# CHARACTERIZATION OF THE OVARY ECDYSTEROIDOGENIC HORMONE AND OVARIAN ECDYSTEROIDOGENESIS IN THE AFRICAN MALARIA MOSQUITO, ANOPHELES GAMBIAE

by

STUART D. THOMAS, JR.

(Under the Direction of Mark R. Brown)

## ABSTRACT

Ecdysteroidogenesis, ecdysteroid titer of hemolymph and yolk deposition over the course of a gonotrophic cycle are quantified for the first time for female *Anopheles gambiae*. The trend of ecdysteroid secretion roughly matched that of *Aedes aegypti*. A cDNA sequence for an ovary ecdysteroidogenic hormone (*AgamOEH*) in *Anopheles gambiae* was also characterized. This peptide also showed similarity to the *Ae. aegypti* OEH in amino acid sequence. The distribution of the *AgamOEH* transcript and peptide are also described for *An. gambiae* at different life stages and during a gonotrophic cycle. We partially purified a putative OEH peptide from 20,800 adult *An. gambiae* heads with multiple HPLC runs. Finally, we were unable to prove bioactivity of the purified peptide but were able to demonstrate the ability of bovine insulin to activate oocyte development *in vivo*.

INDEX WORDS: *Anopheles gambiae*, ecdysteroids, ovary ecdysteroidogenic hormone.

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May 2005

# DEDICATION

This work is dedicated to my family, who have always told me that I would be able to accomplish anything that I want to do. This work is also dedicated to my future wife, Layne, for supporting me every step of the way.

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I would like to thank Dr. Mark Brown for his guidance and mentoring throughout my thesis. I would also like to thank Dr. Michael Riehle, Doug Sieglaff, Dr. Stephen Garczynski, Dr. Joe Crim, and Dawn Stanek for their help and suggestions. My work would not have been possible without their assistance. Finally, I would also like to thank my committee members, Dr. Donald Champagne and Dr. Nancy Hinkle for their advice and guidance.

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

Mosquito-borne diseases impact human populations around the world by causing illness and death in millions of people each year. The most prevalent of these mosquito-borne diseases is malaria, which is caused by protists of the genus *Plasmodium*. The World Health Organization estimates that nearly 40 percent of the world's population is at risk to malaria infection (WHO fact sheet 94, online: http://mosquito.who.int/cmc_upload/0/000/015 /372/RBMInfosheet_1.htm). The majority of these infections occur in sub-Saharan Africa, where an estimated 90 percent of all reported malaria deaths occur. Over the past decades, governments, health agencies, and private industry have invested significant resources in an effort to provide adequate and consistent means of controlling mosquito populations, as well as curbing the impact of malaria and other mosquito borne illnesses. Unfortunately, the reliance on a small number of effective pesticides over several years has contributed to the selection of mosquito populations resistant to these pesticides, making population control difficult in some regions (Balkew et al. 2003). Furthermore, resistance of some pathogens to medical treatment and the lack of effective vaccines makes the need for novel control methods even more desperate (Laufer and Plow 2004). A better understanding of mosquito physiology with respect to reproduction could prove to be instrumental in developing effective mosquito control to reduce the impact of mosquitoes on human and animal health. Mosquito reproduction is a highly regulated event that allows females to produce and deposit synchronously developing batches of

eggs from proteinaceous blood meals taken from vertebrate hosts. *Aedes aegypti*, the yellow fever mosquito, has long been established as the model for the anautogenous type of mosquito reproduction. Anautogenous mosquitoes require a separate blood meal for each batch of eggs produced. In contrast, autogenous mosquito species are able to produce an initial batch of eggs by utilizing their larval energy reserves, but then must procure a blood meal for each successive egg batch produced.

Mosquito reproduction is composed of three distinct stages: the previtellogenic, vitellogenic, and the postvitellogenic phases (Klowden 1997). The first previtellogenic phase begins after eclosion of the adult mosquito and following oviposition of an egg batch and ends once the female has taken a blood meal. After adult eclosion, females require approximately three days for the organs used during oogenesis, namely the ovaries and the fat body, to reach reproductive competency.

Mosquitoes have polytrophic meroistic ovaries consisting of fewer than 50 to more than 500 ovarioles, each of which contains a primary follicle, secondary follicle, and germarium covered with a layer of epithelial cells (Clements 1992). The germarium is the source of follicles for each ovariole and produces a secondary oocyte while the primary oocyte matures. Maturation of the secondary oocyte is held in stasis until the primary oocyte has been oviposited by one or more oostatic factors produced by the ovary (Klowden 1997, Sappington and Raikhel 1999).

In the primary follicle, nutritive nurse cells are connected to the primary oocyte by a number of cytoplasmic bridges. The follicular epithelium performs several functions, including the production of ecdysteroids and secretion of the egg shell. The presence of 20 hydroxyecdysone (20-HE) at the beginning of the previtellogenic phase causes the primary

follicles to separate from their germaria (Beckemeyer and Lea 1980). A number of explanations for the presence of 20-HE during this time have been proposed, such as residual amounts of 20-HE being present from either a previous gonotrophic cycle or left over from the pupal-adult molt of the newly emerged female, a time when 20-HE titer in the hemolymph is elevated (Whisenton et al. 1989).

Following separation from the germarium, the primary follicles continue to develop for another 48-72 hours, at which point they reach a resting stage where no further development occurs. Follicular development to this resting stage is dependent on the action of juvenile hormone III (JH III). The organelles of the follicular epithelial cells surrounding the oocyte are dependent on JH III for development (Raikhel and Lea 1991), and the oocyte requires the presence of JH III in order to create the cellular machinery necessary for the uptake of vitellogenin (Raikhel and Lea 1985). During this time of ovarian development, the fat body also differentiates in the presence of JH III and becomes able to synthesize the yolk protein precursors (YPPs) used by the egg to form yolk (Flanagan and Hagedorn 1977). JH III was found to increase both the number of ribosomes and the ploidy in fat body trophocyte cells (Dittman et al. 1989, Raikhel and Lea 1990). JH III also prepares the ovaries to become responsive to the ovarian ecdysteroidogenic hormone (OEH), to be discussed during the vitellogenic phase (Shapiro and Hagedorn 1982). Although JH III is required for the female to become sexually competent, the presence of elevated JH levels following adult eclosion suppresses female sexual receptivity (Gwadz and Spielman 1973). In the absence of a blood meal, JH titer in the female decreases slowly following sexual maturation, and she eventually becomes responsive to males. However, if the female receives a blood meal soon after maturation, JH titer drops quickly, and she becomes responsive quickly (Shapiro et al. 1986).

Ingestion of a blood meal by a female ends the previtellogenic phase of the gonotrophic cycle and begins the vitellogenic phase. During this phase, the blood meal taken by the female is digested and the fat body begins to synthesize and secrete large amounts of YPPs into the hemolymph, which are then incorporated to form the yolk in the primary oocytes.

Gut distention (Klowden 1987) and amino acids (Uchida et al. 1992) in the hemolymph from the digesting blood meal trigger the release of the ovarian ecdysterogenic hormone (OEH). OEH is synthesized by a few pairs of medial neurosecretory cells in the brain and is secreted into the hemolymph from the corpora cardiaca (Lea 1972). OEH stimulates the ovaries to synthesize and secrete ecdysone, which is converted into 20-HE and triggers the fat body to begin synthesizing YPPs. In addition, an uncharacterized hormone released from the ovaries, termed the OEH releasing factor (OEHRF) (Lea and Van Handel 1982), must first be present to allow OEH to be released from the corpora cardiaca.

The discovery by Hagedorn (Hagedorn et al. 1975) that mosquito ovaries secreted ecdysone was the first evidence that this hormone was produced by a tissue other than the prothoracic glands in insect larvae. Ecdysone is quickly converted to the biologically active 20-HE by the enzymatic action of a monooxygenase. This enzyme has been found in many tissues of the female mosquito, most notably the ovaries and to a lesser extent in the fat body (Smith and Mitchell 1986). The converted 20-HE moves into the hemolymph and causes the fat body to begin producing YPPs.

The fat body's metabolic response to 20-HE is orchestrated by the activity of the ecdysteroid (EcR) and ultraspiracle (USP) nuclear receptors (Raikhel et al., 2005). These receptors together form a heterodimer, the ecdysteroid receptor complex, in the cytoplasm of fat body cells and bind to 20-HE. The ligand/ receptor then moves to the cell nucleus and binds to

the ecdysteroid responsive element (EcRE) sequence upstream of specific genes. These binding events directly activate or inhibit the transcription of a small group of early ecdysone responsive genes as well as activate transcription of the vitellogenin (*Vg*) gene (Martin et al. 2001). These early genes encode transcription factors that activate a larger set of late ecdysone responsive genes that modulate vitellogenesis.

YPPs produced by the fat body are secreted into the hemolymph of the vitellogenic female. YPPs move to the primary oocytes for uptake by passing through gaps between the follicular epithelial cells surrounding the oocytes, a physiological state termed patency. Secretion and uptake of YPPs continues until approximately 30 hours PBM

The final stage of mosquito reproduction, the postvitellogenic phase, begins once YPP synthesis has been halted in the fat body and ends after the female mosquito has laid her egg batch. At around 30 hours PBM, vitellogenic activity in the fat body is halted, and the biosynthetic machinery in the organ is degraded, returning the fat body to its previous non-vitellogenic state (Raikhel 1992). Additional uptake of YPPs by the oocytes is prevented with the loss of patency.

At the cellular level, lysosomes begin to degrade the machinery responsible for producing YPPs in the fat body trophocytes (Raikhel 1986). It is speculated that what triggers this event may be the fat body's response to prolonged exposure to 20-HE (Bohm et al. 1978, Ma et al. 1987) or by a possible negative feedback effect of vitellogenin on its own production (Borovsky 1981). The uptake and storage of the YPPs by the primary oocytes is also halted during this stage and occurs in two ways: the intercellular channels that allowed passage of YPPs into the oocytes during the vitellogenic phase are blocked (Anderson and Spielman 1971) and the deposition of the endochorion layer around the oocyte also physically prevents further uptake

(Raikhel and Lea 1991). Until the primary oocyte has been oviposited, uptake of YPPs by the secondary oocyte is prevented by the presence of one or more factors produced by ovarioles containing fully developed oocytes (Klowden 1997, Sappington and Raikhel 1999).

The female's responsiveness to host animal stimuli and the drive to blood feed, referred to as host-seeking behavior, is suppressed after taking a blood meal and returns only after she has oviposited her egg clutch. The inhibition of these behavioral responses is mediated by neuronal and hormonal stimuli that act in a biphasic manner. Immediately following a blood meal, distention of the midgut inhibits host-seeking behavior (Klowden 1990). Beginning around 30 hours PBM, *Aedes* head peptide (AeaHP) (pQRPPSLKTRFa) (Brown et al. 1994) inhibits the host-seeking behavior in the female mosquito. Neuroendocrine and midgut cells in *Ae. aegypti* were found to contain AeaHP (Stracker et al. 2002). Host-seeking behavior returns only after the female has laid her egg batch.

In the 1950's, Gillett (Gillett 1956) and Clements (Clements 1956) first hypothesized that a humoral factor released from the brain was responsible for initiating egg development in mosquitoes. Lea (Lea 1967) found that this factor, which he called the egg development neurosecretory hormone (EDNH), was synthesized in the medial neurosecretory cells of the brain and released into the hemolymph via the corpora cardiaca in response to blood meal stimuli. Midgut distention (Lea 1972) and amino acids derived from the digested blood meal (Uchida et al. 1992) were found to stimulate release of EDNH into the hemolymph. Hagedorn (Hagedorn et al. 1979) demonstrated that EDNH was the stimulus that triggered the ovaries to release ecdysone, which then initiates the synthesis and secretion of vitellogenin in the fat body. To better reflect the function of the EDNH peptide, it was later renamed the ovarian ecdysteroidogenic hormone (OEH) (Matsumoto et al. 1989, Brown et al. 1995).

There have been several attempts at characterizing and purifying an OEH peptide (Borovsky and Thomas 1985, Whisenton et al. 1987, Matsumoto et al. 1989). The *Ae. aegypti* OEHI (AaeOEHI) is a 149 amino acid prepropeptide that is processed to an 86 residue peptide with a molecular weight of 8803 (Brown et al. 1998). Brown and Cao (Brown and Cao 2001) provided immunohistochemical evidence that an OEH was present in the brain, ventral nerve cord, midgut, and perivisceral organs of *An. gambiae* and *Ae. aegypti* larvae and adults.

