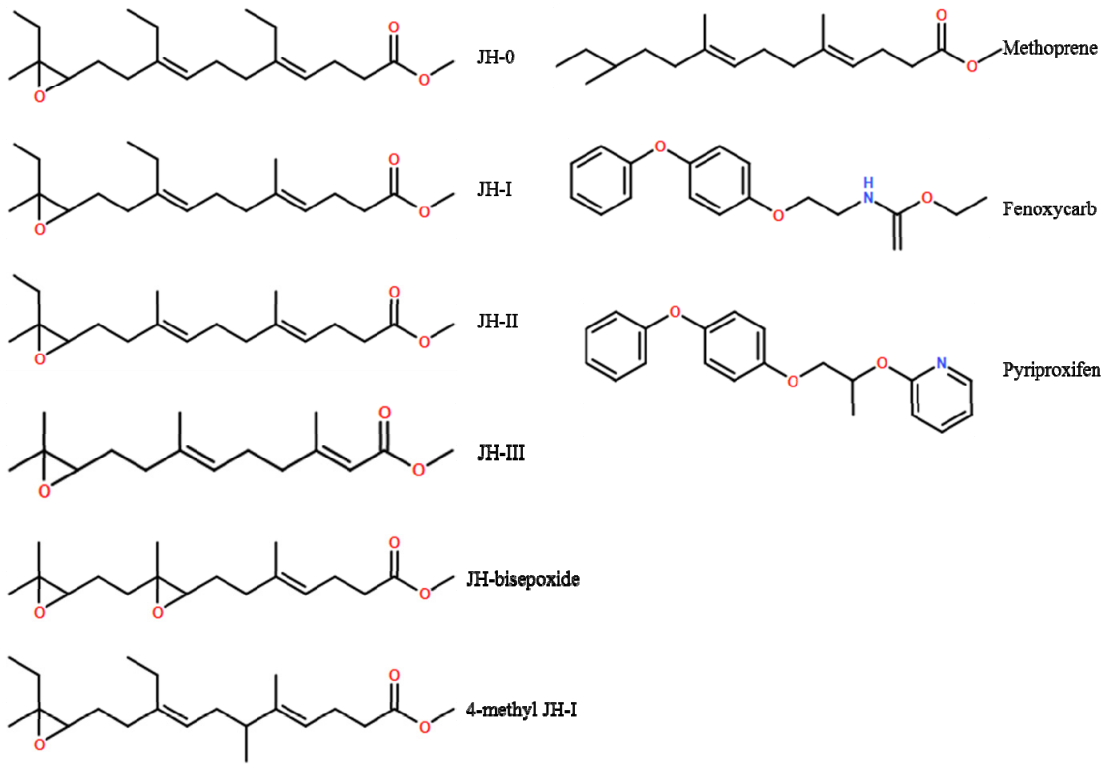

Figure 1. Chemical structures of examples of naturally occurring juvenile hormone (JH) homologues and three JH analogs (agonists).

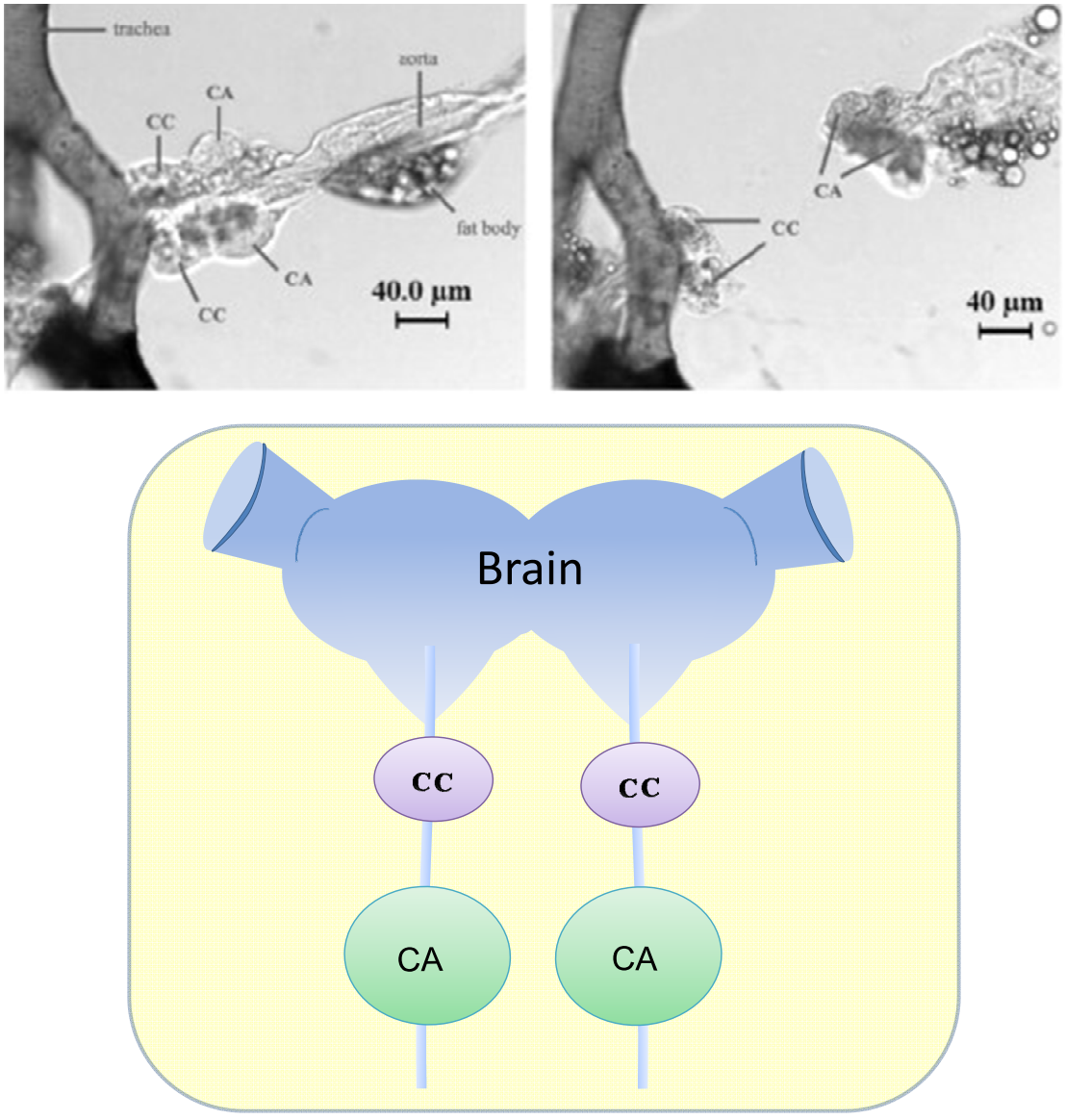

Figure 2. Morphology of the retrocerebral complex of *Aedes aegypti*. Schematic dorsal view depicting retrocerebral complex comprised by the: brain, corpora cardiaca (CC); and corpora allata (CA). Neurosecretory cells from the brain project their axons to the CC and CA.

