ANALYSIS OF OVARIAN ECDYSTEROIDOGENESIS IN THE YELLOW FEVER MOSQUITO, *AEDES AEGYPTI* (LINNAEUS) (DIPTERA: CULICIDAE): ECDYSONE QUANTIFICATION, NEUROPEPTIDE SIGNALING, AND CHOLESTEROL MOBILIZATION

by

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(Under the Direction of Mark R. Brown and Michael R. Strand)

ABSTRACT

The synthesis of ecdysone in insects is fundamental to their development and reproduction. Understanding how the production of this hormone occurs is vital to understanding these processes in insects, and the quantification of this hormone is a key component of this. With the dominant older method of routine ecdysteroid quantification becoming unavailable, a new, refined enzyme-linked immunoassay is developed in parallel with superior extraction methods for insect steroid hormones. Activation of ecdysteroid synthesis occurs in mosquito ovaries following a blood meal, mediated primarily by the binding of the neuropeptides ovary ecdysteroidogenic hormone (OEH) and insulin-like peptide 3 (ILP3) to their respective receptors in follicle cell membranes of the ovary. Both neuropeptides activate ecdysteroidogenesis through the protein kinase Akt, but the relationship of calcium to this activation is unknown. Given that calcium is an important component of ecdysteroid production in the prothoracic glands, it was predicted to have import for ovarian ecdysteroidogenesis. This work demonstrates a regulatory role of calcium in ecdysteroidogenesis. Another important aspect of ecdysone production by the

ovaries is that of the cholesterol precursor for steroid synthesis. Since cholesterol is stored in esterified form, it is necessary to mobilize it for ecdysteroid production. The mobilization of cholesterol is relatively unexplored in insect hormone production, which is surprising, given the inability of insects to produce cholesterol de novo. The work here shows that the mosquito ovary, and specifically the follicle cells of the ovary, is proportionally richer in cholesterol than most of the other tissues in the mosquito. The mobilization of cholesterol was confirmed in that the same signals that activate ecdysteroidogenesis in the ovaries increase the amount of free cholesterol in isolated ovaries. This indicates that the source of cholesterol for such synthesis is contained within the ovaries. Additionally, a stringent requirement for follicle cell plasma membrane cholesterol was observed in cholesterol depletion and in vitro activation experiments. This suggests the role of increased free cholesterol in the follicle cell membranes is related to membrane receptor function.

INDEX WORDS: Ovary ecdysteroidogenic hormone (OEH), insulin-like peptides (ILPs), *Aedes aegypti*, egg maturation, cholesterol, ecdysone, enzyme-linked immunoassay (ELISA), calcium signaling

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DEDICATION

To my wonderful wife Kimberly, whose gracious patience and loving endurance have made this journey feasible.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Mosquitoes are highly relevant insects of human concern. These dipterans in the family Culicidae occur globally, and because many species require one or more blood meals per egg maturation cycle, they are the dominant arthropod vectors (Gubler, et al., 2014; Caminade, et al., 2014). Mosquitoes are the vectors for numerous human and important agricultural animal pathogens. In particular, *Anopheles* mosquitoes transmit the *Plasmodium* protozoan parasites that are the causative agent of malaria, which kills hundreds of thousands of people annually, with more than 350,000 malaria-related deaths of children in 2013 (Liu, et al., 2015). The yellow fever mosquito, *Aedes aegypti*, studied in this work, is a cosmopolitan species that effectively transmits viruses that are current public health concerns, such as yellow fever (Paules and Fauci, 2017), dengue (Akiner, et al., 2016), chikungunya (Diallo et al., 2016), and Zika (Yakob and Walker, 2016). Less apparent, but no less important, mosquitoes are key models for understanding insect physiology in the areas of reproduction (Smykal and Raikhel, 2015) particularly in hematophagous insects and steroid hormone production (Steel and Davey, 2013).