The AaeOEH peptide shows amino acid similarity to the neuroparsins, a small family of neurohormones characterized in locusts**.** Neuroparsins A (NPA) and B (NPB) were originally described in *Locusta migratoria* (Girardie et al. 1987a). Similar to OEH, NPA is synthesized and stored as in neurosecretory cells within the locust brain. NPA is cleaved to form the NPB monomer, which is secreted into the hemolymph from the corpora cardiaca. In *Schistocerca gregaria*, four neuroparsin cDNAs, *SgNPP1-4*, have been characterized. *SgNPP1* and *SgNPP2* were found to occur only in the brain, with *SgNPP1* encoding both NPA and NPB hormones (Janssen et al. 2001). *SgNPP*3 and *SgNPP4* mRNA were detected in the brain as well as the fat body, ventral nerve cord, testes, and male accessory glands (Claeys et al. 2003). The neuroparsins have been shown to exhibit anti-diuretic (Fournier and Girardie 1988), anti-juvenile (Girardie et al. 1987b), trehalosemic and hyperlipemic (Moreau et al. 1988) effects as well as cause neurite outgrowth (Vanhems et al. 1990) in locusts as well as other insects and invertebrates. Thus, despite their apparent similarities concerning amino acid composition, there is no evidence of any functional similarity between OEH and the neuroparsins.

In addition to PTTH, OEH shares its function as an ecdysteroidogenic hormone with the bombyxins. The bombyxins, originally described in the silkworm moth, *Bombyx mori*, were shown to cause ecdysteroidogenesis in this species (Nagasawa et al. 1986). Due to their

structural resemblance to insulin, the bombyxins have been placed in the insulin like (ILP) family of hormones (Jhoti et al. 1987). Graf *et al.* (Graf et al. 1997) then demonstrated that vertebrate insulin was capable of causing ecdysteroidogenesis in ovaries of non-blood fed mosquitoes *in vitro*. Later, Riehle *et al.* (Riehle and Brown 1999) demonstrated that bovine insulin triggered ecdysteroidogenesis in *Aedes aegypti* ovaries through an ILP signaling pathway.

Hormones also mediate insect egg maturation by inhibiting oocyte development (Adams 1998). Crude extracts prepared from *Musca domestica* were able to prevent oocyte development and lower ecdysteroid secretion in *Aedes atropalpus*. Similarly, an ovarian ecdysteroidostatin (OES) from *Musca domestica* was shown to inhibit ecdysteroid secretion by *Musca* ovaries incubated with OEH (Adams and Li 1998). OES is believed to interfere with ecdysteroid release rather than interrupting the OEH signal pathway.

**PURPOSE OF STUDY**

The majority of information concerning mosquito reproduction has come from work done with *Ae. aegypti*. Considering the various reproductive strategies that mosquitoes employ, it is difficult to generalize the physiological events of a particular species based on only a few examples. Given the impact that *An. gambiae* has on the human population, it surprising that very little is known about the physiology of this species. The research goals for this thesis, given below, are aimed at characterizing several important events that occur during *An. gambiae* reproduction.

Chapter two presents the results of experiments from objectives one to four.

1. Determine whether *An. gambiae* ovaries produce ecdysteroids. Radioimmunoassay confirmed that *An. gambiae* ovaries were capable of producing ecdysteroids *in vitro*.
2. Characterize the secretion of ecdysteroids by *An. gambiae* ovaries following a blood meal. Ecdysteroid release reached its maximum between 18 and 24 hours PBM and declines to low levels soon afterwards.
3. Characterize the titer of ecdysteroids present in the hemolymph in *An. gambiae* during a gonotrophic cycle. The peak hemolymph ecdysteroid titer occurred at the same time during the gonotrophic cycle as that observed for ovarian ecdysteroid release.
4. Yolk deposition was measured in *An. gambiae* eggs during a gonotrophic cycle. Yolk deposition in blood-fed *An. gambiae* females was measurable by 12 hours PBM. Yolk length continued to increase throughout the gonotrophic cycle to 72 hours PBM.

Chapter three presents the results for objectives five to nine.

5. Characterization of a cDNA that encodes an OEH peptide in *An. gambiae*. A cDNA was characterized from *An. gambiae* that shows similarity to AeaOEH and the locust neuroparsins.
6. Determine the expression of *AgamOEH* transcripts in different body regions during both *An. gambiae* development and a gonotrophic cycle. Transcripts were present in eggs and in the heads and thoraces of fourth instar larvae, but only in adult heads. During a gonotrophic cycle, transcripts were present in the heads and thoraces of females but not the abdomens.
7. Determine the presence of AgamOEH peptides in different body regions during both *An. gambiae* development and a gonotrophic cycle. A peptide believed to be an OEH was

detected in eggs as well as in heads and thoraces of fourth instar larvae, pupae, and adults.

8. Purification of AgamOEH peptide from extracts of adult *An. gambiae* heads and testing biological activity of the purified AgamOEH. A peptide showing immunoreactivity to an AaeOEH antibody was purified but did not have biological activity in an *in vivo* assay.
9. Demonstrate the bioactivity of bovine insulin in *An. gambiae*. Bovine insulin was shown to cause egg development in blood fed females in the absence of an OEH signal.

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# CHAPTER 2

# OVARIAN ECDYSTEROIDOGENESIS AND YOLK ELONGATION DURING A GONOTROPHIC CYCLE IN THE AFRICAN MALARIA MOSQUITO, *ANOPHELES GAMBIAE* (DIPTERA: CULICIDAE)[1]

[1] Thomas, S. D. and M. R. Brown. To be submitted to *Journal of Medical Entomology*.

**ABSTRACT**

A radioimmunoassay was used to quantify the secretion of ecdysteroids by ovaries of *Anopheles gambiae in vitro* before and during a gonotrophic cycle. Hemolymph retrieved from these individuals was measured for ecdysteroid content. Finally, yolk length of the primary oocytes from these females was quantified as a measure of egg development. Ovary ecdysteroidogenesis and hemolymph titer roughly match the previous profile of whole body ecdysteroids described in *Aedes aegypti*, which peaked between 18 and 24 hours post-blood meal. However, the patterns of both species were found to be different from other mosquito species investigated previously.

INDEX WORDS: *Anopheles gambiae*, ecdysteroids, ovary, hemolymph.

## INTRODUCTION

The ingestion of a blood meal by a female mosquito triggers a carefully regulated series of physiological events that results in a clutch of mature eggs ready to be oviposited. According to the model developed for the yellow fever mosquito, *Aedes aegypti*, within a few hours of taking a blood meal, the medial neurosecretory cells (MNC) of the brain release ovarian ecdysteroidogenic hormone (OEH I) into the hemolymph via the corpora cardiaca (Lea 1967, Brown et al. 1998). OEH then stimulates the secretion of ecdysone by the ovaries (Brown et al. 1998). In *Ae. aegypti* whole body extracts, ecdysteroids begin to rise sharply around six hours after a blood meal and continue to increase until reaching its maximum between 18 and 24 hours post-blood meal (PBM) (Hagedorn et al. 1975). Following this peak, ecdysteroid levels begin to decrease, reaching non-blood fed levels by 30 hours PBM.

Soon after its synthesis, ecdysone is converted into the biologically active 20-hydroxyecdysone (20-HE) by a 20-monooxygenase enzyme found in the ovaries, fat body, and peripheral tissues of the female mosquito (Smith and Mitchell 1986). The fat body begins synthesizing the yolk protein precursors (YPP) in response to the presence of 20-HE. The response of the fat body to 20-HE is coordinated by the activity of the ecdysteroid receptor (EcR) and ultraspiracle (USP) nuclear receptors (Raikhel et. al, 2005 ). EcR and USP form a heterodimer, the ecdysteroid

receptor complex, which bind 20-HE and move together into the cell nucleus. The ligand/ receptor complex then binds to an ecdysteroid responsive element DNA sequences (EcRE) on specific genes. This binding event directly activates the transcription of a small group of early

ecdysone responsive genes, including the vitellogenin gene. Some of these early genes encode transcription factors that multiply the endocrine effect of 20-HE by activating a larger group of late ecdysone responsive genes. Their gene products act to mediate vitellogenesis by the fat body (Raikhel et al. 2005). Yolk components produced by the fat body are secreted into the hemolymph and are absorbed by the primary oocytes by receptor mediated endocytosis and condensed to form the egg yolk (Raikhel and Dhadialla 1992). The synthesis and uptake of the yolk components continue until approximately 30 hours after the blood meal. After this time, the loss of patency by the oocytes physically blocks the passage of any additional yolk components (Raikhel et al. 2005).

Once her eggs have matured sufficiently and are ready for oviposition, the female mosquito then begins to search for a suitable oviposition site. After the female has deposited her egg batch, she is then physiologically able to respond to host cues once and begin another reproductive cycle (Brown et al. 1994).

Ecdysone is known as the insect molting hormone because its increased titer in the hemolymph induces molting between larval instars and the metamorphosis of pupae into adults. The primary source of ecdysteroids in immature insects is the prothoracic gland and the ring gland in dipteran species (Klowden 1997). Taking into consideration the role of ecdysteroids in insect development, it is not surprising that evidence for the presence of ecdysteroids in adult insects was often discounted or disregarded when it was first reported. Providing convincing proof that ecdysteroids were present in adult insects was made even more difficult due to the fact that the prothoracic gland is degraded during the pupal stage or very early in adulthood, thereby removing the then only recognized source of ecdysteroids. Nonetheless, ecdysteroids have been

found in the ovaries, eggs, or female hemolymph of many insect orders, including Isoptera, Orthoptera, Lepidoptera, Coleoptera, and Diptera (Hagedorn 1983).

Hagedorn (Hagedorn et al. 1975) demonstrated that the ovaries of *Ae. aegypti* females were a source of ecdysteroids. Ecdysteroids are released in response to a blood meal and are responsible for controlling vitellogenesis. Since these discoveries, ovaries of other dipteran species have also been found to be capable of synthesizing ecdysteroids; including the fruitfly, *Drosophila melanogaster* (Richard et al. 1998), the common housefly, *Musca domestica* (Adams et al. 1992), the blowfly, *Phormia regina* (Maniere et al. 2000), and the blackfly, *Simulium vittatum* (Noriega et al. 2002). Mosquitoes other than *Ae. aegypti* have also been studied to determine the trend of ecdysteroid secretion occurring during their reproductive cycle. These species include the northern house mosquito, *Culex pipiens* (Baldridge and Feyereisen 1986) and *Anopheles stephensi* (Redfern 1982). Dipterans are still the only insect order known in which ecdysteroids play a central role in egg development by mediating vitellogenesis (Raikhel et al. 2005).

In this study, we demonstrate for the first time that the ovaries of adult *An. gambiae* females are a source of ecdysteroids *in vitro*. We also quantified the secretion of ecdysteroids by female *An. gambiae* ovaries throughout a gonotrophic cycle using a radioimmunoassay (RIA). We then used the RIA to determine the titer of ecdysteroids secreted into the hemolymph of the same individuals. Finally, we observed yolk deposition in the primary oocytes in these individuals by measuring the longitudinal axes of the egg yolks. The results we present are an initial step in understanding some physiological events of the reproductive cycle of a major vector of human disease.

## MATERIALS AND METHODS

*Mosquitoes: Anopheles gambiae* (G3 strain) mosquitoes were obtained from the Centers for Disease Control and Prevention in Atlanta, Georgia. All life stages were maintained at ~27° C on a 16 h light: 8 h dark photoperiod. Larvae were reared in groups of 200 each in covered, shallow aluminum trays with 6 ounces of deionized water and fed a finely ground mixture of IAMS® weight control formula cat food and TetraMin® Rich Mix fish food at a ratio of 3:1, respectively. Pupae were separated from larvae prior to adult eclosion by transferring them into an Erhlenmeyer flask filled with deionized water and collecting pupae that rose to the surface. Pupae were kept in eight ounce plastic cups containing deionized water in ventilated acrylic enclosures (12 in x 9 in x 6.5 in) in preparation for adult eclosion during the next 24-hour period. Any uneclosed pupae after this period were moved to a new enclosure to ensure that similarly aged adults were used in all experiments. Adult mosquitoes were maintained on 8% fructose water solution and rehydrated raisins. Adult *An. gambiae* females were placed in the dark for a minimum of one hour prior to blood feeding on Sprague-Dawley strain rats (Charles River Laboratory) that were narcotized with a combination of Xyla-ject® (Phoenix Pharmaceutical Inc.) and Ketaset® (Fort Dodge Animal Health) anesthetics. Following all blood meals, the sugar water was replaced and freshly rehydrated raisins were made available to the mosquitoes on which to feed.