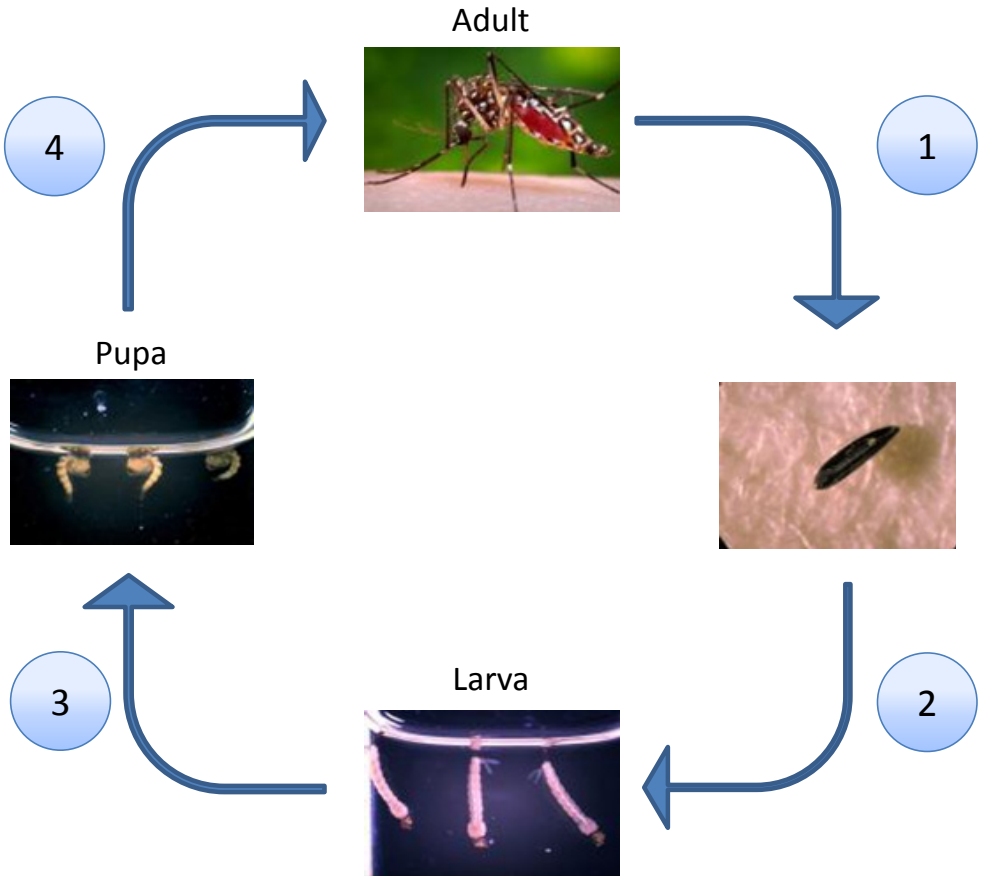

Figure 3. Diagram depicting the life cycle of *Aedes aegypti*. (1) Female mosquitoes lay their eggs, eggs hatch into larva when water inundates the eggs. (2) After hatching larva undergo four larval instar molts. (3) At the end of the fourth instar, larva pupate, molt in to a pupa. (4) ~ 48 hours later, pupa emerge into the adult mosquito.

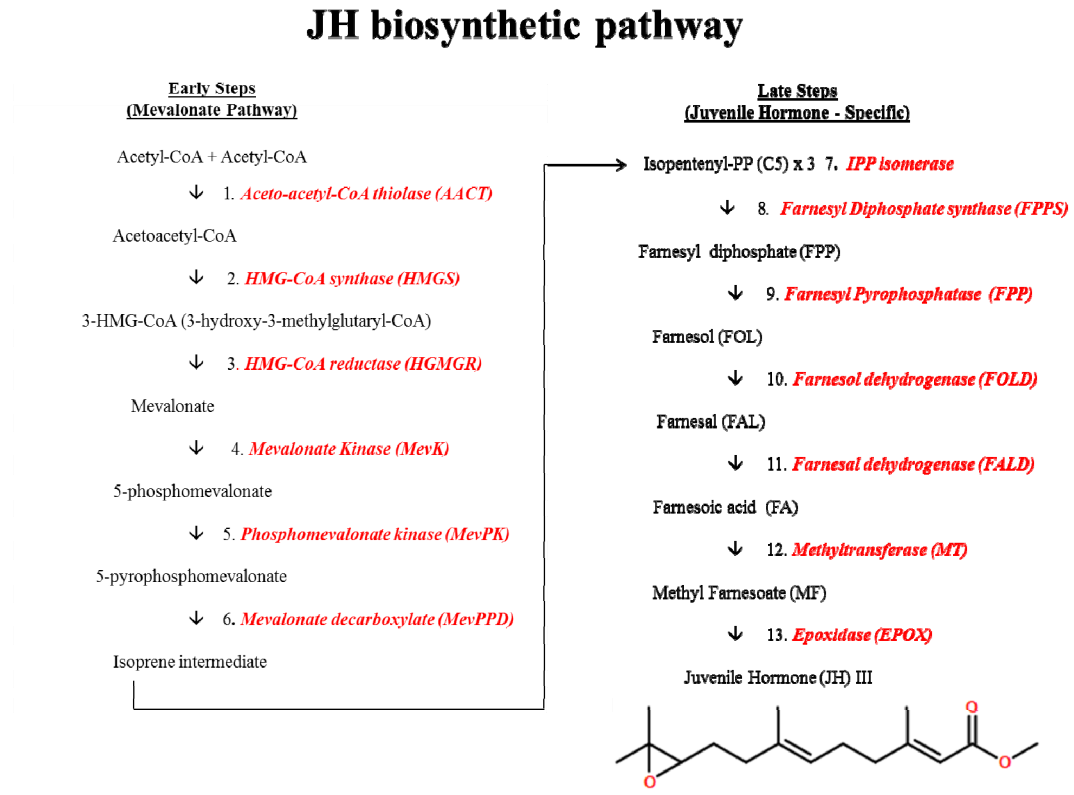

Figure 4. The Juvenile Hormone biosynthetic pathway. The pathway is divided into two steps: early and late steps which involve 13 sequential enzymatic reactions. The early steps go by the way of the mevalonate pathway; the later steps are unique to JH-biosynthesis.

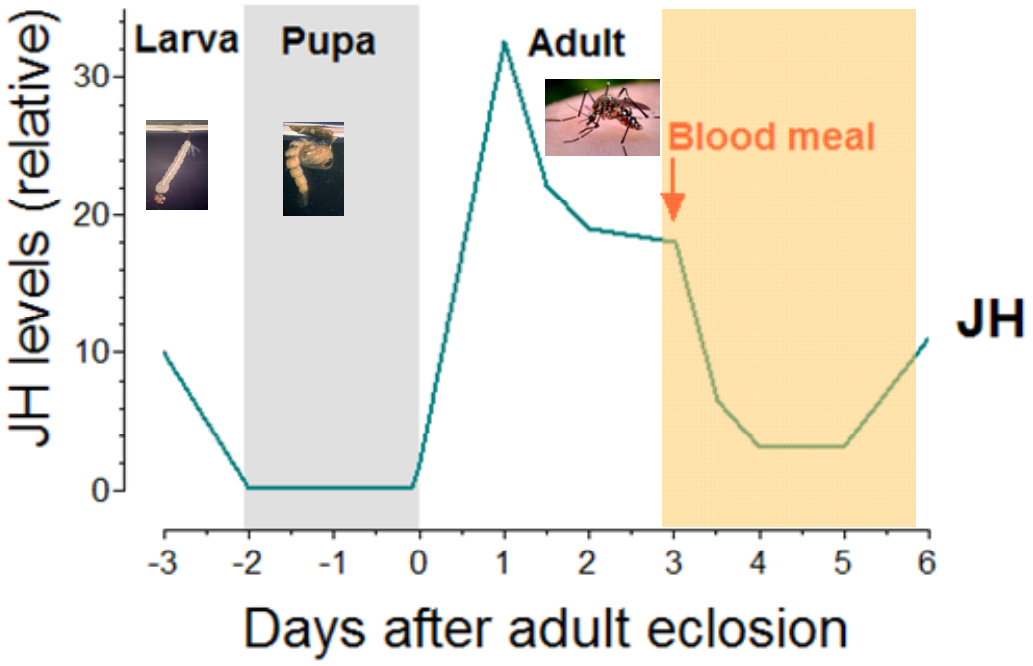

Figure 5. Diagram depicting the JH III (JH) levels in Aedes aegypti. JH levels drop at the last larval stadium which allows pupation to take place. In the last six hours of the pupal stage, the CA synthesizes basal levels of JH. Newly emerged females synthesize low amounts of JH that increase during the first day. After a blood meal, JH levels begin to decrease and reach their lowest point 24 h later. Forty-eight hours post blood meal, JH levels begin to rise and return to a high level on the third day.