1.1. Introduction

1.1.1. Mosquito reproductive physiology

A. aegypti is an anthropophilic (human-associated) species that breeds in small ephemeral water sources like cans, tires, and rain barrels. Eggs are laid at the edge of the air/water interface, and can last weeks or months without water. Upon hydration, larvae emerge and feed on detritus and bacteria in the water. Larval development progresses through four instars and, while the

timeframe is variable, typically lasts for 9 to 12 days (Couret, et al., 2014). Larvae pupate, and then eclose to adults. During a brief maturation period, the ovaries are primed for activation by a pulse of juvenile hormone (Gwadz and Spielman, 1973; Hernández-Martínez, et al., 2015), and held in a state of arrest until a blood meal is acquired (Lea, et al., 1978; Clements and Boocock, 1984). Females may mate with males at breeding sites or in close proximity to hosts, as males also exhibit host-seeking behavior. Females seek out a host for blood feeding because they require a blood meal to complete a gonadotropic cycle. Host-seeking involves detection of long-range cues, such as carbon dioxide, and short-range cues, like host volatiles (Bowen, 1991; Takken, 1991), by the antenna of the mosquito (Zwiebel and Takken, 2004; Suh, et al., 2014). Following a blood meal, an unknown signal induces the release of neuropeptides from the neurosecretory cells of the brain that stimulate egg development. Two types that have been identified are ovary ecdysteroidogenic hormone (OEH: Dhara, et al., 2013) and insulin-like peptides (ILPs: Riehle and Brown, 1999). The ovaries of the mosquito are a paired organ with 50-70 ovarioles arising radially from the distal oviduct such that they appear similar to clusters of grapes. Each ovariole is covered by a thin layer of muscle and an acellular matrix, and consists of a primary follicle and a germarium. The follicle is made up of a single layer of epithelial cells, called follicle cells, which surrounds large nurse cells and the oocyte (Clements, 2002). The follicle cells are the receptor-bearing cells that respond to OEH and ILP3 through their respective pathways. Both receptors are receptor tyrosine kinases (RTKs) and activate a shared signaling pathway (Vogel, et al., 2015). The ovaries respond to these signals, and likely others, by follicle cell proliferation and ecdysone synthesis (Mattee, 2015). Follicle cells increase in number for about 12 hours following a blood meal, while ecdysteroidogenesis begins after 4 to 6 hours, and reaches a maximum at 18 to 24 hours (Clements, 1992). The rest of the mosquito's

body is not quiescent, and the fat body responds to the ecdysteroid signal by the activation of vitellogenesis, the production of vitellogenin for oocyte uptake (Roy et al., 2016). Other cell types in the ovary also respond, albeit indirectly, with oocyte maturation and increased metabolic activity in the nurse cells (Anderson and Spielman, 1973). It takes about 48 hours for the completion of oocyte development. Following this, oviposition sites are carefully selected by the gravid female, which will lay as many as 200 eggs (Bentley and Day, 1989; Clements, 1992; Day, 2016) before returning to host-seeking. The early steps of oocyte development in blood fed mosquitoes are the focus of understanding the regulation of reproduction in these vectors. The highly regulated control of reproduction in mosquitoes is reliable and amenable to experimental manipulation. For this reason, it has been the subject of inquiry for more than half a century (e.g., Curtin and Jones, 1961).

1.1.2. Adult ecdysteroid synthesis

A major component of mosquito reproduction is the synthesis and release of the steroid hormone ecdysone (ECD) from the ovaries following a blood meal (Klowden, 1997). *A. aegypti* is a model for adult ECD synthesis, herein ecdysteroidogenesis, because its synchronous follicular development is regulated by a blood meal, and hence so is its ecdysteroidogenesis. This creates a reliable and easily interrogated experimental system (Clements, 1992). Steroid hormone production in the follicle cells of the ovary is directly activated by neuropeptides, specifically OEH and ILPs, that are released following a blood meal. ECD is synthesized by, and released from, the follicle cells into the hemolymph. ECD is converted to its active form, 20-hydroxyecdysone (20ECD), in target tissues (Hagedorn, et al., 1975). The 20ECD serves to activate vitellogenin synthesis (Hagedorn, et al., 1975; Borovsky, et al., 1985), as well as preparing the secondary follicles for another gonadotropic cycle (Beckemeyer and Lea, 1978).

1.1.3. Significance of ecdysteroidogenesis

The production of ECD is necessary for mosquito reproduction. Beyond this, it is also crucial for embryonic development and larval development. The importance of ECD is not limited to mosquitoes, as it plays equally vital roles in all insects and many non-insect invertebrates, including crustaceans (Chang and Mykles, 2011) and spiders (Sawadro, et al., 2017), and there are strong implications of functioning similarly in nematodes (Ondrovics, et al., 2016). While ECD is best known for its role in regulating the molting process as the climax of developmental transitions (Yamanaka, et al., 2013), it has many other equally important functions in embryogenesis (Jarvela and Pick, 2017), behavior (Ishimoto, et al., 2013), and reproduction (Meiselman, et al., 2017). However, despite the wide-ranging importance of ECD, researchers have used different methods for identification and quantification.