*In vitro ecdysteroid secretion, hemolymph titer and yolk length measurement:* Ovaries and hemolymph were collected from sugar fed (three-day old) and blood fed female *An. gambiae* at 6, 12, 18, 24, 30, 36, 48, and 72 hours post-blood meal**.** Mosquitoes were immobilized with cold and placed into 100 µl of modified Beyenbach saline media (0.139 M NaCl, 0.00405 M KCl, 0.00185 M $CaCL_2$, 0.0125 M HEPES, 0.0025 M trehalose, 0.0003 M $MgCl_2$ and 0.009 M

$NaHCO_3$ in sterile $H_2O$) in depression well glass slides. With two pairs of forceps, the two terminal abdominal segments were removed into the saline media along with the Malpighian tubules, reproductive system, and a portion of the gut. The ovary pair was then dissected from the other internal organs and abdominal segments with forceps. The longitudinal axis of yolk deposited in one representative primary oocyte in each ovary pair was measured using an Olympus SZ-60 stereo microscope outfitted with an ocular micrometer (20 µm increments). Following yolk length measurement, the ovary pair was removed from the saline media and temporarily stored in a separate pool of the same media until a set of four ovary pairs was obtained**.** The ovary pairs were transferred into 60 µl of saline media in a 500 µl polypropylene microcentrifuge tube cap placed into 24 well, flat bottom RIA plates (Costar®) containing 200 µl deionized water. The ovary pairs were incubated for 6 hours at 37° C in a rotating water bath. Following incubation, 50 µl of saline media was removed from each cap by pipette and stored at –80º C for later measurement of ecdysteroids by RIA in 500 µl polypropylene microcentrifuge tubes. Any remaining hemolymph in the carcasses was collected by squeezing the thoraces and abdomens in the saline media. The carcasses were removed and 50 µl of the saline media, equal to 2 female hemolymph equivalents, was transferred to a 500 µl polypropylene microcentrifuge tube and stored at –80º C for later ecdysteroid quantification by RIA. For all experiments, a minimum of three different generations (cohorts) of females were collected and analyzed. Each of these cohorts consisted of three replicate sets of four females.

*Ecdysteroid radioimmunoassay:* Modified procedures for *in vitro* tissue ecdysteroid production and the ecdysteroid RIA were followed (Riehle and Brown 1999). The anti-ecdysteroid rabbit serum (AS 4919, a gift from P. Poncheron, Université P. et M. Curie, Paris, France) recognizes ecdysone and 20-hydroxyecdysone equally (Porcheron et al. 1989) as verified with our RIA. For

RIA, each tube contained 50 µl of a stock [23,24-3H(N)]ecdysone solution (= [3H]ecdysone; 12,000-13,000 counts/minute (cpm)/50 µl; PerkinElmer, Boston, MA), 50 µl of antiserum diluted to 1:35,000-45,000 (final dilution for bound to free [3H]ecdysone cpm ratio (B/F) = 1), and 50 µl of sample or 20-hydroxyecdysone standard. Separate, triplicate tubes were set up for total cpm [3H] ecdysone, non-specific binding of [3H]ecdysone, diluted antibody and [3H]ecdysone alone to monitor B/F, and each of the 20-hydroxyecdysone standards (1, 5, 10, 25, 50, 100, 250, 500, and 1000 pg) for every RIA. After overnight incubation at 4° C, bound and free radiolabeled ecdysone were separated in tubes by the ammonium sulfate method, and pellets in tubes were dispersed in water and scintillation fluid and counted in a scintillation counter (Beckman). For each RIA, a standard curve was plotted from the averaged B/F (Y axis) and log values for the 20-hydroxyecdysone standards (X axis). The quantity of immunoreactive ecdysteroids in samples was calculated from a regression equation for the linear portion (10-250 pg) of the standard curve; samples were diluted when necessary to stay within this range. Sample values reported for each tissue treatment are presented as "ecdysteroid pg", because the secreted ecdysteroid species are unknown, and the values are means of triplicate treatments from three experiments.

*Statistical analysis:* Regression analysis of standard line samples was done using Microsoft Excel software. Statistical analysis of all data was performed with JMP 5.1 statistics program suite (SAS).

**RESULTS**

*In vitro release of ovary ecdysteroids:* The ability of ovaries from sugar- and blood-fed *An. gambiae* mosquitoes to secrete ecdysteroids was determined *in vitro* and measured with an ecdysteroid RIA. Ovaries of sugar fed *An. gambiae* were found to secrete low levels of ecdysteroids *in vitro* (mean = 8.2 ecdysteroids (pg)/ovary pair/6 hours ± 4.6 s.e.m., n=9) (Figure 2.1). Ovarian ecdysteroid secretion began to increase within six hours after the blood meal. Ecdysteroid secretion peaked between 18 and 24 hours PBM, with the highest secretion measured at 24 hours PBM (66.89 ecdysteroids (pg)/ovary pair/6 hours ± 3.95, n=12). Ecdysteroid release decreased steadily after the peak, reaching levels similar to non-vitellogenic ovaries by 48 hours PBM.

*Hemolymph ecdysteroid titer:* The amount of ecdysteroids present in the hemolymph of *An. gambiae* females was measured prior to and during a gonotrophic cycle (Figure 2.2). Ecdysteroid titer levels were elevated in sugar-fed controls (mean =76.3 ecdysteroids (pg)/female ± 15.43 s.e.m., n=9). At 6 hours PBM, ecdysteroid titer had fallen but began increasing by 12 hours PBM. Ecdysteroid titer in hemolymph peaked between 18 and 24 hours PBM, with the highest titer measurement at 18 hours PBM (126.81 ecdysteroids (pg)/female ± 17.49, n=7). Following this peak, hemolymph ecdysteroids began to decrease, reaching previtellogenic level before conclusion of the gonotrophic cycle.

*Oocyte yolk length:* Deposition of egg yolk in *An. gambiae* was quantified by measuring the longitudinal axis of yolk in the primary oocytes of sugar- and blood-fed individuals (Figure 2.3). Oocytes of sugar-fed females contained no yolk. Measureable amounts of yolk were detected at 12 hours PBM (mean =27.22 µm ± 4.5, n= 36). The deposited yolk was disk shaped, with the length of the longitudinal axis being less than the width of the yolk disk and the yolk being

slightly transparent in appearance. At 18 hours PBM, additional yolk deposition had occurred, increasing yolk length (61.43 µm ± 5.10, n=28). Yolk at this time was dense and its overall shape in the oocyte was spherical. At 24 hours PBM, average yolk length had again increased significantly (105 µm ± 3.90, n=48). The shape of the yolk had become more oval, with the end proximal to the nurse cells being somewhat flattened. This trend of substantial yolk length increase between time points continued throughout the gonotrophic cycle, with average length reaching its maximum 72 hours PBM (402 µm ± 3.48, n=60).

**DISCUSSION**

In this study, we confirmed that the ovaries from *An. gambiae* females are a source of ecdysteroids and that the release of these ecdysteroids into the hemolymph occurs in response to a blood meal stimulus. We quantified the secretion of ecdysteroids by *An. gambiae* ovaries during a first gonotrophic cycle. Ovarian ecdysteroid release was quantified by incubating ovaries at various times during a gonotrophic cycle in saline media and measuring the secreted ecdysteroids. The amount of circulating ecdysteroids in the hemolymph of these same females was also measured with an RIA.

Our study found ovarian ecdysteroidogenesis in blood-fed *An. gambiae* females reaches a maximum between 18 and 24 hours PBM. Following this peak, production steadily declines to non-blood-fed levels before the end of the gonotrophic cycle and remains at a relatively low level, presumably until another blood meal has been taken. The timing of peak ovarian ecdysteroidogenesis during the gonotrophic cycle matches that observed by Hagedorn in *Ae. aegypti* (24 hours PBM) (Hagedorn et al. 1975) but differs greatly from the observations of peak

levels in *Cx. pipiens* (36 hours PBM) (Baldridge and Feyereisen 1986) and *An. stephensi* (30-34 hours PBM) (Redfern 1982).

We also measured the titer of ecdysteroids in female hemolymph during a gonotrophic cycle. The peak of the ecdysteroid titer occurs between 18 and 24 hours PBM and then falls to pre-vitellogenic levels. Thus, the data observed matches well with ovary steroidogenesis data. Both of these experiments illustrate very clearly that the trends of ecdysteroid synthesis and secretion in *An. gambiae* are similar to the trend of whole body ecdysteroid levels described in *Ae. aegypti* (Hagedorn et al. 1975). In this study, the physiological range of hemolymph ecdysteroids was $6 \times 10^{-8}$ to $2.7 \times 10^{-7}$ M.

In mosquitoes, elevated ecdysteroid levels in the hemolymph trigger the fat body's synthetic machinery to begin producing YPPs. The YPPs are secreted into the hemolymph and incorporated into the primary oocytes in response to ecdysteroids in the hemolymph (Raikhel and Dhadialla 1992). Our experiments found no measurable yolk content in ovaries of sugar-fed control individuals. Following the blood meal, measurable deposition of yolk components was not observed until 12 hours PBM. Although the length of the egg yolk continued to increase throughout the gonotrophic cycle to 72 hours PBM, any lengthening after 30 hours PBM is most likely due to elongation from chorion development and not addition of yolk (Clements 1992).

Much of what has been learned about the endocrinology of mosquito reproduction over the past decades comes mostly from work with *Ae. aegypti*. The discovery of the central role that ecdysteroids play in mosquito vitellogenesis has led to novel approaches in controlling their population, such as ecdysteroid agonists developed to control both immature and adult mosquitoes (Beckage et al. 2004). The possibility of physiological differences between *Ae. aegypti* and other medically important mosquitoes makes investigation into these species very

important. The data we present here are a significant step to quantifying key endocrinological and physiological events occurring during the reproduction process in another anautogenous mosquito species, *An. gambiae*. This work also goes further in describing ecdysteroid synthesis in *An. gambiae* than previous work in *Ae. aegypti*. Instead of only measuring ecdysteroids in the whole body, we were able to quantify the amount of ecdysteroids that the ovaries alone were capable of producing. We also were able to measure the amount of ecdysteroids present in the hemolymph of those same individuals. In the future, researchers will be better able to answer questions concerning the possible changes in these phenomena in response to such conditions as repeated gonotrophic cycles, the nutritional status of the immature or adult mosquito, and infection by disease pathogens.

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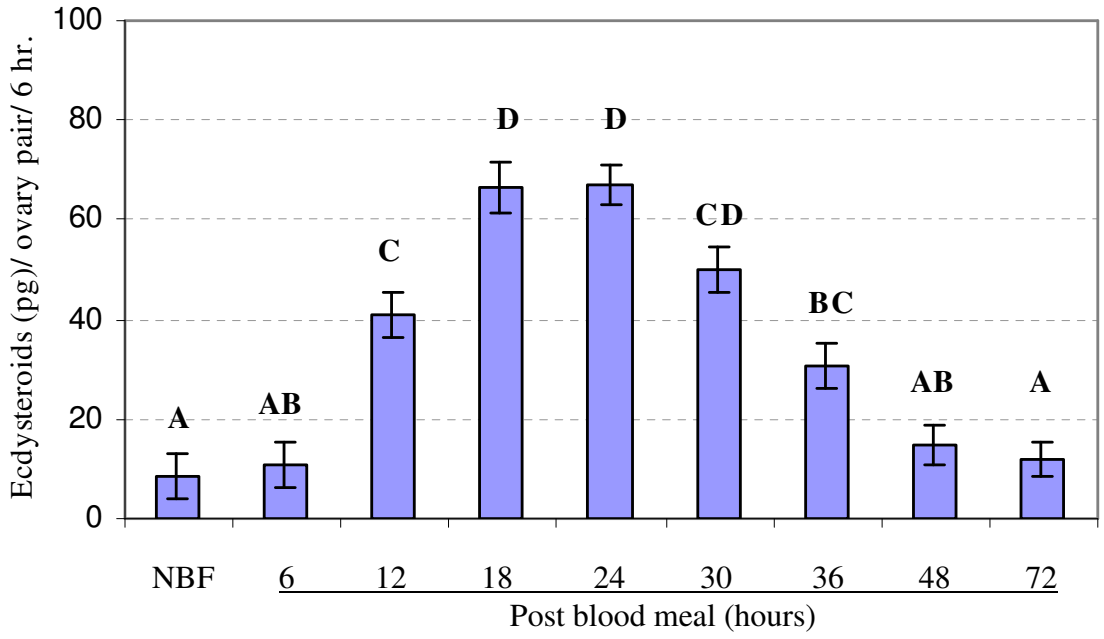


Figure 2.1. *In vitro* secretion/ production of ecdysteroids by *An. gambiae* ovaries during a gonotrophic cycle. Ovary pairs were dissected from *An. gambiae* females fed sugar water (NBF) and at times after receiving a blood meal. Ovary pairs were incubated as sets of four in 60 µl modified Beyenbach saline solution for 6 hours at 27º C. At least three cohorts containing three replicate sets were measured for each time point. Secreted ecdysteroids were measured by assaying 50 µl of the saline solution using a radioimmunoassay. Results represent average ecdysteroids (pg) secreted/ovary pair/6 hours. Error bars represent standard errors. Statistically similar results are denoted with the same letter (Tukey-Kramer HSD, $p \leq 0.05$). Sample number for each time point ranges from 7 to 15.