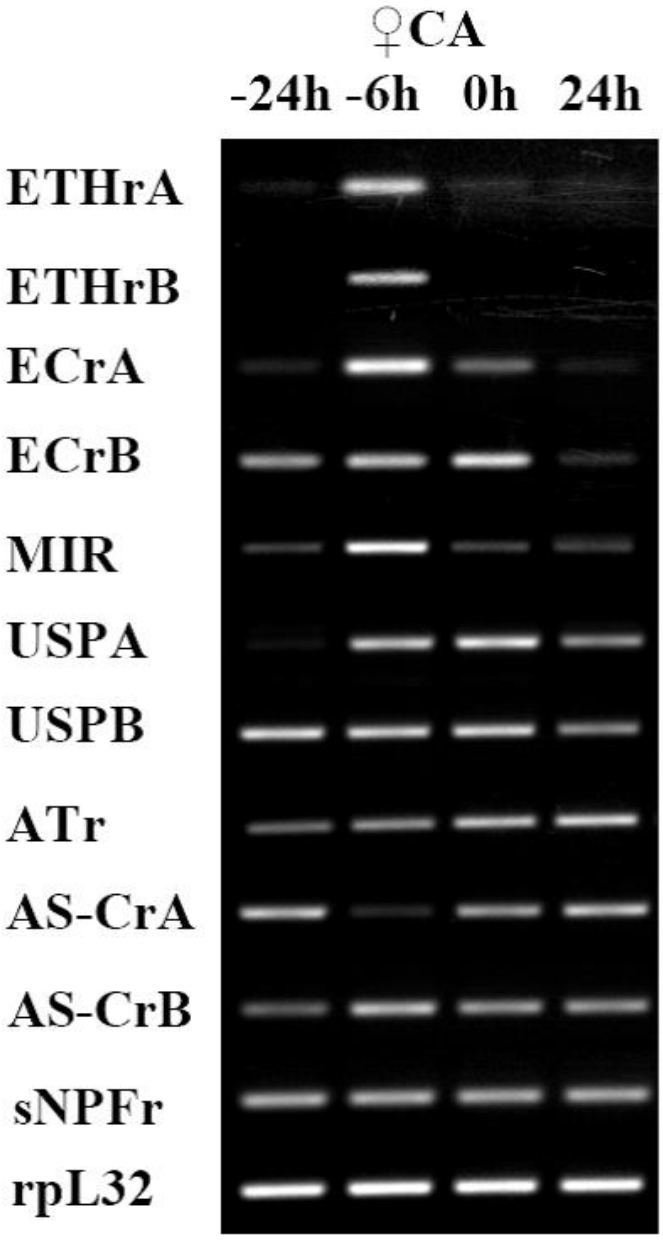

Figure 6. Expression of various receptor transcripts in the CA before and after adult emergence. The genes studied include: ecdysis triggering hormone receptors A and B (ETHrA and B), ecdysone receptors A and B (ECrA and B), mosquito insulin receptor (MIR), Ultraspiracle receptor A and B (USPA and B), allatotropin receptor (ATr), AS-C receptor A and B (AS-CrA and B) and a short neuropeptide F receptor (sNPFr). Ribosomal protein L32 (rpL32) was used as a control.

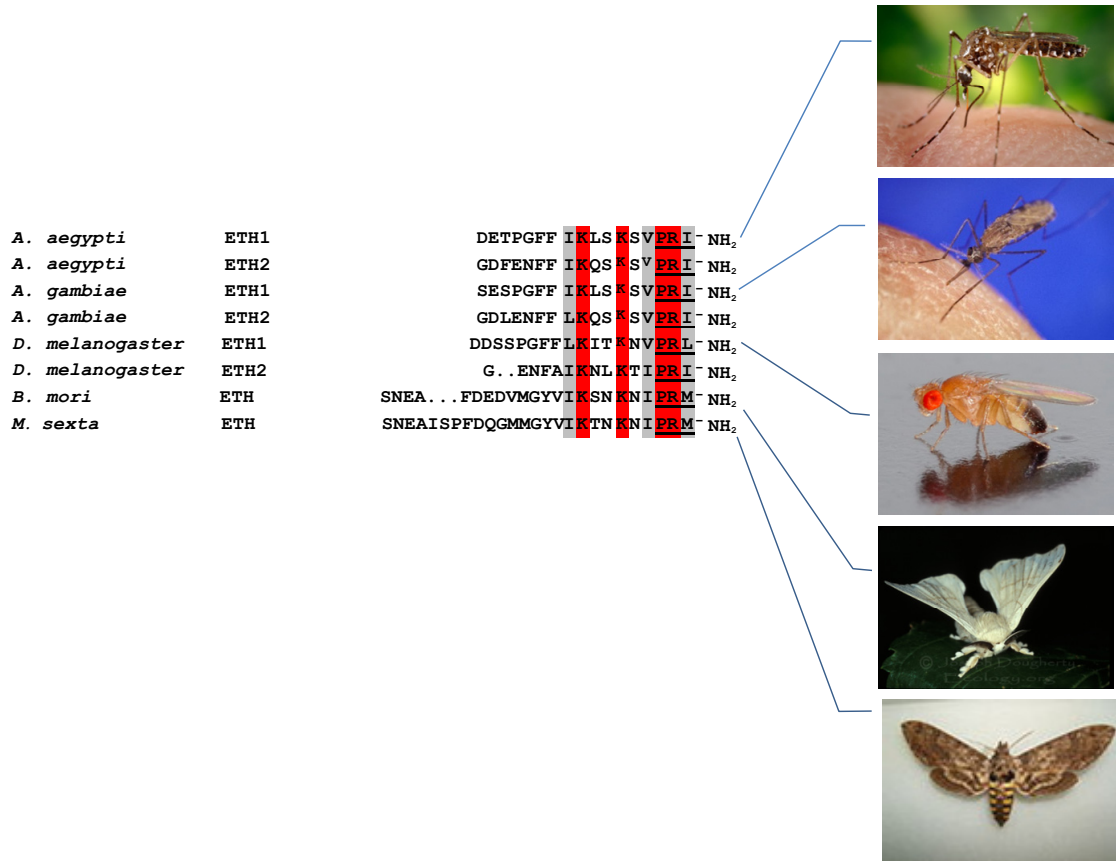

Figure 7 .Amino acid sequence alignment of sequences of ecdysis triggering hormones in insects. Conserved amino acid alignment is indicated in red; semiconservative amino acid alignment is indicated in grey.