1.1.4. Summary of what is known for mosquitoes

Synthesis of ECD in the follicle cells appears to be canonical (Sieglaff, et al., 2005; Brown, et al., 2009), in that it follows a similar biosynthetic pathway as what is known to occur in the larval prothoracic glands of *Drosophila melanogaster* and lepidopteran larvae. As in all other insects, cholesterol is the precursor to steroid hormones in *A*. *aegypti* larvae, which actively convert related dietary plant sterols to cholesterol, as demonstrated by the work of Svoboda, et al. (1982). Cholesterol in adult female *A*. *aegypti* prior to blood feeding comes from larval stores. Vertebrate blood is a rich source of cholesterol, and in *A*. *aegypti*, cholesterol is present in the follicle cells in sufficient quantities to allow for ECD production by isolated ovaries activated in vitro. Following neuropeptide activation, cholesterol is transported intracellularly to the initial site of steroidogenesis, where it is converted to 7-dehydrocholesterol (7-DC) by the 7,8-dehydrogenase known as neverland (Yoshiyama-Yanagawa, et al., 2011). The 7-DC is then

converted to ecdysone through a series of hydroxylation by cytochrome p450s (Rewitz, et al., 2006).

1.2. Characterization of insect steroid hormones past and present

Steroid hormones regulate a wide array of processes in multicellular animals. One unified characteristic of their activity is that their physiological functions are a matter of changes in titer, and not presence or absence. This practically necessitates quantification if finer aspects of insect physiology are to be understood. The characterization of ecdysteroid hormones in a variety of contexts is still necessary for understanding the physiology state that they are connected with. Whether this is embryological development (Sumiya, eta al., 2016; Abidi, et al., 2017), regulation of molting (Israni and Rajam, 2017; Lin, et al., 2017), or reproduction (Ameku, et al., 2017; Nuss and Brown, 2017), measuring the level of ecdysteroids secreted by or in the blood or tissues of insects is a direct way to assess these physiological processes.

Since the discovery of a molting hormone in the early 1900s by Kopec and Wigglesworth (Karlson, 1996), its isolation by Butenandt and Karlson (1954), and its structural elucidation by Karlson, et al. (1965), ECD and its related steroids have been the subject of much study. Specific to mosquitoes, ecdysteroid production is a direct and rapid readout of the activation of the ovaries (Hagedorn, et al., 1975). It allows for an understanding of not just reproductive state as active or inactive, but of specific components that can alter mosquito reproduction. A number of methods have been used or proposed for the quantification of ecdysteroids, and here they will be reviewed briefly.

1.2.1. Biological approaches to ecdysone quantification

The first methods used to demonstrate ECD-like activity (e.g., molting) were fairly sensitive, operating in the low nanogram range (Thomson, et al., 1970), but mostly qualitative

bioassays. The first method established for monitoring ecdysteroid activity was the *Calliphora* bioassay developed by Becker (1941) for monitoring ECD during isolation, involving injection of unknown material into the abdomens of ligated last instar *Calliphora* species (blow fly larvae). The percentage of individuals that formed puparia were an indication of the ECD-like activity of the unknown material. A number of other insects have been used in this type of assay. More modern bioassays have been proposed, although none of them ever came into wide use.

Insect cell-based bioassays range from semi-quantitative to quantitative. A semi-quantitative method was developed and refined by Clément and Dinan (1991; Clément, et al., 1993) and uses a ph cell line (BII; *D. melanogaster*) that clumps in the presence of ecdysteroids, giving a change in light absorbance that provides detection down to the high picogram level. Another more complicated bioassay was developed where quantification is accomplished through a reporter system in an insect cell line. In these systems, ecdysteroid hormone induces transcription that ultimately leads to a fluorescent signal proportional to the concentration of the hormone. Through comparison with a known hormone amount, precise quantification can be obtained in the low nanogram range. This type of bioassay has been developed independently by two different labs, but functions similarly for both (Swevers, et al., 2004; Kamimura, et al., 2014). Without exception, these methods have not been adopted for routine ECD quantification. However, some classical methods are still used in special circumstances (Sláma and Zhylitskaya, 2016).