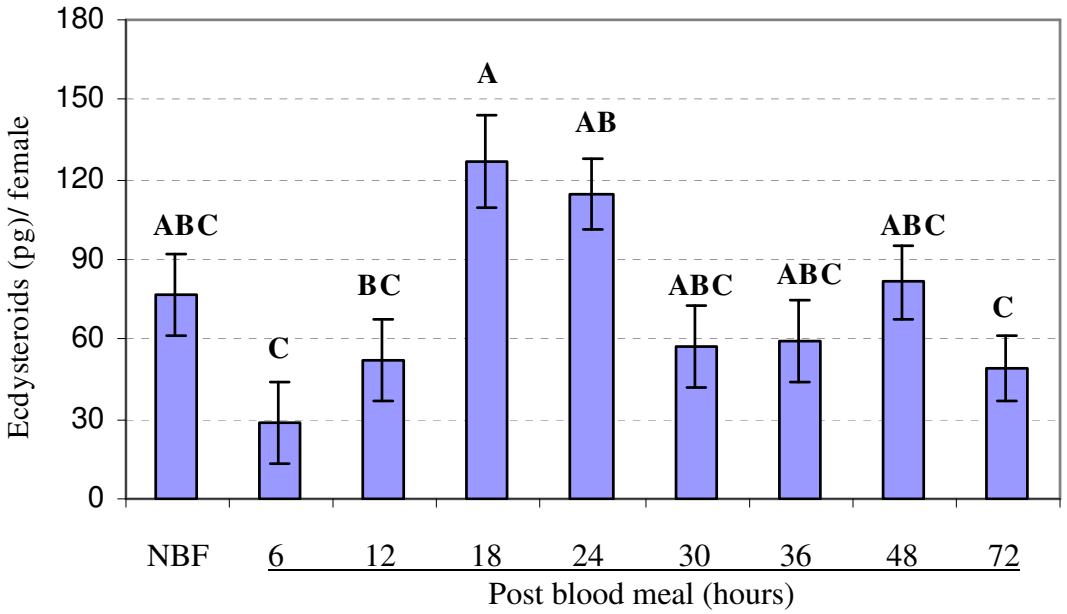


Figure 2.2. Hemolymph ecdysteroid titer for *An. gambiae* females during a gonotrophic cycle. Hemolymph from groups of four sugar-fed (NBF) or blood-fed (PBM) *An. gambiae* females was allowed to diffuse into 100 µl modified Beyenbach media and 50 µl of the media/hemolymph solutions were removed for quantification of ecdysteroids in a radioimmunoassay. At least three cohorts containing three replicate sets were measured for each time point. Results represent average hemolymph ecdysteroids per female. Error bars represent standard errors. Statistically similar results are denoted with the same letter (Tukey-Kramer HSD, $p \leq 0.05$). Sample number for each time point ranges from 7 to 15.

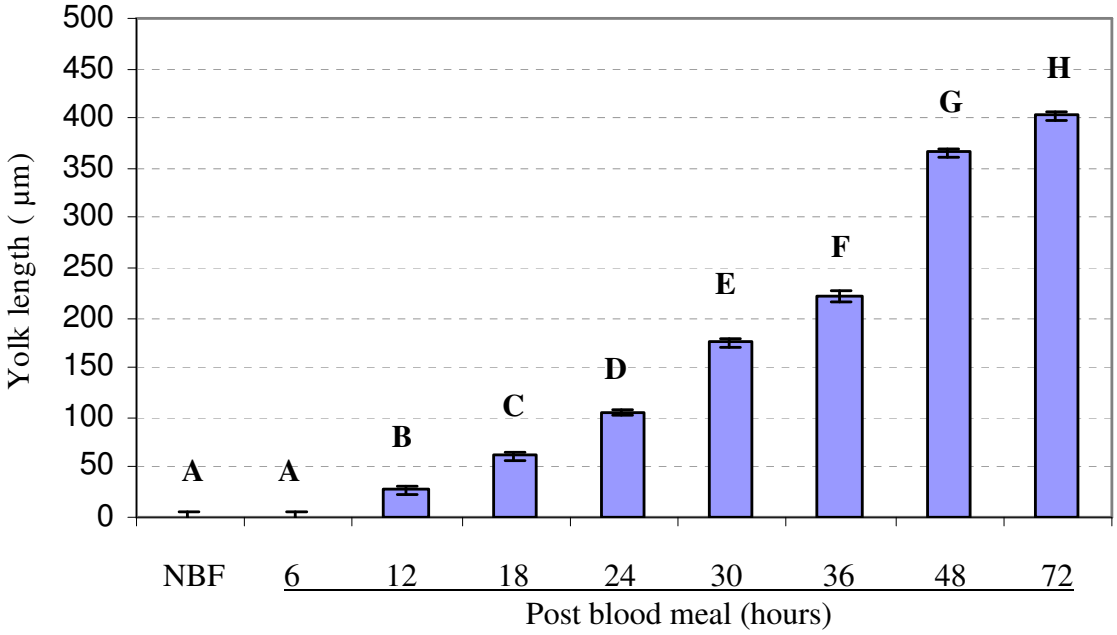


Figure 2.3. Yolk length in *An. gambiae* oocytes during a gonotrophic cycle. Representative oocytes from ovaries of sugar-fed (NBF) and blood-fed females were measured along their longitudinal axes as a representation of yolk deposition before and during a gonotrophic cycle. Ovary pairs from which oocytes were measured were used in the *in vitro* assay. Error bars represent standard errors. Statistically similar results are denoted with the same letter (Tukey-Kramer HSD, $p \leq 0.05$). Sample number for each time point ranges from 28 to 60.

# CHAPTER 3

# CHARACTERIZATION OF THE OVARIAN ECDYSTEROIDOGENIC HORMONE IN *ANOPHELES GAMBIAE* (DIPTERA: CULICIDAE)[1]

[1] Thomas, S. D. and M. R. Brown. To be submitted to *Journal of Medical Entomology*.

## ABSTRACT

We characterized a cDNA sequence that encodes a putative ovarian ecdysteroidogenic hormone (*AgamOEH*) in *Anopheles gambiae*. The translated open reading frame has an amino acid sequence similar to the *Aedes aegypti* OEH. Both OEHs also show some similarity to the locust neuroparsins. The distribution of the *An. gambiae* OEH transcript and peptide are characterized in *An. gambiae* during different life stages and during a gonotrophic cycle. A putative OEH peptide was purified from 20,800 adult *An.gambiae* heads using HPLC. Although we were unable to show bioactivity of this purified peptide *in vivo* or *in vitro*, we were able to demonstrate the ability of bovine insulin to activate oocyte development *in vivo*.



INDEX WORDS: *Anopheles gambiae*, *Aedes aegypti*, ovary ecdysteroidogenic hormone, ovary.

## INTRODUCTION

The reproductively competent female mosquito uses various cues to locate a potential host. Once an appropriate host has been located, the female mosquito then maneuvers to take a blood meal within a few minutes and escape unharmed. The ingested blood meal triggers an intricate cascade of physiological events that occur over the next few days and conclude with excretion of the digested blood meal and oviposition of eggs. Much of what we have learned about the processes involved in mosquito reproduction primarily comes from work with one of the approximately 3,000 mosquito species. The model species for anautogenous reproduction is the yellow fever mosquito, *Aedes aegypti*. The extensive use of *Ae. aegypti* is primarily due to its medical importance as a major disease vector and because of its ease of rearing (Klowden 1997). Within a few hours after taking a blood meal, the medial neurosecretory cells (MNC) in the female brain release a neuropeptide hormone, ovarian ecdysteroidogenic hormone (OEH), from axons terminating in the insect neurohemal organ, the corpora cardiaca (Lea 1972, Brown et al. 1998). This neuropeptide stimulates the ovaries to synthesize and release ecdysone, commonly known as the insect molting hormone (Hagedorn et al. 1975). Almost immediately after being secreted, ecdysone is converted into the biologically active 20 hydroxyecdysone (20-HE) by the action of a 20-monooxygenase, an enzyme distributed in the ovaries and peripheral tissues of the female mosquito (Smith and Mitchell 1986). The converted 20-HE stimulates the biosynthetic machinery in the fat body cells to begin manufacturing yolk components, primarily yolk protein precursors, lipids and other molecules. The various yolk constituents are then secreted into the hemolymph and are taken into the developing primary oocytes by receptor

mediated endocytosis and condensed to form the yolk granules (review: Raikhel, et al., 2005). Around 30 hours PBM, adequate yolk provisions have been stockpiled in the primary oocytes and the ovaries begin to lose patency. The loss of ovarian patency physically blocks any additional yolk uptake. The synthetic machinery responsible for yolk synthesis in the fat body is broken down and metabolism returns to the non-vitellogenic state in preparation for the next reproductive cycle. The female then begins the search for a suitable oviposition site once her eggs have matured. Only after oviposition has taken place is the female mosquito able to take another blood meal and begin the reproductive process anew.

Gillett (Gillett 1956)and Clements (Clements 1956) showed that a humoral factor released from the brain was necessary for egg development in mosquitoes. Later, Lea (1967) demonstrated that this factor, which he named the "egg development neurosecretory hormone" (EDNH), was produced by the MNC and was released into the hemolymph through the corpora cardiaca in response to signals from a blood meal. Hagedorn et al. (1979) showed that EDNH was the stimulus that triggered the release of ecdysone from the ovaries. Several attempts were made to purify and characterize EDNH from *Aedes aegypti* (Borovsky and Thomas 1985, Whisenton et al. 1987, Matsumoto et al. 1989). The term ovarian ecdysteroidogenic hormone (OEH) later replaced the EDNH in order to better describe the hormone's function (Matsumoto et al. 1989). Finally, a neuropeptide was isolated, and a cDNA characterized that encodes a prepropeptide that is processed into a 8803 MW peptide that shows sequence similarity to the locust neuroparsins (Brown et al. 1998).

In respect to functional similarity, OEH does share functional similarity with PTTH and the bombyxins, both of which cause ecdysteroid secretion by the prothoracic gland. The bombyxins were originally characterized in the silkworm moth, *Bombyx mori* (Nagasawa et al.

1986), and belong to the insulin like peptide (ILP) family of hormones based on their tertiary structure (Jhoti et al. 1987). Due to the structural similarity of bombyxins to insulin, the ecdysteroidogenic function of vertebrate insulins has been tested and confirmed *in vitro* in *Aedes aegypti* (Graf et al. 1997, Riehle and Brown 1999).

Using the recently completed *Anopheles gambiae* genome database (Holt et al. 2002), we identified and characterized a cDNA sequence that encodes a putative homolog to the *Aedes aegypti* OEH I neuropeptide in the African malaria mosquito, *An. gambiae* (Riehle et al. 2002). This OEH is also the first neuropeptide to be characterized in *An. gambiae*. We also demonstrated the tissue and temporal distribution of the OEH transcript and peptide during both development and the gonotrophic cycle in *An. gambiae*. We then purified a putative OEH from extracts of adult *An. gambiae* heads. The bioactivity of the purified OEH and bovine insulin were tested with an *in vivo* assay and OEH only was tested with an *in vitro* assay.

## MATERIALS AND METHODS

*Mosquito rearing: Anopheles gambiae* (G3 strain) were obtained from the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia. All life stages were maintained at 27° C with a 16 hour light: 8 hour dark photoperiod. Larvae were reared in groups of approximately 200 in four ounces of deionized water in covered, shallow aluminum trays and fed finely ground TetraMin® Rich Mix fish food (Tetra) until pupation. Pupae were separated from larvae prior to adult eclosion by transferring both into an Erhlenmeyer flask filled with deionized water and collecting pupae as they rose to the surface. Pupae were kept in one ounce plastic cups containing deionized water in ventilated acrylic enclosures (12 in x 9 in x 6.5 in) in preparation for adult eclosion within the following 24-hour period. Any uneclosed pupae after this period

were moved to a new enclosure(s) to ensure that similarly aged adults were used for all experiments. Adult mosquitoes were fed an 8% fructose water solution as a sole means of nutrition previous to a blood meal. Females were allowed to blood feed for approximately 20 minutes on CD1 mice (Charles River Laboratory) narcotized with a combination of Xyla-ject® (Phoenix Pharmaceutical Inc.) and Ketaset® (Fort Dodge Animal Health) anesthetics.