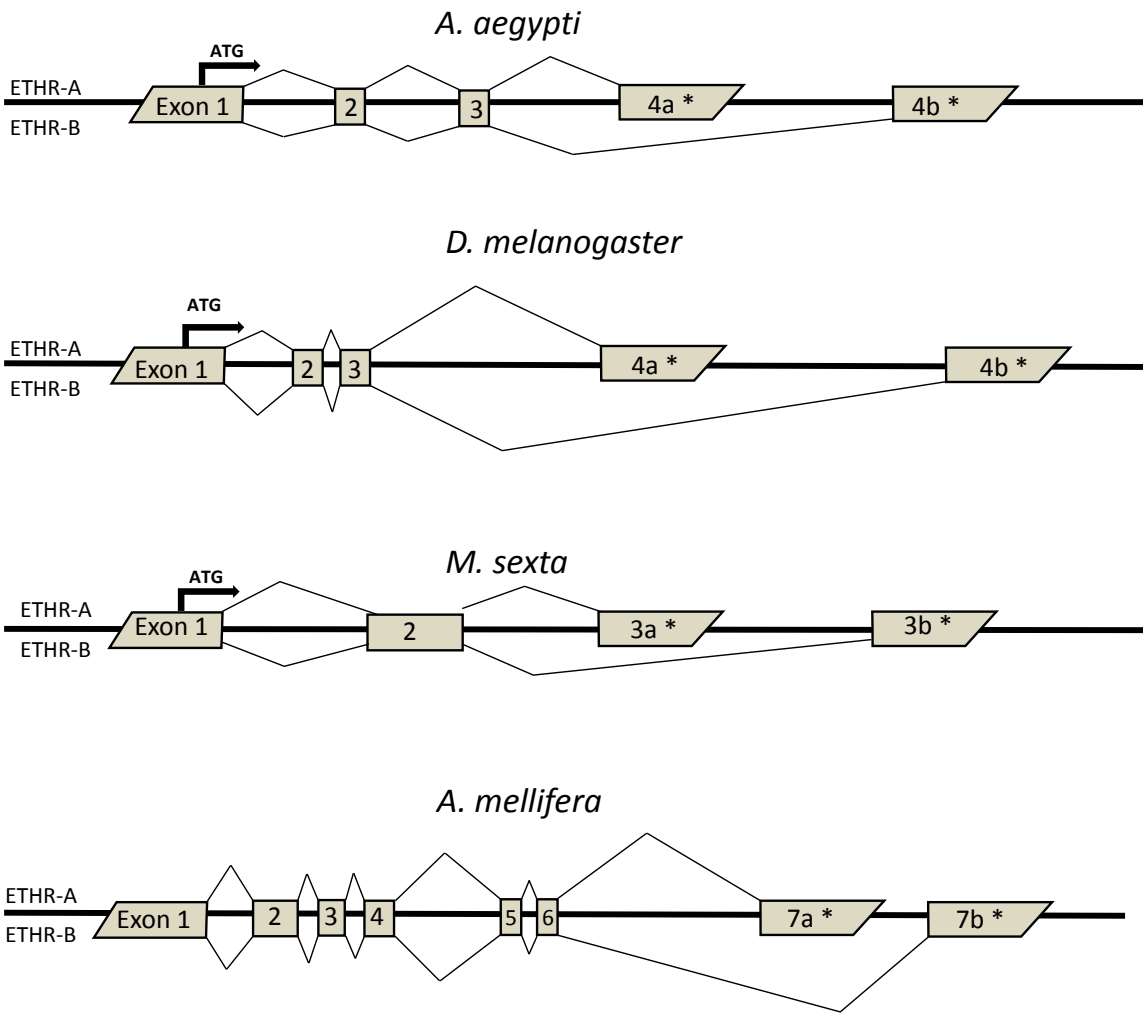

Figure 8. Genomic map of ETH receptors. Structure of ETHrs in *A.aegypti* (Dai and Adams, 2009), *D. melanogaster* (Park *et al.*, 2003), *M. sexta* (Kim *et al*., 2006) and *A. mellifera* (Roller *et* al., 2010). Alternative splicing of the 3' exon results in expression of two ETHR subtypes subtypes (ETHR-A and ETHR-B) and are depicted as slanted ends. The ATG indicates the putative translation initiation site and the asterisks indicate location of the stop codon.

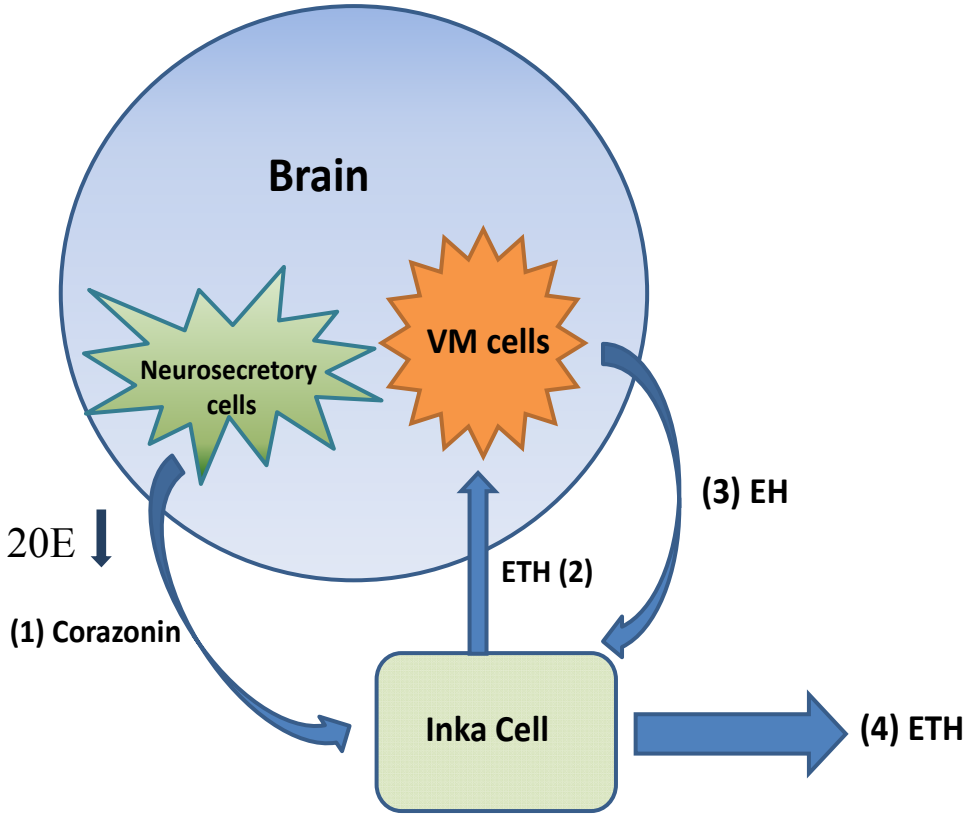

Figure 9. Model for initiation of ETH release. (1) Declining titers of 20E triggers the release of corazonin into the hemolymph by neurosecretory cells. (2) Corazonin acts on receptors of Inka cells resulting in low level release of ETH. (3) ETH acts on the ventromedial cells (VM) in the brain triggering release of eclosion hormone (EH) into the hemolymph.(4) EH acting on Inka cells is responsible for the massive ETH release.

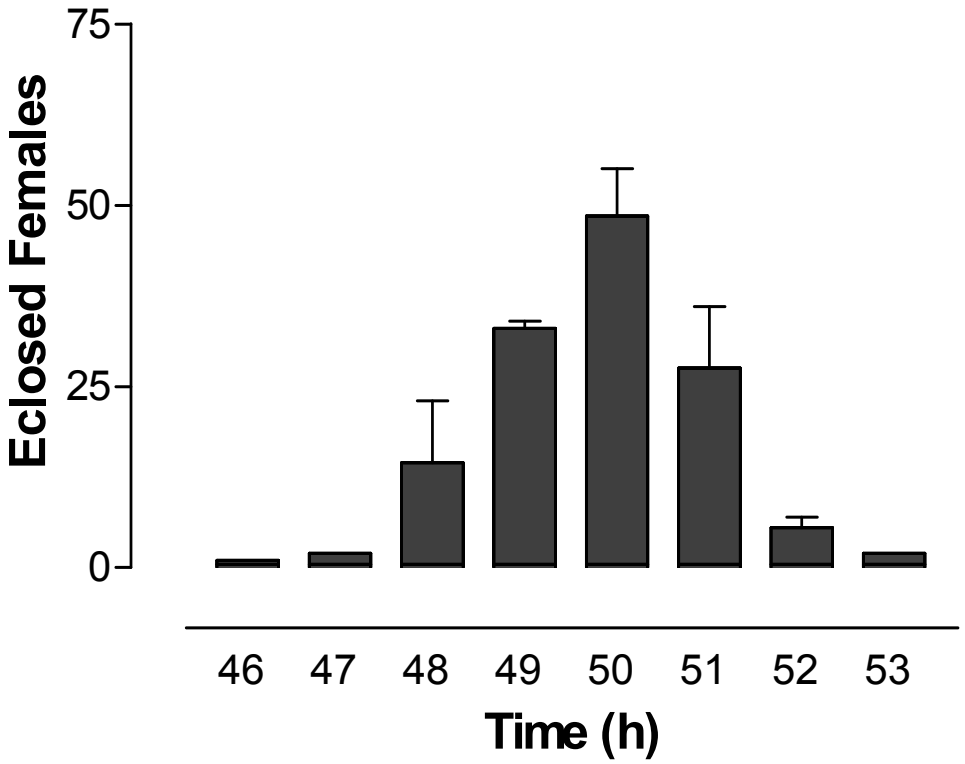

Figure 10. Eclosion profile for pupal stage of *Aedes aegypti***.** The data is expressed as a percent of eclosed mosquitos during hourly intervals. Comparisons among the eclosion percentage for 48 h, 49 h, 50h,51 h, and 52 h. (n= 263)