1.2.2. Chromatographic methods

During characterization of different ecdysteroid forms in insects, early workers found that ecdysteroids were readily separated from other material through chromatographic methods, either thin-layer chromatography (TLC) or, more commonly, liquid chromatography (LC). TLC

was introduced in the analysis of ecdysteroids in the 1960s (e.g., Stahl, 1962). Detection of ecdysteroids is by means of either UV absorbance on a fluorescent background or chemical conversion of ecdysteroids to a colored compound, with similar levels of detection in the high nanogram to low microgram level (Ruh and Black, 1976; Mayer and Svoboda, 1978). Modern quantification of 20ECD with so-called high-performance thin-layer chromatography (HPTLC) permits reliable quantification down to about 10 ng (Muchate, et al., 2017). Alternatively, liquid chromatography has also been used to separate and quantify ecdysteroids, with reverse phase high-performance liquid chromatography (HPLC) being first implemented by Horn, et al. (1968), for purification. HPLC detection by UV absorption is limited to around 30 nanograms of ecdysteroids (Lafont, et al., 1982), and reliable direct quantification is possible, albeit in the hundreds of nanogram range (Holman and Meola, 1978; Serra, et al., 2012). Traditionally, HPLC is coupled with some other means of quantification, like radioimmunoassay (RIA) or enzyme-linked immunoassay (EIA), which are covered later, or more recently, directly coupled to mass spectrometry (Hikiba, et al., 2013), or even indirectly with gas chromatography coupled mass spectrometry (GC-MS: Nirde, et al., 1984). Notably, the LC-MS-MS method is accurate down to the single picogram range (Venne, et al., 2016).

The use of GC was first applied to ecdysteroid work by Katz and Lensky (1970). However, while this method is far more sensitive than simple HPLC (picogram range), to get ecdysteroids to ionize for GC, it is necessary to first derivatize the steroid (VandenHeuvel, et al., 1960; Bielby, et al., 1986). This makes GC analysis of ecdysteroids impractical for high throughput or routine analysis. The addition of MS coupled to GC quantification also reaches the low picogram range (Evershed, et al., 1987) with the benefit of identification of unknowns, but this instrumentation is not directly available to most laboratories.

Chromatographic methods remain in common use for the purification of ecdysteroids from raw material like macerated plants or insects, but remain uncommon for quantification, likely as a consequence of the requirement of nanograms of material for reliable quantification with most common chromatography configurations, or the requirement of highly specialized configurations (Hikiba, et al., 2013). The only lab to use HPLC regularly for ECD quantification is that of Kataoka, with the LC-MS-MS protocol developed in his lab (Hikiba, et al., 2013; Enya, et al., 2014; Iga, et al., 2014; Ogihara, et al., 2015; etc.). While it is still too early to say what may occur, this method has not yet come into common use. This is due to the availability of sensitive equipment needed to perform such analysis. Additionally, there appears to be uncertainty in how data from this method should be presented, which makes it difficult to connect the amount of ECD in the insect to the interpolated data. For example, values like pg/mL/mg are difficult to compare with previous literature (Kloss, et al., 2017). Of the six published articles from authors reliant on his method other than the Kataoka lab, there are four non-interchangeable types of data formats for the exact same kind of data (Rodenfels, et al., 2014; Lavrynenko, et al., 2015; Li, et al., 2016; Saito, et al., 2016; Venne, et al., 2016; Kloss, et al., 2017).

1.2.3. Immunodetection approaches

A sensitive and commonly used technique to quantify ecdysteroids was the RIA. The assay was first applied to ecdysteroids by Borst and O'Connor (1972). In this method, a radioisotope-labeled ecdysteroid (known standard), often ECD, is used as a competitive target for an unknown ecdysteroid amount in a relatively pure sample. An antiserum, generated against an ecdysteroid conjugate immunogen (Horn, et al., 1976), is added, and competition in solution between the labeled and unlabeled ecdysteroid for the binding sites of the immunoglobulins in

the antisera occur. Unbound labeled ecdysteroid is separated from bound ecdysteroid through precipitation of the antisera. This method of quantification was reliable into the low picogram range, but was dependent on the quality and availability of antisera. In recent years (circa 2013), commercial production of a radiolabeled ECD with high specific activity stopped. Without reliable access to a radiolabeled ECD, the ability to perform ecdysteroid radioimmunoassay as a routine method for quantification is no longer a viable option.