*AgamOEH gene characterization*: Heads from 3 to 4 day old sugar fed *An. gambiae* females (G3 strain) were dissected with forceps and microscissors treated with RNaseZap (Ambion) and stored immediately in 50 µl RNAlater (Sigma) at 4°C or used directly for genomic DNA purification with the E.Z.N.A. Insect DNA kit (Omega Bio-Tek). Total RNA was purified with the RNeasy® mini kit (Qiagen) along with an on-column DNase digestion with the RNase-free DNase kit (Qiagen). Quantification of total RNA in samples was done using a Spectronic® GeneSys 5 spectrophotometer. Synthesis of cDNA from total RNA was done with SuperScript II™ (Invitrogen), Advantage™ RT-for-PCR (BD Biosciences) or iScript™ (Bio-Rad) cDNA synthesis kits. 5'and 3' UTR of cDNA were prepared using the 5'/3' RACE kit, 2nd generation (Roche). Gene specific primers were synthesized for PCR amplification of cDNA based on *Agam*OEH EST sequences found in the *Ano*DB sequence database (*Ano*DB ESTs: BX 055247, BX 608380, BX 066701, BX 612237, BX 066702, and BX 055248). PCR amplification protocol was as follows: 95° C for 5 minutes, 95° C for 15 seconds, 55-69° C for 15 seconds, 72° C for 30-45 seconds (30-35 cycles), 72° C for 7 minutes. PCR products were separated by agarose gel electrophoresis with 1% agarose TAE gels. PCR products were excised from agarose gels and purified of ethidium bromide with GenElute™ Minus EtBr spin columns (Sigma). PCR products were inserted into pCR4-TOPO or pCRII-TOPO plasmid vectors (Invitrogen), which were then transfected into Top 10® *Escherichia coli* (Invitrogen) via

chemical transformation. Colonies containing inserts were cultured overnight in LB broth (100 µg /ml Ampicillin). Plasmid extraction of overnight cultures was done with QIAprep® Spin miniprep kit (Qiagen) and sequenced by Molecular Genetics Instrumentation Facility (UGA, Athens, GA) or Retrogen, Inc (San Diego, CA).

*PCR Analysis of AgamOEH transcripts during development and a gonotrophic cycle:* Total RNA for the developmental timecourse was collected from 36 hour post-oviposition eggs (300), 10 first instar larvae whole bodies, 10 each of heads, thoraces, and abdomens from fourth instar larvae, pupae, and three day-old sugar-fed male and female adult *An. gambiae*. Samples for gonotrophic cycle experiments were the heads and thoraces from 3 day-old sugar-fed females and females at 2, 6, 12, 18, 24, 30, 36, 48, and 72 hours post-blood meal. All necessary dissections were conducted in RNAlater™ with forceps and microscissors treated with RNaseZap® (Ambion). Samples were then stored in 50 µl RNAlater (Sigma) and stored at 4°C prior to RNA purification. Total RNA was purified from all samples with Qiagen's RNeasy Mini Kit along with on-column DNase digestion with the RNase-free DNase kit (Qiagen). Quantification of total RNA was performed using a Spectronic® GeneSys 5 spectrophotometer. Up to a maximum of 2.0 µg of total RNA was used for the synthesis of cDNA for each sample using Advantage™ RT-for-PCR (BD Biosciences). PCR amplification of *Agam*OEH product was conducted with an Eppendorf Mastercycler thermocycler using a gene specific forward primer: 5'-CAA AAT TGC AAT GTC GTT CGG-3' (ORF position -10 to 10) and reverse primer: 5'-CTT CTT CTT CTT TGC TCG ATC GG-3' (ORF position 528 to 506). The PCR amplification protocol was as follows: 95° C for 5 minutes; 95° C for 15 seconds, 62° C for 15 seconds, 72° C for 30 seconds (30 cycles); 72° C for 5 minutes. Single primer and no template control samples were also prepared. PCR products were gel purified on a 1% agarose TAE gel

and imaged with a GeneGnome (Syngene Bio Imaging). Tissues from two separate replicates were processed for the developmental time course and three replicates were analyzed for the gonotrophic cycle experiment.

*Immunoblot analysis of AgamOEH during development and a gonotrophic cycle:* Peptides for the developmental timecourse were extracted from 36 hour post-oviposition eggs (300), first instar larvae whole bodies, and heads, thoraces, and abdomens from fourth instar larvae, pupae, and three day-old sugar-fed male and female adult *An. gambiae*. Peptides for the gonotrophic cycle were extracted from heads and thoraces of 3 day-old sugar-fed females and females at 2, 6, 12, 18, 24, 30, 36, 48, and 72 hours post-blood meal. All samples were collected in 400 µl cold 0.2 N acetic acid, ground with plastic pestles, sonicated with a Branson Sonifier (20% duty cycle, 2 output level, 8 pulses), centrifuged at 13,000 x g at 4° C for five minutes, and the supernatant removed by pipette and frozen at -80°C. Samples were later lyophilized, resuspended in reducing Tris-tricine sample buffer (200 mM Tris-HCl (pH 6.8), 40% glycerol, 2% SDS, 0.04% Coomassie Blue R-250, 125 mM DTT) and incubated at room temperature for 10 minutes. The experimental samples, *Aedes aegypti* OEH standard (*Aae*OEH) and Kaleidoscope polypeptide standard (Bio-Rad) were heated at 65°C for 45 minutes, iced quickly, then centrifuged at 13,000 x g for two minutes at 4° C.

For SDS/PAGE, samples and standards were loaded into 10-20% Tris-tricine/ peptide Criterion™ gels (Bio-Rad). Samples were electrophoresed in a Criterion gel apparatus (Bio-Rad) for 2.75 hours at 90 V, 4°C in 1x Tris-tricine running buffer (100 mM Tris-HCl, 100 mM Tris-base, 0.1% SDS). The samples were then transferred onto Immuno-Blot™ (Bio-Rad) PVDF membrane for 30 minutes at 45 V, 4°C in transfer buffer (12 mM Tris-base, 96 mM glycine, 20% MeOH). PVDF membranes were blocked in 40 ml 2% bovine serum albumin (BSA)/ 2%

non-fat dry milk/ 1% goat serum TBS-T (20mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) for one hour at room temperature. After blocking, membranes were incubated overnight at 4°C with primary antibody (rabbit anti-AaeOEH 304C) (Brown and Cao 2001) at a 1:10,000 dilution. Membranes were washed with TBS-T twice briefly, once for 15 minutes, then three additional times for 5 minutes each. Membranes were incubated for three hours at room temperature with an anti-rabbit antibody conjugated to horseradish peroxidase diluted to1: 40,000 in 40 ml TBS-T with 2% BSA/ 2% non-fat dry milk/ 1% goat serum, and washed again as described earlier. For chemiluminescent imaging, membranes were treated with 1.2 ml (600 µl of each reagent) of Renaissance Western Blot Chemiluminescense Reagent Plus™ or ECL Advance™ (Amersham Biosciences) western detection reagents per membrane, incubated at room temperature, and imaged using GeneGnome (SynGene) imaging equipment. Estimation of peptide molecular weight was performed using Gene Tools software (SynGene). Tissues from three separate cohorts of mosquitoes were processed and analyzed for both experiments.

*Peptide extraction from An. gambiae heads:* An estimated 20,800 adults previously collected and stored at -80°C were flash frozen in liquid nitrogen, and then shaken vigorously to break the heads from the bodies. Heads were sorted through a #40 hand sieve, weighed, and moved to a glass beaker stored on ice. The heads were then homogenized in 20 ml of cold (4°C) 0.2 N acetic acid with an Ultra-Turrax homogenizer (Tekmar Co.) at maximum power for one minute. An additional 3 ml of acetic acid was combined to the homogenate from washing the homogenizer tip to reclaim any residual extract. The homogenized head solution was centrifuged (15 minutes at 20,000x g, 4°C), the supernatant was decanted and stored at 4ºC. Another 20 ml 0.2 N cold acetic acid was added to the pellet and the pellet was homogenized for an additional minute. Any extract residue was cleaned from the homogenizer tip with another 3

ml 0.2 N acetic acid. This extract solution was centrifuged a second time (15 minutes at 20,000x g, 4°C), and the supernatant was added to the previous supernatant for a total volume of 50 ml. The supernatant and pellet were then lyophilized.

*HPLC purification of peptides from* An. gambiae *head extracts:* To begin the purification of AgamOEH, the crude head extract solution was divided into twelve samples that were separately subjected to cation exchange HPLC (TSK Spherogel SP-5PW column; 7.5 x 75 mm; mobile phase solvents A: 0.02 M ammonium acetate, pH 4.5, with 10% $CH_3CN$; and B: 1 M ammonium acetate, pH 4.5; 0 to 100%B gradient over 50 min; monitored at 275 nm). From one HPLC run, aliquots of five sequential fractions were pooled and processed for Tris-tricine electrophoresis and immunoblotting, as previously described, and the pooled fraction sets 26-45 contained OEH-like material. Based on this result, fractions 26-45 from the above HPLC runs were pooled (ca. 120 ml) and extracted three times with 60 ml of hexane. The aqueous solution was divided into 6, ~20 ml samples that were lyophilized down to ~0.5 ml each.

Each of the six samples were subjected to reversed phase (RP) HPLC (Phenomenex Jupiter C5 column, 5 µm, 300Å material, 4.6 x 250 mm; mobile phase solvents A: 0.01% trifluoroacetic acid (TFA) (Pierce) in HPLC grade water; and B: same in 90% $CH_3CN$; gradient 20 to 60%B, 40 min, and 60 to 100%B, 10 min; monitored at 220 nm). From one HPLC run, aliquots of individual fractions were tested by immunoblotting, as above, and fractions 26-30 contained OEH-like material. Fractions 26-30 from the six HPLC runs were pooled and lyophilized for two subsequent HPLC steps on the same RP column: 1) mobile phase solvents A: 0.01% heptafluorobutyric acid (Pierce) in HPLC grade water; and B: same in 90% $CH_3CN$; gradient 20 to 100%B, 50 min; monitored at 275 nm; and 2) same mobile phase solvents with TFA as above; gradient of 25 to 55%B, 50 min, 55 to 100%B, 10 min; monitored at 275 nm. As

determined from the immunoblots of fraction aliquots from step 1, fractions 33 and 34 contained OEH-like peptide in the penultimate HPLC step were pooled, lyophilized, and subject to step 2. In this final purification step, fractions 19-21 contained a peptide (~7700 MW) that was immunoreactive to the AeaOEH antibody (Figure 3.7). From these fractions, 50 µl of each were combined and sent for amino acid analysis at UTMB (Galveston, TX) to measure the total amount of peptide purified. The analysis determined that the 150 µl submitted contained 3 µg of peptide.

In vitro *assay of purified AgamOEH:* Triplicate sets of four ovary pairs were dissected from three to five day-old sugar-fed *An. gambiae* females and placed into 1:1 *Aedes*: Beyenbach saline media (*Aedes* solution: 128 mM NaCl, 4.7 mM KCl, and 1.9 mM $CaCl_2$; Beyenbach solution: 0.139 M NaCl, 0.00405 M KCl, 0.00185 M $CaCL_2$, 0.0125 M HEPES, 0.0025 M Trehalose, 0.0003 M $MgCl_2$ and 0.009 M $NaHCO_3$) held in inverted 500 µl microcentrifuge tube caps placed into 24 well flat-bottomed RIA plates (Costar®) filled with 200 µl deionized water (to maintain humidity). After ovary dissections were completed, HPLC purified product from the *An. gambiae* head extracts was added to the incubating ovaries (total volume of 60 µl) and incubated for 6 hours at 27°C in a shaking water bath. Following incubation, 50 µl of each sample was removed and stored at -80° C prior to ecdysteroid quantification by radioimmunoassay (RIA).

*Ecdysteroid radioimmunoassay:* Modified procedures for *in vitro* tissue ecdysteroid production and the ecdysteroid (RIA) were followed (Riehle and Brown 1999). The anti-ecdysteroid rabbit serum (AS 4919, a gift from P. Poncheron, Université P. et M. Curie, Paris, France) recognizes ecdysone and 20-hydroxyecdysone equally (Porcheron et al. 1989), as verified with our RIA. For RIA, each tube contained 50 µl of a stock [23,24-3H(N)]ecdysone solution (= [3H]ecdysone;

12,000-13,000 counts/minute (cpm)/ 50 µl; PerkinElmer, Boston, MA), 50 µl of antiserum diluted to 1:35,000-45,000 (final dilution for bound to free [3H]ecdysone cpm ratio (B/F) = 1), and 50 µl of sample or 20-hydroxyecdysone standard. Separate, triplicate tubes were set up for total cpm [3H]ecdysone, non-specific binding of [3H]ecdysone, diluted antibody and [3H]ecdysone alone to monitor B/F, and each of the 20-hydroxyecdysone standards (1, 5, 10, 25, 50, 100, 250, 500, and 1000 pg) for every RIA. After overnight incubation at 4° C, bound and free radiolabeled ecdysone were separated in tubes by the ammonium sulfate method, and pellets in tubes were dispersed in water and scintillation fluid and counted in a scintillation counter (Beckman). For each RIA, a standard curve was plotted from the averaged B/F (Y axis) and log values for the 20-hydroxyecdysone standards (X axis). The quantity of immunoreactive ecdysteroids in samples was calculated from a regression equation for the linear portion (10-250 pg) of the standard curve; samples were diluted when necessary to stay within this range. Sample values reported for each tissue treatment are presented as "ecdysteroid pg", because the secreted ecdysteroid species are unknown, and the values are means of triplicate treatments from three experiments.