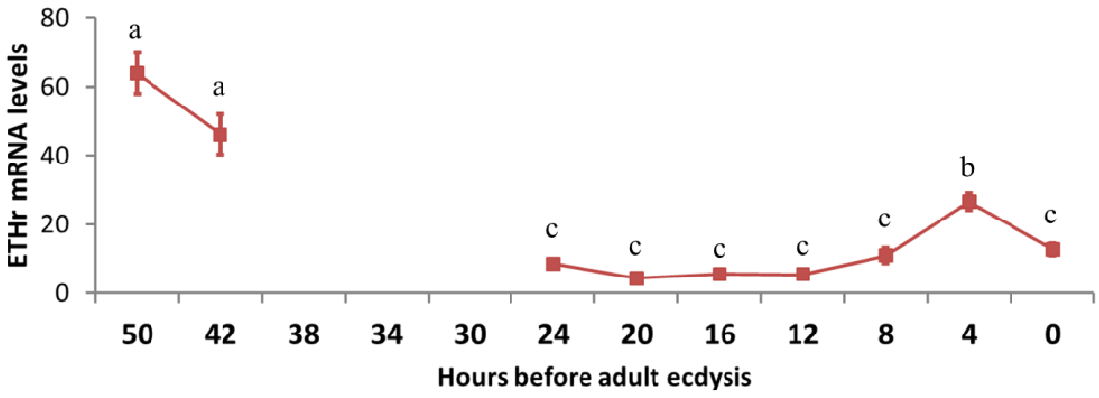

Figure 11. Levels of ETH receptors follow ecdysteroid levels in pupal stage. Transcript levels of ETH receptors (ETHR-A and ETHR-B) in the CA of female pupa during different developmental stages; from newly pupated -50 h (50) up to newly emerged 0 h (0). Expression levels of ETH receptors are expressed as a copy number / 10,000 copies of rpL32 mRNA. Each RT-PCR data point is average of three independent biological replicates of groups of 20 CAs.

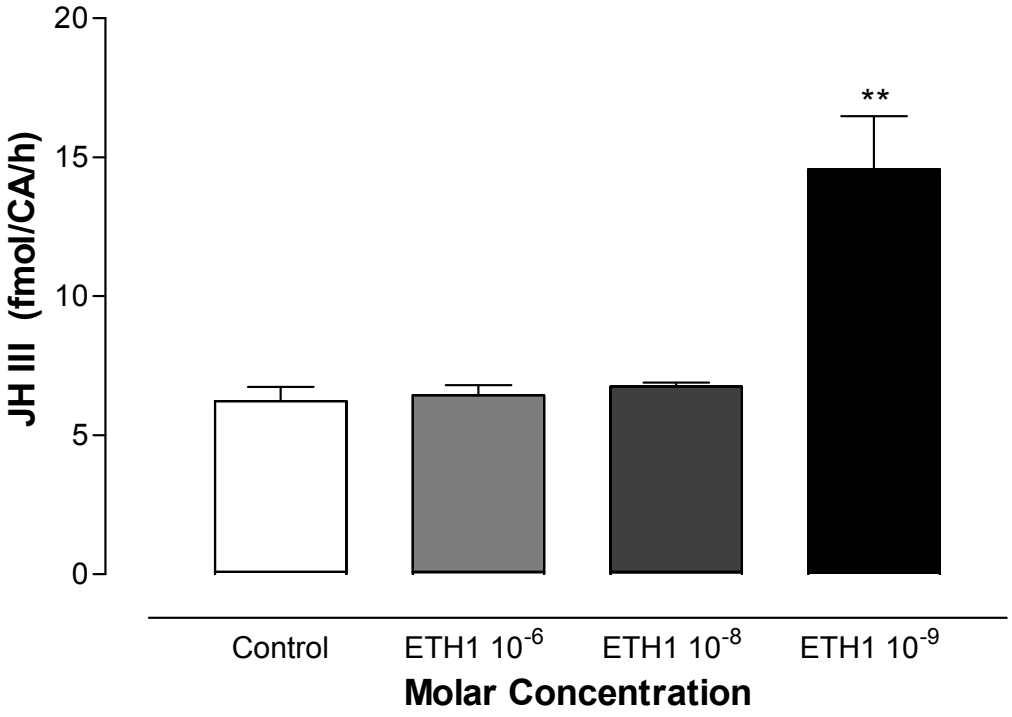

Figure 12. Optimization of ETH1 Molar Concentrations for experiments in pupa. Comparisons among the four ETH treatment concentrations for each variable were done using one way ANOVA (with Tukey's post hoc test at $p < 0.05$). Asterisks denote significant difference among the treatments.

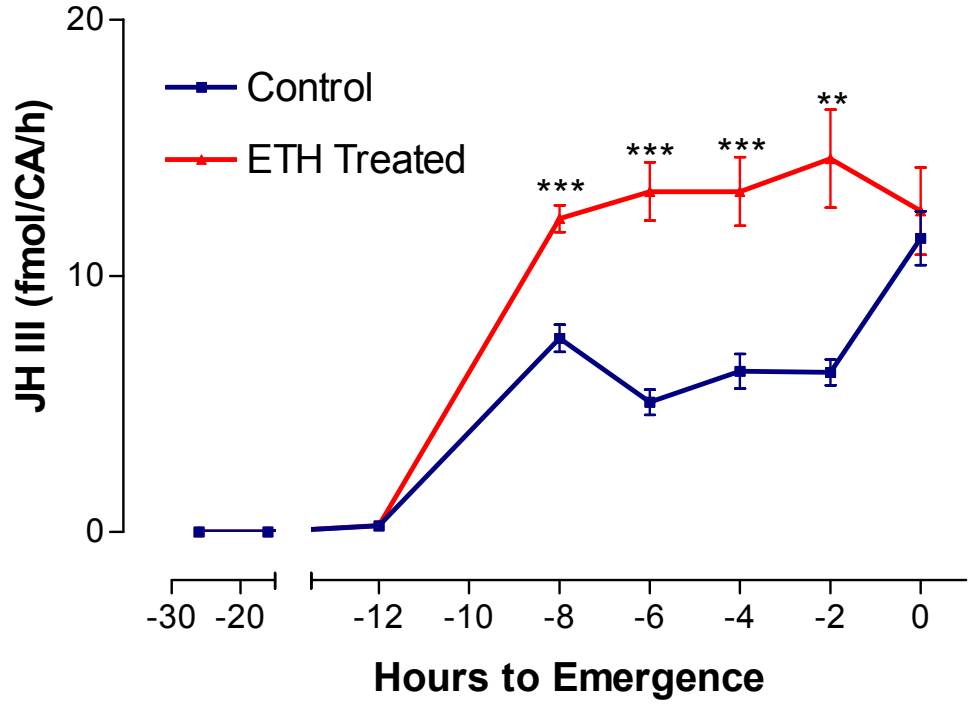

Figure 13. Stimulatory effect of ETH1 JH biosynthesis during the pupal stage. JH synthesized *in vitro*: BR-CA-CC complex were dissected from female pupa at: -26 h, -16 h, -12 h, -8 h, -4 h, -2 h before emergence and newly emerged at 0 h and incubated *in vitro* for 4 h with ETH1 (10^{-9} M). JH levels were measured with HLPC-FD. Each data point represents the means ± S.E.M. of 3-8 independent determinations of individual CA complex. Asterisks denote significant difference (unpaired t-test; ** $P \leq 0.05$; *** $P \leq 0.001$)