Currently the most universal method for small molecule quantification is EIA, which does not required specialized equipment or training to perform (Aydin, 2015). This method was first applied to ecdysteroids by Kingan (1989), and uses antisera generated against a conjugated steroid target. This methodology has a wide array of variations, but most commonly, antisera are immobilized onto the bottoms of a polystyrene 96-well plate, and ecdysteroids are mixed with an enzyme-linked ecdysteroid, which competes for binding sites on the antisera (e.g., Margam, et al., 2006). There is experimental evidence that when tested in parallel with a related method (e.g., EIA vs. HPLC), the quantification values are statistically indistinguishable (Pascual, et al., 1992; Kamimura, et al., 2014). However, it is true that the level of agreement between EIA and other methods will vary with experimental system and antisera used. Presently, a single supplier offers a kit for 20ECD, produced by BertinPharma under the SpiBio brand and distributed by Cayman Chemical, using the methods pioneered by Porcheron, et al. (1989). A lesser-known method, the time-resolved fluoroimmunassay (TR-FIA) has also been applied to quantification of ecdysteroids (Mizoguchi, et al., 2013), and is mentioned here for completeness. Both EIA and TR-FIA have quantification limits in the low picogram range, dependent almost entirely on the affinity of the antisera to the ecdysteroid being analyzed.

1.2.4. Mosquito applications

In the context of studying mosquito physiology, most quantification of ecdysteroids has historically utilized RIA and, currently, commercially available EIA. The current commercial EIA uses a proprietary 20E-linked acetylcholinesterase as a competitive tracer against ecdysteroids.

One major problem in ecdysteroid quantification, both in mosquito work and more broadly, is the lack of any kind of uniform extraction procedure. This is important, given that solvents and extraction methods alone can alter sterol structure, and thus detection, from any method that is structure-dependent, which includes nearly all currently used quantification methods (Scott and Ellis, 2007). Additionally, based on this work, it appears that the use of methanol, which occurs in most extraction procedures, can alter the structure of the 20E, the dominant ecdysteroid.

1.3. Regulation of insect steroid hormone production

Regulation of ecdysteroid synthesis in insect ovaries is poorly understood compared to the larval counterpart, the prothoracic glands (PG), as best known for the model systems of the silkworm *Bombyx mori* and and *D. melanogaster* (Marchal, et al. 2010; Lafont, et al., 2012; Yamanaka, et al., 2013; Ou, et al., 2016). In mosquitoes, ovarian steroidogenesis is better understood than larval, which appears to be tied to the body wall rather than the PG in last instars (Jenkins, et al., 1992; Telang, et al., 2007). The activation of ecdysteroid synthesis is closely associated with blood feeding in mosquitoes (Hagadorn, et al., 1975), and thus is of direct import to disease transmission. For the purpose of this introduction, ecdysteroid synthesis will be broken up into three major phases.

1.3.1. Activation of ecdysteroid production

Activation of the production of ecdysteroids is accomplished primarily through neuropeptide signaling (Yamanaka, et al., 2013). In PGs, the canonical signal for ecdysteroidogenesis is the prothoracicotropic hormone (PTTH), which was discovered by Kopec (1922), and identified as a protein by Ichikawa and Ishizaki (1963), and fully characterized by Kawakami, et al. (1990). This hormone has been experimentally validated in Diptera (Roberts, et al., 1984), Lepidoptera (Kelly, et al., 1986; Bollenbacher, et al., 1993), and Hemiptera (Vafopoulou and Steel, 1997). The peptide as well as the ecdysteroid-producing gland has been demonstrated separately in Hymenoptera (Simões and Hartfelder, 1993; Hartfelder, 1993). Ecdysteroid production apart from PTTH confirmation has been seen in Coleoptera (Romer, et al., 1974), Blattodea (Borst and Engelmann, 1974), and Orthoptera (Hirn, et al., 1979). Activation of the PG by PTTH is presumably functional in most insect taxa (Smith and Rybczynski, 2012). Activation can be described as a linear series of events beginning with PTTH binding its RTK torso, which initiates a Ras-Raf-ERK signal cascade (Rewitz, et al., 2009). However, this signal cascade is branched, and Ras can activate a phophoinositol-3-kinase (PI3K)-Akt-target of rapamycin (TOR) cascade, which contributes, among other things, a nutrient-dependent sensitivity to the activation of ecdysteroidogensis (Layalle, et al., 2008; Gu, et al., 2012). Less understood are other aspects of signaling in prothoracic gland, such as the well-documented role of calcium and cAMP as prominent secondary messengers (Birkenbeil, 1996; Gu, et al., 1998; Birkenbeil, 2000; De Loof, et al., 2015), a feature also common to vertebrate steroidogenesis (Yamazaki, et al., 1998; Manna, et al., 1999; Weitzel, et al., 2014; Mukherjee, et al., 2017). However, a number of other signals have been shown to activate ecdysteroid synthesis in the PG (Orme and Leevers, 2005; Smith, et al., 2014), and other tissues

have been shown to be capable of synthesizing ecdysteroids (Sakurai, et al., 1991; Chávez, et al., 2000). Beyond receptor binding, activation of ECD biosynthesis is in part through upregulation of the transcripts of the enzymes involved in ecdysteroidogenesis (Rewitz, et al., 2009). However, not all of the signaling pathway is characterized, nor are all the downstream targets known (Ou, et al., 2016).