In vivo *assay of purified AgamOEH and bovine insulin:* Female mosquitoes were allowed to blood feed on narcotized CD1 mice for approximately 20 minutes, decapitated with microscissors within one to two hours, and injected with HPLC purified AgamOEH or bovine insulin (Sigma). Lyophilized, HPLC purified AgamOEH was resuspended with *Aedes* saline media to a concentration of 0.5 µg/ 0.5 µl and sequentially diluted to a final concentration of 0.062 µg/ 0.5 µl. Bovine insulin lyophilate was resuspended in purified water with the aid of 0.1 M HCl to a stock solution of 2.5 nmol/ 0.5µl. The stock solution was diluted with saline media in half steps from a range of 1.25 nmol/ - 0.078 nmol/ 0.5 µl. Injections were performed by

piercing the venter of the thorax near the abdomen of each mosquito with a glass needle and injecting 0.5 µl of sample with mouth pressure. Mosquitoes were then kept at 27° C under high humidity until egg yolk measurements were taken 22-26 hours PBM to measure OEH-like activity of the samples.

**RESULTS**

*Nucleotide and amino acid sequence of AgamOEH:* An *AgamOEH* expressed sequence tag (EST) was identified in the *An. gambiae* database by conducting a tblastx search using the *AeaOEH* open reading frame (ORF) sequence. The query returned an *AgamOEH* cDNA that contained a 411 bp ORF encoding a 137 amino acid prepropeptide with a predicted molecular weight of 15,534 (Figure 3.1). The resulting peptide produced by signal peptide cleavage after $A_{22}$ has a predicted molecular weight of 13,134 Daltons (Da) (SignalP 3.0 software). AgamOEH shares 46% amino acid sequence indentity and 56% similarity with AaeOEH. Likewise, AgamOEH shares similarity with the grasshopper neuroparsins (Figure 3.2). The predicted *AgamOEH* genomic sequence contained two introns, 107 and 74 bp in length, respectively. The *AgamOEH* sequence was confirmed by sequencing cloned PCR products amplified with gene specific primers from cDNA and gDNA template from heads of 3-4 day old sugar fed *An. gambiae* females.

*AgamOEH transcript expression during* An. gambiae *development:* The presence or absence of *AgamOEH* transcript was tested in different life stages and body regions of *An. gambiae*. Gene specific primers were used to PCR amplify a 521 bp product from cDNA template from eggs and heads, thoraces, and abdomens of larvae, pupae, male and female adults (Figure 3.3). Amplicons 521 bp in length were detected in eggs as well as in the heads and thoraces of fourth instar larvae

and pupae. *AgamOEH* transcripts were also present in heads of adult male and female mosquitoes, but not in the thoraces or abdomens. Amplification of gDNA in samples produced a 702 bp amplicon and was detected in various samples.

*AgamOEH peptide localization during development:* Gel electrophoresis was performed with extracts of eggs and the heads, thoraces, and abdomen of larvae, pupae, male and female adults (Figure 3.4). Immunoblotting detected a single immunoreactive peptide with a predicted molecular weight of 13,000 Da in eggs and the heads and thoraces, but not abdomens of larvae and pupae. In adult mosquitoes, an additional peptide was detected in the heads and thoraces that had an approximate molecular weight of 19,000 Da. Density of bands detected in thoracic samples was less than that detected in head samples.

*AgamOEH transcript expression during a gonotrophic cycle:* The predicted 521 bp amplicon of *AgamOEH* cDNA was amplified by PCR from heads, but not thoraces or abdomens (data not shown) of sugar-fed females and at all times during the gonotrophic cycle in blood-fed individuals (Figure 3.5).

*AgamOEH peptide localization during a gonotrophic cycle:* Immunoblots were conducted with peptides collected from the heads, thoraces, abdominal walls, and hemolymph of sugar-fed females and blood-fed females at 2, 6, 12, 18, 24, 30, 36, 48, and 72 hours PBM. Immunoblot analysis detected two immunoreactive peptides (13,000 and 19,000 Da) in the heads and thoraces, but not abdominal walls (data not shown) of sugar-fed females and blood-fed females at all time points examined during the gonotrophic cycle (Figure 3.6**).**

Hemolymph samples collected from sugar-fed females did not contain any immunoreactive peptides. However, a peptide with an estimated molecular weight of 49,000 Da was detected at

2 hours PBM and was still present until 24 hours PBM (Figure 3.7). After 24 hours PBM the peptide was no longer detected in the hemolymph.

*HPLC purification of AgamOEH peptide:* Crude extract obtained from the heads of *An. gambiae* mosquitoes was purified with four HPLC steps. Following each HPLC step, immunoblotting of the resulting fractions revealed those that contained immunoreactive peptides (11,000 Da) with a mass close to that predicted for AgamOEH. The positive fractions were combined and used for the next HPLC step. Purification continued in this manner until we were able to concentrate the peptide into a few fractions. Following the final HPLC purification, immunblotting detected a peptide with a molecular weight around 7,700 Da in three fractions (Figure 3.8). Aliqouts were prepared that contained a total of 200 µl from the three fractions, with each aliquot containing 12 µg of peptide, based on amino acid analysis data. The bioactivity of the peptide was later tested with both *in vivo* and *in vitro* assays.

In vivo *assay of purified AgamOEH and bovine insulin:* Bovine insulin and OEH peptide purified from *An. gambiae* heads were tested for egg development activity with an *in vivo* assay (Figure 3.8). The purified AgamOEH was unable to cause egg maturation at any of the dosages tested**.** However, the largest dosages (0.3 –1.25 nmol/ 0.5 µl) of bovine insulin were able to produce egg maturation similar to that of non-decapitated, sham injected individuals.

In vitro *assay of purified AgamOEH:* Purified OEH was also unable to cause ovary ecdysteroidogenesis at any concentration tested with the *in vitro* assay (data not shown).

**DISCUSSION**

The work we have presented here is a thorough characterization of the first neuropeptide identified for *An. gambiae*: AgamOEH. This homolog to AeaOEH was identified and characterized in *An. gambiae*. A sequence homology search of the *An. gambiae* genome

returned a genetic sequence encoding an OEH. This was confirmed by sequencing gDNA and cDNA from the heads of *An. gambiae* females. The *AgamOEH* open reading frame is 411 bp in length and encodes a prepropeptide 137 amino acids in length. Removal of the signal peptide produces a 115 residue peptide with an estimated molecular weight of 13,134 Da. The AgamOEH peptide shares 46% identity and 56% similarity with the amino acid sequence of AaeOEH. This includes the position of 10 cysteine residues and up to five disulfide bridges. Both OEH peptides exhibit some sequence similarity to the locust neuroparsins.

*AgamOEH* transcript was present in eggs, heads and thoraces of fourth instar larvae and pupae, and only in the heads of male and female adults. Immunoblots of similar samples detected an immunoreactive band with a molecular weight similar (13,000 Da) to the predicted AgamOEH peptide (13,134 Da) in eggs, heads and thoraces but not abdomens of larvae, pupae, male and female adults. In addition, a larger band with a molecular mass of 19,000 Da was also detected in male and female adult heads and thoraces. These results contrast with previous data from Brown and Cao (2001) in which they immunohistochemically detected OEH in neurosecretory cells, endocrine cells in the midgut and cardiac valve, perivisceral organs, and axons around the pyloric valve of *An. gambiae* larvae and adults. A possible reason for this discrepancy in the distribution of AgamOEH from the earlier work and our data may be in part due to crossreactivity of the primary antibody to other peptidergic cells. The absence of *AgamOEH* transcript and presence of the peptide in the thoracic region of adult mosquitoes may be a result of sample contamination from poor dissection technique which resulted in the inclusion of some portion of the corpora cardiaca, where OEH is released, remaining in the thorax. The presence of both the transcript and peptide in non-adult life stages as well as male adults hints at the possibility of an alternate function of OEH yet to be described.

PCR of *An. gambiae* cDNA found that *AgamOEH* transcripts were expressed in the heads, but not thoraces or abdomens of sugar-fed females and blood-fed females at all times observed after a blood meal. Immunoblot analysis of proteins extracted over the same time period detected two peptides in the heads and thoraces, but not abdomens, of sugar-fed and blood-fed females. The smaller of the peptides (13,000 Da) was approximately the molecular weight of that predicted for AgamOEH (13,134 Da) while the larger peptide had a molecular weight of 19,000 Da. These results match well with the observations of similar peptides in the development experiment. No changes in the levels of either the transcript or peptide were readily apparent during the gonotrophic cycle.

Immunoblot assay of female hemolymph during a gonotrophic cycle detected a single immunoreactive peptide from 2 to 24 hours PBM, but it was not present in hemolymph from sugar-fed females or after 24 hours PBM. However, this peptide had a larger mass (49,000 Da) than that of AgamOEH. Several possible explanations exist for the discrepancy in peptide mass in this experiment. The increased size may be the result of multiple OEH molecules combined acting as a unit, the addition of a carrier molecule which helps transport the peptide through the hemolymph, or the presence of another peptide that displayed artifactual immunoreactivity to the antibody used. The presence of a conglomerate of molecules may be unlikely because of the use of reducing compounds during the denaturation process. We believe that OEH is stored in preparation of the blood meal event and is released within two hours of the blood meal. OEH release ends and the peptide is removed from the hemolymph around 24 hours PBM. The timing of this event coincides with the peak of ecdysteroid secretion by the ovaries previously measured in chapter two.

Bovine insulin and a putative OEH purified from *An. gambiae* heads were tested for egg development activity with an *in vivo* assay. Although the purified AgamOEH was unable to cause egg development, bovine insulin was able to do so (Figure 3.8). At higher concentrations (0.3 –1.25 nmol/0.5 µl), bovine insulin was able to cause egg development similar to that of non-decapitated, sham injected individuals. Likewise, the purified AgamOEH was also unable to cause yolk deposition at any concentration tested with the *in vitro* assay (data not shown). The lack of OEH peptide bioactivity may in part be attributable to degradation (from 11,000 to 7,700 Da) that appeared to occur during the purification process.

Despite the threat that *An. gambiae* poses to human populations by transmitting deadly disease agents like *Plasmodium*, relatively little research has been concentrated on understanding the physiology or ecology of this species, especially compared to the body of work for *Ae. aegypti*. The data presented here are an initial step in establishing important parameters of reproductive physiology that can be used to compare *An. gambiae* to other mosquitoes, dipterans, and insects.

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5′ 1      TTTCACCATCACCATCAACCTGAATGTATCAGTAACCTTTCGCCAGGATCGTACCTGTGT

5′ 60     GCCTGCGTTCGTTTTACCGTGTGCGCGTCCTTGGAGCCAGTTGCTTGTCCACGGTCCTGC

5′ 120    TGTGATTAAGTGTTCTTTTGAGTGTCTCTGTGTGTAGTAAGATAGTTCTAACAAAAATTG

5′ 180    CAATGTCGTTCGGAAACGTTCTGCTGCTAACCGCACTGCTCGTGGGATATTCCTACTGGA
            M  S  F  G  N  V  L  L  L  T  A  L  L  V  G  Y  S  Y  W  S
            1                          10                          20

              Intron 1: 107 bp
5′ 240    GCTTAGCCCAACCAACGAACCTGATCGAGATCCGGTGCAAGATGCAGCGCGACGAAGACA
            L  A  Q  P  T  N  L  I  E  I  R  C  K  M  Q  R  D  E  D  I
                                       30                          40
                                                    Intron 2: 74 bp
5′ 300    TCAGCACGTGTAAATACGGCTTCTACATGACCACCTGCAAAACGTTTGCCTGTTGGAAGG
            S  T  C  K  Y  G  F  Y  M  T  T  C  K  T  F  A  C  W  K  G
                                       50                          60

5′ 360    GCCCGAACGAACCGTGCGGCGGCGAGCTGAGCAACAACATGCTGTACGGCAAGTGCAAGT
            P  N  E  P  C  G  G  E  L  S  N  N  M  L  Y  G  K  C  K  S
                                       70                          80

5′ 420    CCGGGCTGCGGTGCTGCAACGGCCGGTGCACCGGCTGCCTGAACGGGGTGTGCAGCGACT
            G  L  R  C  C  N  G  R  C  T  G  C  L  N  G  V  C  S  D  S
                                       90                          100

5′ 480    CCTGCCACCCGTCCAAGCTGCACAGCTTCCAGCATCGCTCCGATCGTACATGGTGGAGGC
            C  H  P  S  K  L  H  S  F  Q  H  R  S  D  P  Y  M  V  E  E
                                       110                         120

5′ 540    AGCGCCACCTGAGCCCGCTCTACCGGTTCTTCGACTACTACAGCAACGAGTAAGGCGGCT
            R  H  L  S  P  L  Y  R  F  F  D  Y  Y  S  N  E  *
                                       130