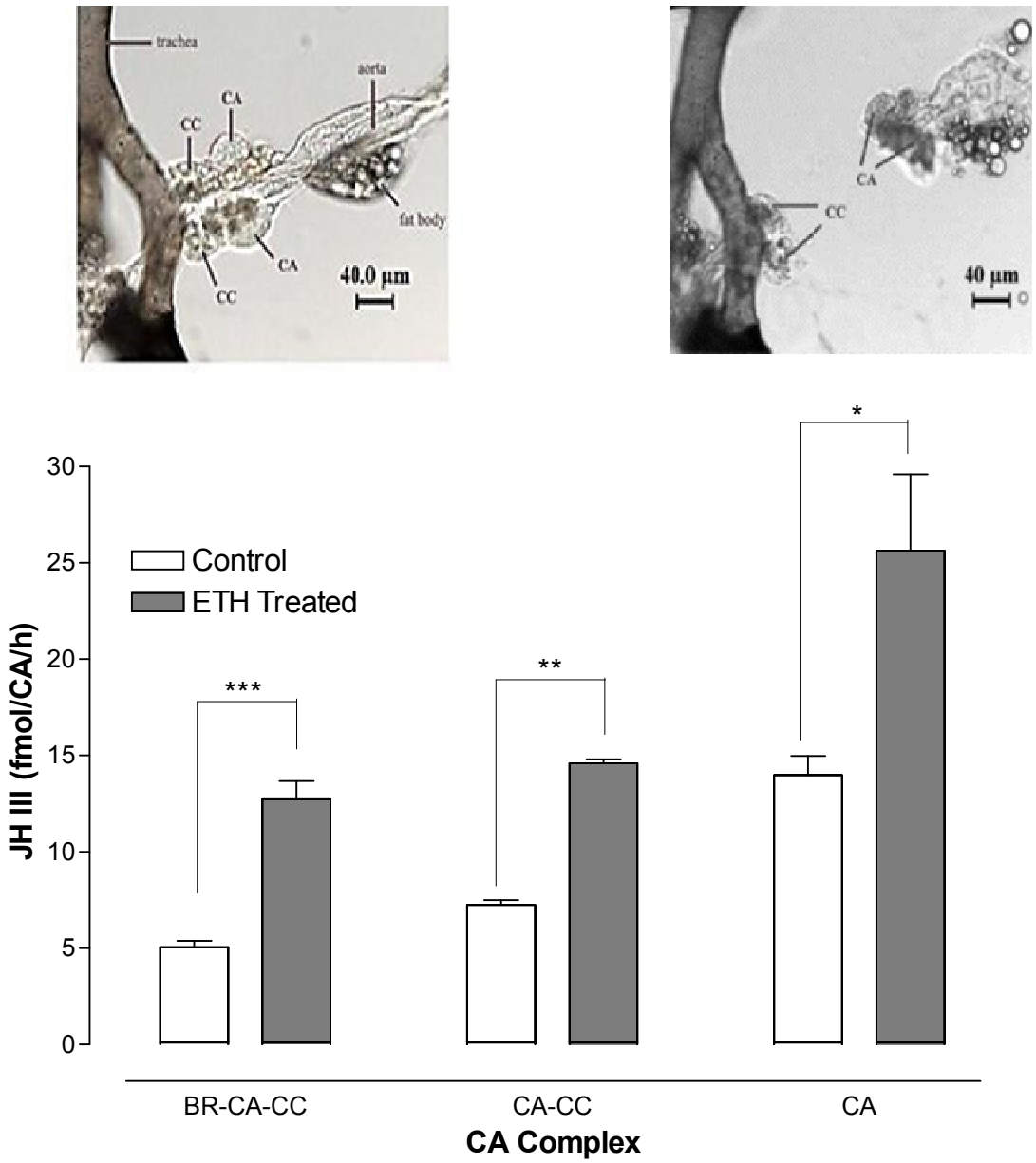

Figure 14. Effect of ETH1 on CA of female pupa. Tissues were dissected from -3 h female pupa (3 hours before emergence) and incubated *in vitro* for 4 h with or without ETH1 (10^{-9} M). Dissections performed: BR-CA-CC (intact, connected to brain). CA-CC (disconnected from brain). CA (isolated CA). JH levels were measured with HLPC-FD. Each data point represents the means ± S.E.M. of 2-4 independent determinations of individual CA complex. Asterisks denote significant difference (unpaired *t*-test; $*P \leq 0.01$;$**P \leq 0.05$; *** $P \leq 0.001$)