5′ 600    GCAGTCACACTCCGCCGATCAACCCGATCACCACAGTCACACAGTCACCCGCACTACCCC

5′ 660    GATACTTACGCCGATCGAGCAAAGAAGAAGAAGAAGAAGAAAAAAATAATTAACGCTTCG

5′ 720    GCCACACAACGTGTCGCAATGGTAGCCACTTCAACGATGCCAGGTGAGCTTTGCCATTCC

5′ 780    GCGCAACGATCTCCGCTCTCCTGTCCGCTGTCCGGAGATCCTGGGAACATTCCACCATCC

5′ 840    GCAGCCCCTCTAGACAGTTTGCTTCTACTTAGATAAGAACACGTATAGCCCTTCAATAAA

5′ 900    GCAGAAAAAAAAGTAATGTAACATCTACATGAACCCAGTGAAAAAAAAAAAAAAAAAAA

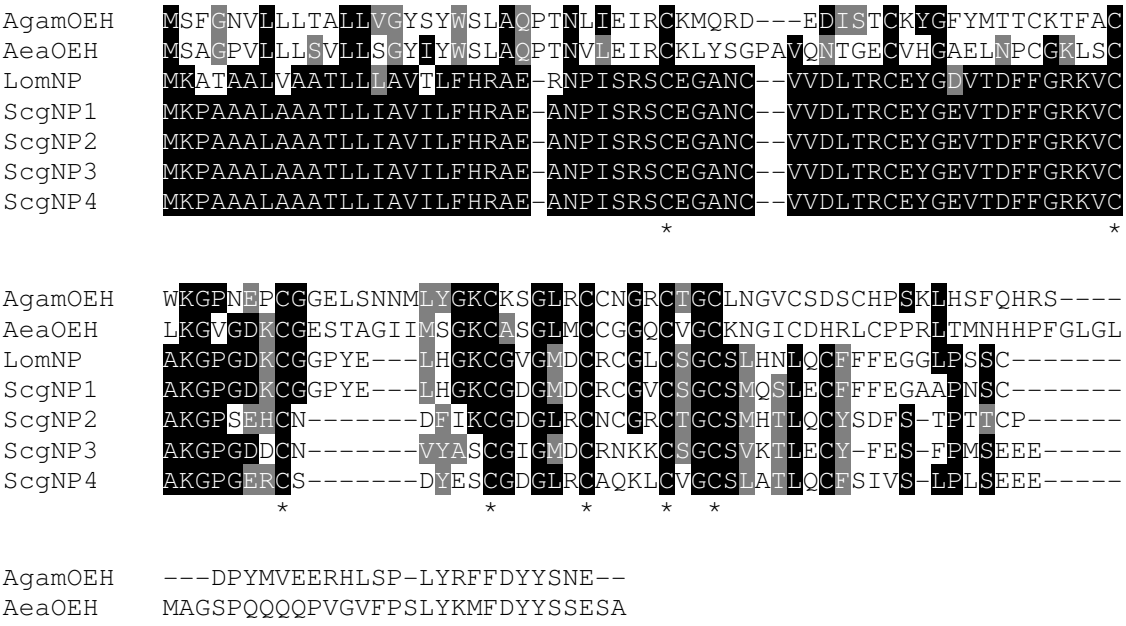
5′ 960    AAAAAAAA


Intron 1
   1      GTTAGTATCTCGGGCACCTGCACTGTCTGGCTTTTGTCCCCTCTTCCTATCGCAAACTCT
   60     GCTAACTCTGTGCCTGCTAAAATTTTTCTCCCCCGCTCGATACTGCAG

Intron 2
   1      GTAGGCCCTCGATTTCAGTGCGCTCTCGTACAATCGTTCACCGTTCCGAATCGCCTCTTC
   60     CTCCTTCCCTTTAG
```

Figure 3.1. Nucleotide sequence and translated open reading frame of *AgamOEH* cDNA. Signal peptide sequence is indicated in bold letters and peptide sequence is underlined. Intron positions are sizes are noted and their respective sequences listed. Polyadenylation signal at 3' end is underlined.

A.




B.


Figure 3.2. A. Comparison of the amino acid sequences of mosquito OEHs and grasshopper neuroparsins. B. *An. gambiae* (AgamOEH) and *Ae. aegypti* OEH (AeaOEH) are compared to neuroparsin sequences of *Locusta migratoria* (LomNP) and *Schistocerca gregaria* (ScgNP1-4). Amino acids shaded black are identical and those shaded gray are considered physiochemically similar by Boxshade software analysis. Amino acid sequences of AgamOEH and AeaOEH are 46% identical and 56% similar to one another. Asterisks (*) denote cysteine residues conserved between OEHs and neuroparsins. OEH signal peptides are noted with +. Putative OEH enzymatic processing site is noted with an arrow.

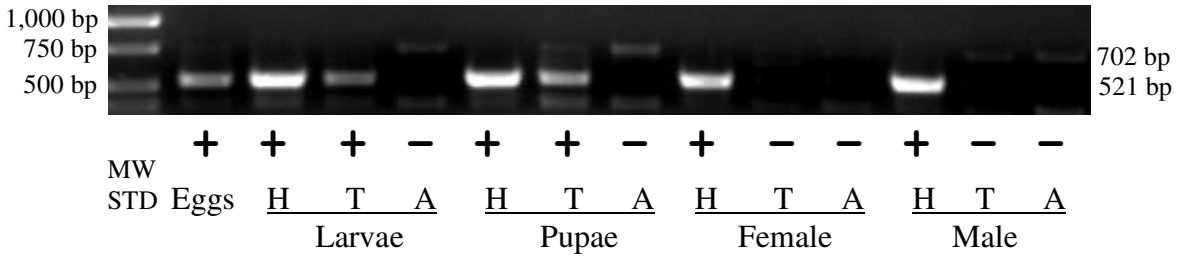



Figure 3.3. Transcript expression of *AgamOEH* at different life stages.
*An. gambiae* cDNA was synthesized from up to 2 µg total RNA collected from eggs, heads (H), thoraces (T), and abdomens (A) of 4th instar larvae, pupae, male and female adults (3 days old, sugar-fed). Below the representative gel of *AgamOEH* PCR products is the summary of RT-PCR results from two cohorts: +, transcripts present in the majority of samples or -, transcripts absent in the majority of samples. *AgamOEH* PCR product from cDNA is 521 bp in length and that from gDNA is 702 bp long.

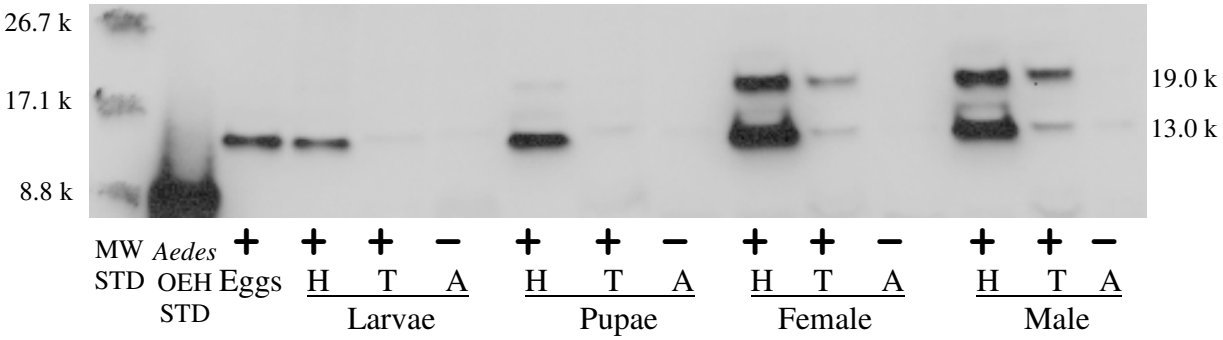



Figure 3.4. Immunoblot analysis of AgamOEH peptide of different life stages of *An. gambiae*. Peptides extracted from eggs, heads (H), thoraces (T), and abdomens (A) of fourth instar larvae, pupae, male and female adults (3 days old, sugar-fed) were electrophoresed on a 10-20% tris-tricine polyacrylamide gel under reducing conditions. Blots were incubated with an anti-AaeOEH antibody for chemiluminescent detection. Molecular weights of standards (MW STD) and *Aedes* OEH standard are displayed to the left. Below each gel is the summary of immunoreactive peptide detection for three cohorts: +, peptide present in majority of samples or -, peptide absent in the majority of samples. An immunoreactive band the approximate size of *Agam*OEH was detected in eggs and the heads and thoraces but not the abdomens of fourth instar larvae, pupae, and male and female adults. Another larger peptide was also detected in heads and thoraces of males and females only.

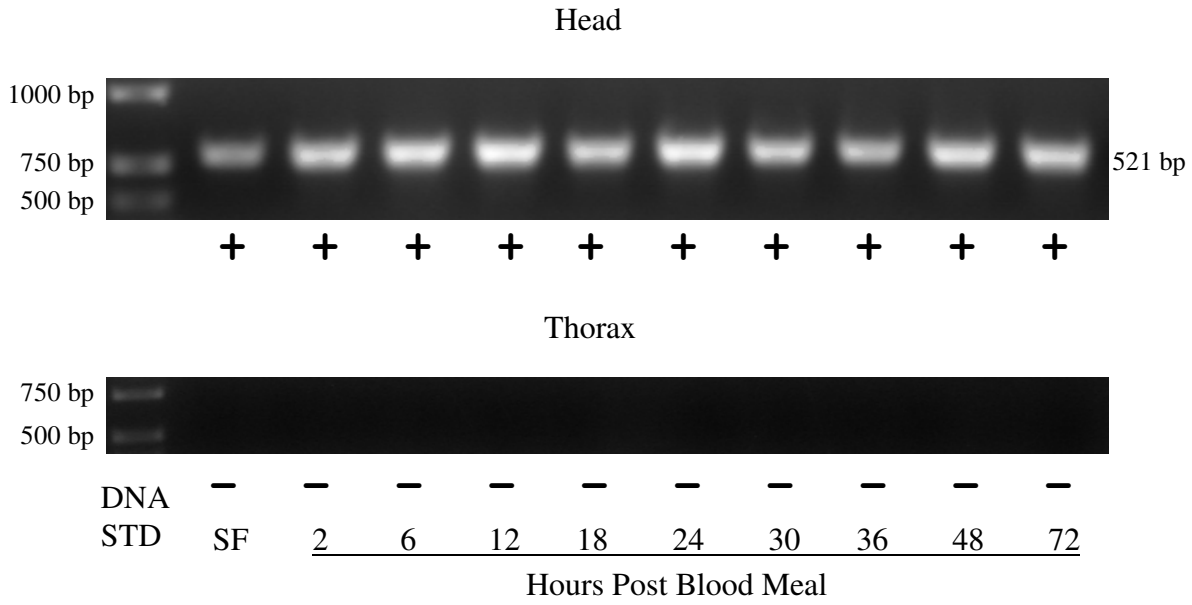



Figure 3.5. Representative gel showing *AgamOEH* transcript expression in female body regions during a gonotrophic cycle. cDNA was synthesized from total RNA (up to 2 µg) collected from heads and thoraces of sugar-fed (3 days old) (SF) and blood-fed females. Below each gel of *AgamOEH* PCR products is the summary of RT-PCR for three cohorts: +, transcripts were present in the majority of samples or -, transcripts were absent in the majority of samples. Nucleotide markers (DNA STD) are listed to the left. The size of the expected PCR product (521 bp, without introns) is given to the right.

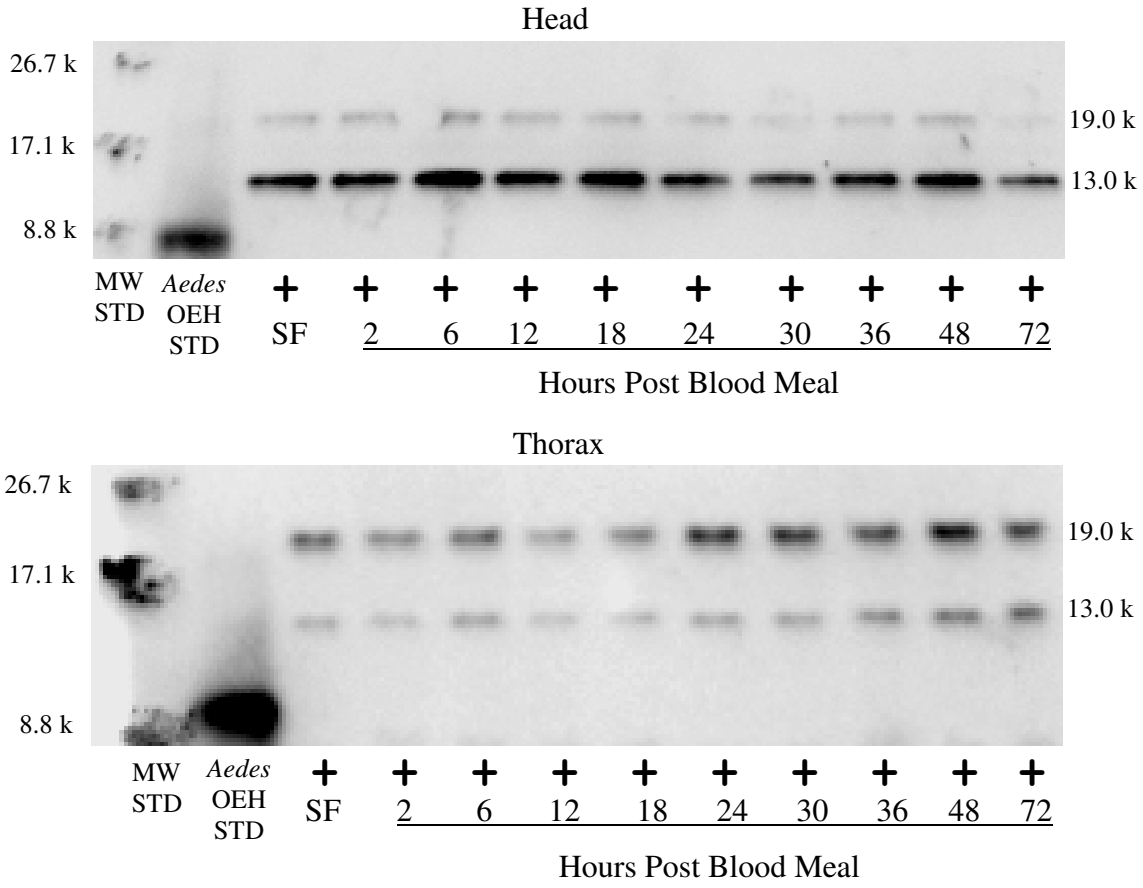



Figure 3.6. Tissue distribution of AgamOEH peptide during a gonotrophic cycle in *An. gambiae*. Lanes were loaded with heads (1 equivalent), and thoraces (2 equivalents) of sugar and blood-fed *An. gambiae* females extracted with a 0.2 N acetic acid solution. Molecular weights of standards (MW STD) and *Aedes* OEH standard are displayed to the left. Approximate size of the immunoreactive bands are listed to the right. Below each gel is a summary of immunoreactive peptides for three cohorts: +, peptide present in majority of samples or -, peptide absent in majority of samples. An immunoreactive band the approximate size of AgamOEH was detected before and during the gonotrophic cycle in heads and thoraces but not abdominal walls (data not shown). Another larger peptide (19,000 MW) was also detected in all samples.

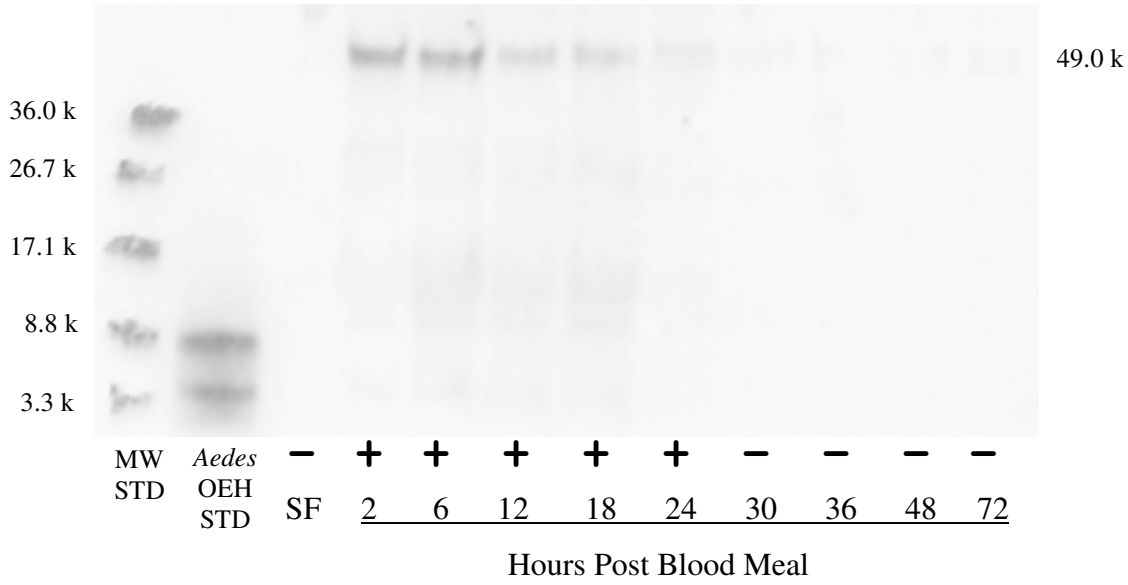



Figure 3.7. Representative immunoblot of AgamOEH in female hemolymph during a gonotrophic cycle. Lanes were loaded with hemolymph from sugar or blood-fed *An. gambiae* females. Molecular weights of standards (MWS) are displayed to the left. Approximate size of the detected bands are listed to the right. Below gel is the summary of immunoreactive peptides from three cohorts: +, peptide present in majority of samples or -, peptide absent in majority of samples. Immunoreactive bands with a predicted molecular weight of 49,000 were detected in hemolymph 2 to 24 hours PBM that were not detected in hemolymph from sugar-fed mosquitoes.

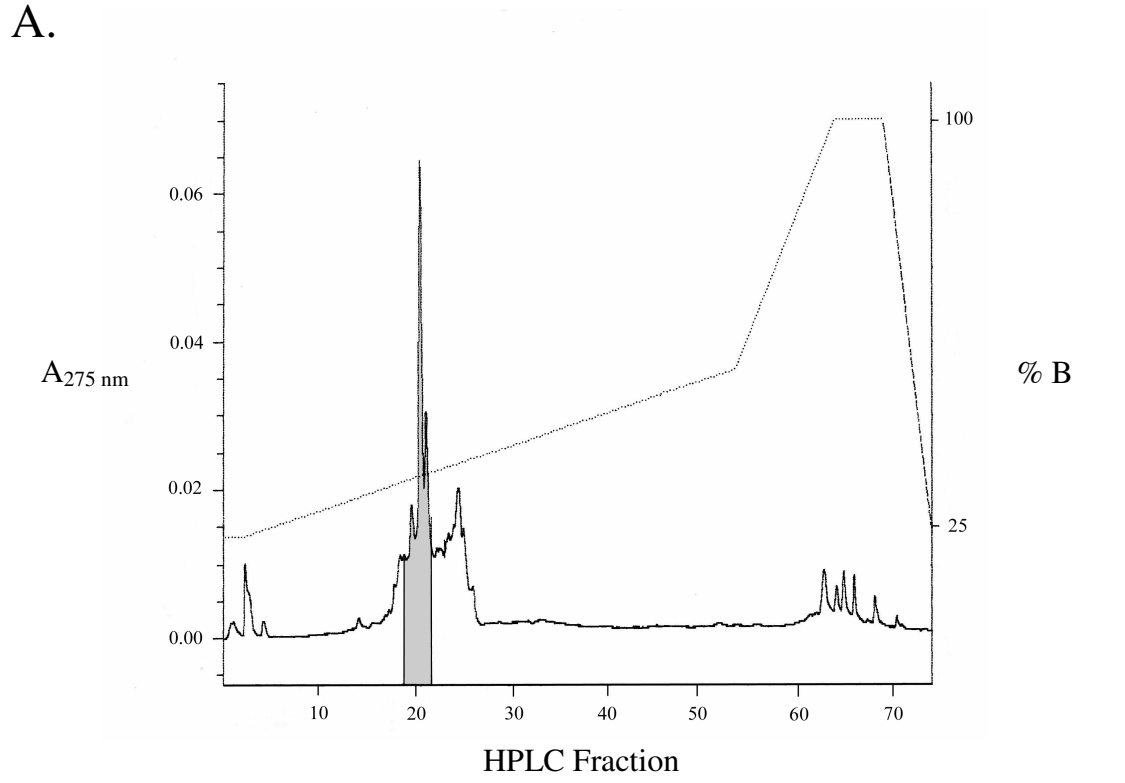



Figure 3.8. A. Final HPLC purification of AgamOEH from heads of *An. gambiae* adults. Material was separated with solvent B 0.01% heptafluorobutyric acid in 90% $CH_3CN$ at a gradient of 25 to 55%, 50 min, 55 to 100%, 10 min; monitored at 275 nm. B. HPLC fractions were assayed for immunoreactivity by SDS/PAGE/ western blot with anti-AaeOEH antibody. Immunoreactive fractions 19-21 in Figure B indicated as shaded area in Figure A. These fractions were collected and processed for *in vivo* assays and are noted with an asterisk.

Table 3.1. *In vivo* egg development assay of bovine insulin and HPLC purified AgamOEH. *An. gambiae* females were decapitated within 2 hours after a blood meal, treated as listed below, and incubated at 27° C (high humidity) for 22-26 hours PBM. Following incubation, yolk length was measured to assess bioactivity of each treatment. At the highest concentrations tested, bovine insulin (B. I.) was able to induce yolk deposition. AgamOEH was inactive at any of the concentrations tested. Treatments with the same letters are considered statistically similar to one another based on Tukey-Kramer HSD ($p<0.05$).

| Treatment | Mean yolk length (µm) | S.E.M.. | n | Similarity |
|---|---|---|---|---|
| No decap., no injection | 204.0 | 7.2 | 35 | A |
| No decap., sham injection | 176.8 | 7.0 | 37 | A, B |
| Decap., no injection | 40.0 | 7.3 | 34 | E |
| Decap., sham injection | 47.2 | 8.6 | 25 | E |
| 0.5 µg AgamOEH/ 0.5 µl | 32.5 | 15.1 | 8 | E |
| 0.25 µg AgamOEH/ 0.5 µl | 38.3 | 12.4 | 12 | E |
| 0.125 µg AgamOEH/ 0.5 µl | 38.6 | 11.4 | 14 | E |
| 0.062 µg AgamOEH/ 0.5 µl | 51.4 | 11.4 | 14 | D, E |
| 0.031 µg AgamOEH/ 0.5 µl | 42.0 | 13.5 | 10 | D, E |
| 1.25 nmol B.I./ 0.5 µl | 142.9 | 16.2 | 7 | B, C |
| 0.625 nmol B.I./ 0.5 µl | 133.8 | 11.9 | 13 | B, C |
| 0.312 nmol B.I./ 0.5 µl | 120.0 | 13.5 | 10 | C |
| 0.156 nmol B.I./ 0.5 µl | 135.4 | 11.9 | 13 | B, C |
| 0.078 nmol B.I./ 0.5 µl | 100.0 | 11.1 | 15 | C, D |

# CHAPTER 4

# CONCLUSIONS

Mosquitoes have a major impact on the health and economic ability of people around the world by acting as both nuisance pests and vectors of disease agents. Despite the exhaustive and expensive efforts designed to curtail the spread of these diseases, mosquitoes continue to negatively impact the human population. Even more disconcerting is the belief by authorities that the threat mosquitoes pose is expected to only increase over the upcoming years. Malaria, for example, is responsible for the sickness and death of millions of people every year and the number of malaria cases continues to rise every year.

Because of its capacity for transmitting malaria and other parasites, *An. gambiae* is considered to be one of the most dangerous insect species in the world. However, very little information is available for this species, due in part, at least, to the difficulty in rearing this species under laboratory conditions. Information concerning *An. gambiae* biology, ecology, or physiology could prove to be instrumental in the development of novel and effective control strategies. Over the course of my thesis, I have employed many techniques from various fields, including but not limited to entomology, molecular genetics, and physiology in order to provide new scientific information about *An. gambiae*.

Using a radioimmunoassay (RIA) that measures ecdysteroids, we were able to determine both ovarian ecdysteroidogenesis before and during a gonotrophic cycle. Our results concluded that ecdysteroid production by *An. gambiae* ovaries after a blood meal peaked between 18 and 24

hours post-blood meal before falling to non-blood fed levels for the remainder of the gonotrophic cycle. This approximates the same pattern of ecdysteroid synthesis described previously in *Ae. aegypti*. Additionally, we determined that the titer of ecdysteroids in the hemolymph of these females followed the trend described for ecdysteroid biosynthesis by the ovaries. The resulting yolk deposition became visually measurable within 12 hours of a blood meal, and that yolk deposition continued in a steady fashion until the primary oocytes reached maturity between 48 and 72 hours post-blood meal.

In the final section of this thesis, we characterized a cDNA encoding a homolog to the *Ae. aegypti* OEH gene in *An. gambiae*. We described the distribution of the *AgamOEH* transcript during mosquito development and a first reproductive cycle by PCR with gene specific primers. The distribution of the AgamOEH peptide was confirmed during mosquito development and the gonotrophic cycle with immunoblots with antibody raised to the AeaOEH peptide. Finally, a putative AgamOEH peptide was purified from the heads of adult *An. gambiae* mosquitoes using HPLC. We were unable to prove bioactivity of the putative AgamOEH in *in vitro* and *in vivo* experiments, but demonstrated that bovine insulin was capable of producing vitellogenesis *in vivo*.

The research presented here is an important beginning in understanding some of the physiological and biochemical processes that orchestrate reproduction in *An. gambiae*. This data provide a significant base of knowledge that will be used to compare *An. gambiae* to other mosquito species and may prove useful in determining possible effects of many variables on *An. gambiae* reproductive capacity.