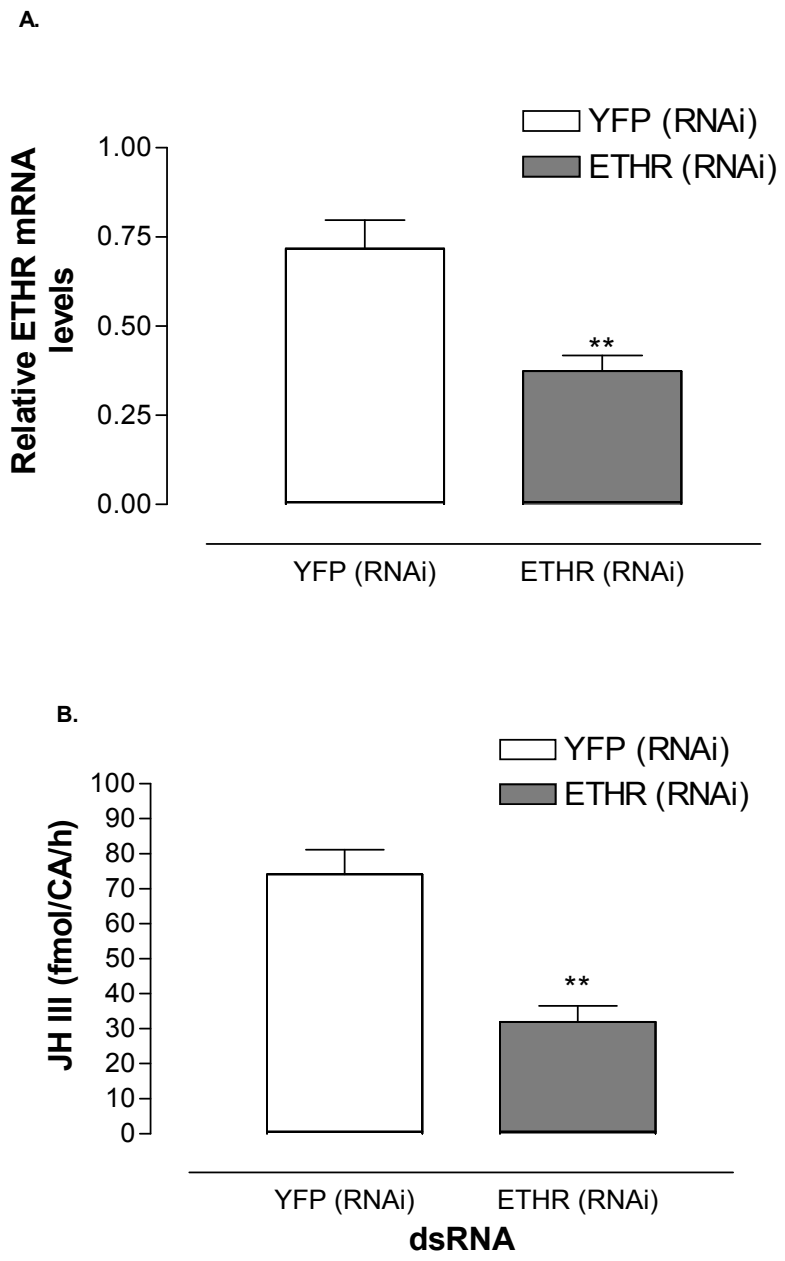

Figure 15. Effect of RNAi-silencing of ETH in late pupa. (a) Effect of ETHR silencing on mRNA levels of ETHR. Abdomens of dsRNA injected females were dissected 18 h after emergence and relative transcript levels were q-PCR. Each data point represents the means ± S.E.M. of 5 biological replicates. Asterisk denotes significant difference (unpaired *t*-test; $^{**}P \leq 0.05$). (b) Effect of ETHR silencing on JH synthesis. BR-CA-CC were dissected 18 h after emergence (~66 h post injection) and incubated for 4 h in tissue culture media. Basal JH levels were measured with HLPC-FD. Each data point represents the means ± S.E.M. of 6 independent determinations of individual CA complex. Asterisk denotes significant difference (unpaired *t*-test; $^{**}P < 0.05$).

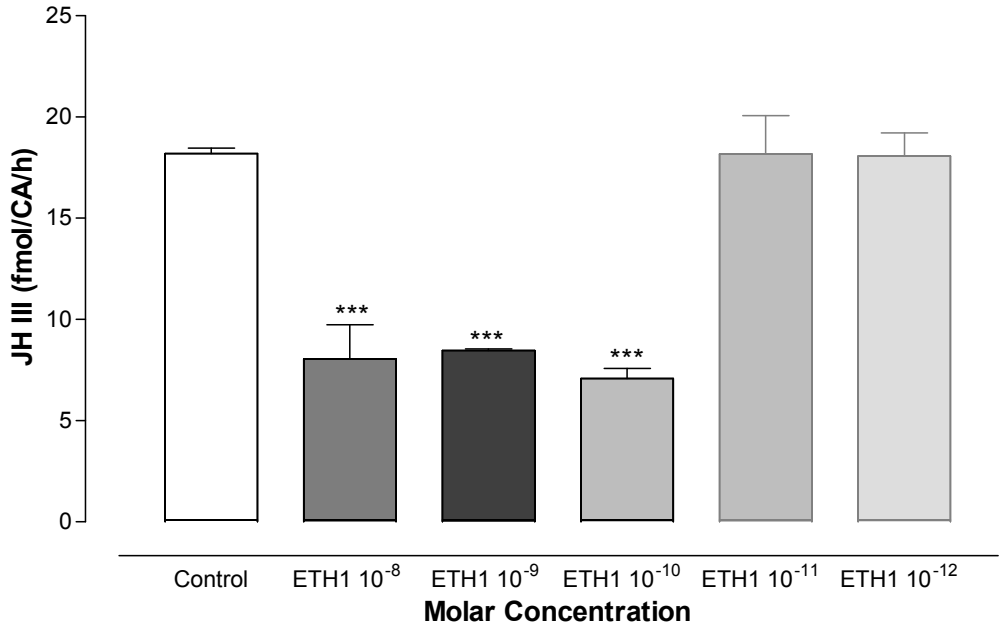

Figure 16. Optimization of ETH1 Molar Concentrations for experiments in blood-feed (BF) adults. Comparisons among the four ETH treatment concentrations for each variable were done using one way ANOVA (with Tukey's post hoc test at $p < 0.05$). Asterisks denote significant difference among the treatments.

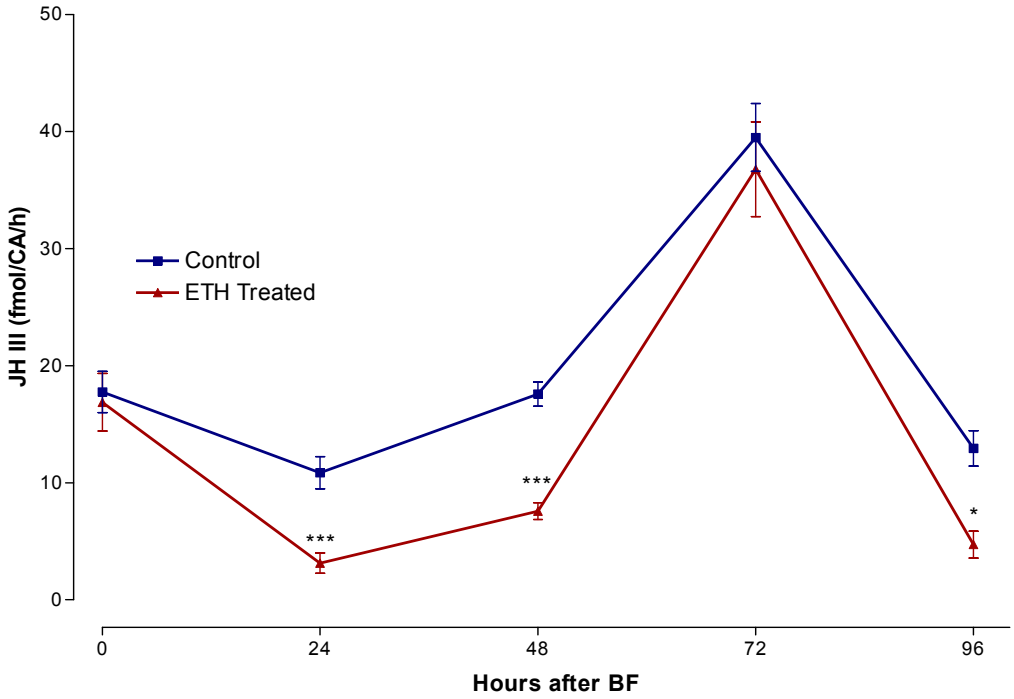

Figure 17. Inhibitory effect of ETH1 on JH biosynthesis of blood-feed females. JH synthesized *in vitro*: BR-CA-CC complex were dissected 4 d sugar fed (0 h) and from blood-fed females at: 24 h, 48 h, 72 h, and 96 h and incubated *in vitro* for 4 h with ETH1 (10^{-9} M). JH levels were measured with HLPC-FD. Each data point represents the means ± S.E.M. of 3-16 independent determinations of individual CA complex. Asterisks denote significant difference (unpaired *t*-test; * $P \leq 0.05$; *** $P \leq 0.001$)

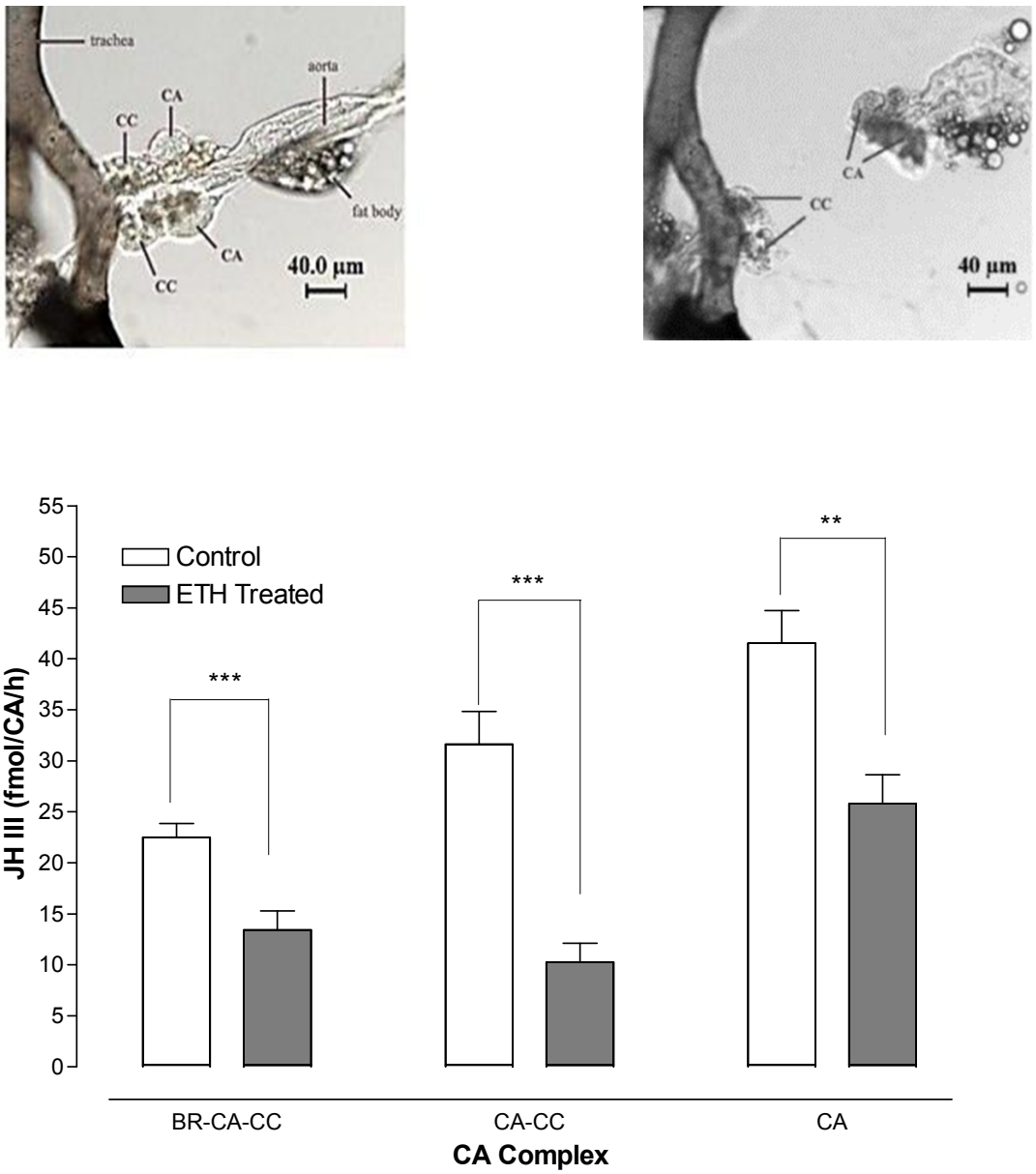

Figure 18. Effect of ETH on CA of blood-fed female. Tissues were dissected from 48 h blood-fed female adults and incubated *in vitro* for 4 h with or without ETH1 (10^{-9} M). Dissections performed: BR-CA-CC (intact, connected to brain). CA-CC (disconnected from brain). CA (isolated CA). JH levels were measured with HLPC-FD. Each data point represents the means ± S.E.M. of 4-7 independent determinations of individual CA complex. Asterisks denote significant difference (unpaired *t*-test;**$P \leq 0.01$; *** $P \leq 0.001$)

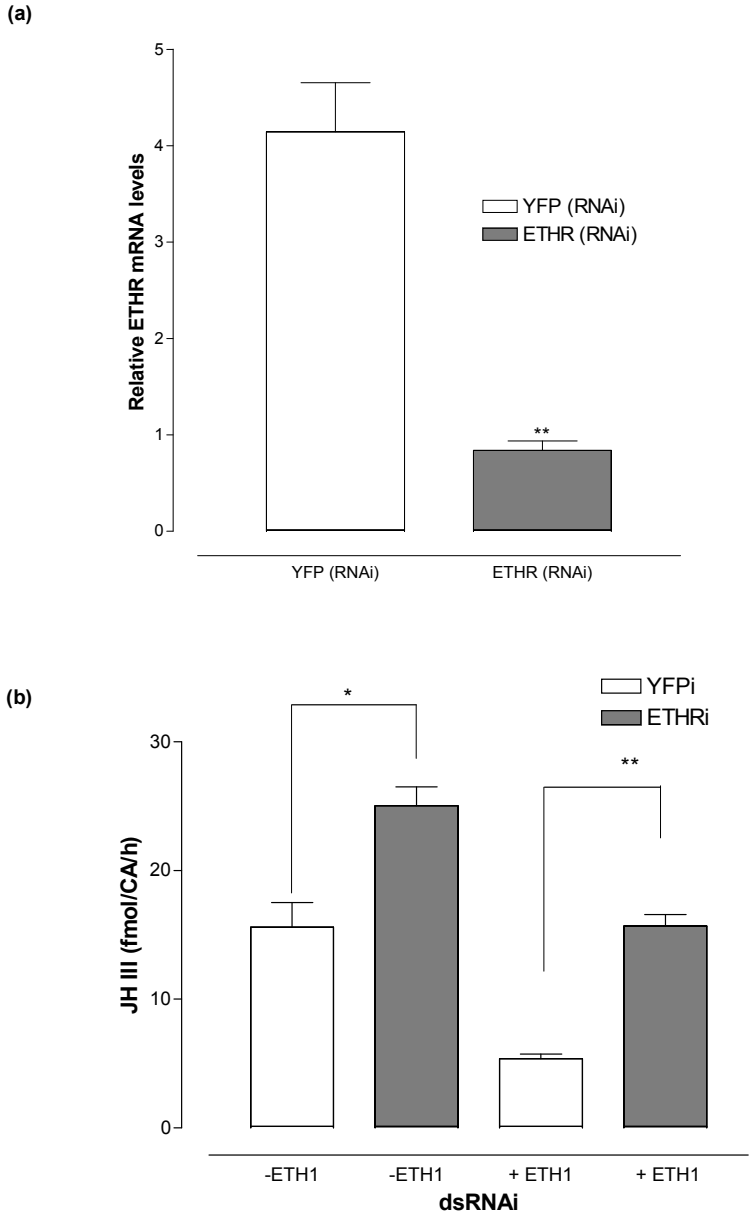

Figure 19. Effect of ETHR silencing in blood-fed female mosquito. (a) Effect of ETHR silencing on mRNA levels of ETH. Thoraxes of 48 h blood-fed female mosquitos were dissected and relative transcript levels were analyzed by qRT-PCR. Each data point represents the means ± S.E.M. of 3 biological replicates. Asterisk denotes significant difference (unpaired *t*-test; $^{**}P \leq 0.05$. (b). Effect of ETHR silencing on JH synthesis. BR-CA-CC were dissected 48 h after blood meal (~3 d post injection) and incubated for 4 h with or without ETH1(10^{-9} M) in tissue culture media. JH levels were measured with HLPC-FD. Each data point represents the means ± S.E.M. of 2-3 independent determinations of individual CA complex. Asterisk denotes significant difference (unpaired *t*-test; $^{*}P \leq 0.05$; $^{**}P \leq 0.001$)

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APPENDICES

Supplemental Table 1: Accession numbers and primer probe sequences for ETHR and housekeeping gene 60s ribosomal protein L32

Gene	**Accession #**	**Primer forward** (5 '→ 3')	**Primer reverse** (5 '→ 3')	**Probe** (5 '→ 3')
ETHR-A	ABI93273	AGGTGTGGGTACTGGGAGAA	CACCGTCAGCTCCACGAA	CCGCTTTACACATTTC
ETHR-B	ABI93274			
rpL32	AAEL003396	CCATCAGTCCGATCGCTATGA	GTTGTCAATACCTTTCGGCTTACG	CAAGCTTGCCCCCAACTG