As for the ovaries of *A. aegypti* and other mosquitoes, the primary activators are neuropeptides released from the neurosecretory cells in the brain following a blood meal (Matsumoto, et al., 1989). OEH was the first to be characterized, and the insulin-like peptides, (ILPs) such as ILP3 (Brown, et al., 2009), are similarly potent. There is some evidence that other neuroendocrine factors may be involved (De Loof, et al., 2001). Both OEH and ILP3 bind to RTKs (Wen, et al., 2010; Vogel, et al., 2015). ILP signaling is transduced through the conserved insulin pathway, while OEH signaling converges at Akt and possibly higher in the pathway (Dhara, et al., 2013; Vogel, et al., 2015). As for PGs and ovaries, there is also a nutrient-dependent signaling component of ecdysteroidogenesis involving target of rapamycin (TOR) (Dhara, et al., 2013). The work presented here investigates the potential role of calcium as a secondary messenger for activation of ecdysteroid synthesis in relationship to OEH, ILP, and potentially PTTH.

1.3.2. Modulation of ecdysteroid production

Ecdysteroid synthesis is not a binary process, and its rate and timing are heavily modulated both in PGs and ovaries. In PGs, nutrient availability (Layalle, et al., 2008) alters the production of the hormones through insulin signaling and the TOR pathway (Smith, et al., 2014). There also appears to be some level of autocrine regulation following activation (Gu, 2007; Vandersmissen, et al., 2007). In mosquito ovaries, it is unknown if calcium plays a role in

modulating the rate of ecdysteroid production, but in a parallel steroid hormone synthesis in vertebrates, a calcium-driven metabolic throttling is observed (Stocco, et al., 2005; Sharthiya, et al., 2017).

1.3.3. Termination of ecdysteroid production

The cessation of ecdysteroid production is probably the least studied aspect of ecdysteroid synthesis. In crustaceans, which also use an ecdysteroid-driven molting system, inhibition of the ecdysteroid secreting gland is accomplished through the molt-inhibiting hormone (MIH). This peptide hormone induces a signaling cascade that results in nitrous oxide/cyclic guanosine monophosphate repression of ecdysteroidogenesis (Covi, et al., 2012). Whether a homolog of this peptide or this pathway plays a role in downregulation of ecdysteroidogenesis in insects is unknown. In PGs it appears to be partially connected to feedback inhibition, where rising ecdysteroid concentrations result in decreased ecdysteroidogenesis (Jiang and Koolman, 1999). This is directly involved in stopping synthesis, likely through several nuclear receptors, including the ECD receptor (EcR), ultraspiricle (USP), hormone receptor 4 (DHR4), and, ultimately, through a number of other transcription factors, leading to changes in the metabolism of the ecdysteroids (Song and Gilbert, 1998; Ou, et al., 2011; Ou and King-Jone, 2013; Rewitz, et al., 2013). Not surprisingly, nothing is known about the termination of ecdysteroid synthesis in ovaries, other than the anecdotal observation that it appears to be some kind of negative feedback mechanism.

1.4. Transport and distribution of cholesterol in insects

The need for sterols in the diet of insects has long been known and was first shown empirically by Hobson (1935), and later supported for other insects (Fraenkel and Blewett, 1954), including *A. aegypti* (Golberg and De Meillon, 1948). The necessity of dietary sterols is

tied to the inability of insects to synthesize sterols de novo, which was established biochemically by Clark and Bloch (1959), and this continues to be understood for all insects (Clayton, 1964; Svoboda and Feldlaufer, 1991; Vinci, et al., 2008). Like vertebrates, insects employ cholesterol to maintain membrane fluidity (Shreve, et al., 2007; Dawaliby, et al., 2016) and lipid raft regulation of receptors and channels (Gimpl, et al., 1997; Eroglu, et al., 2003; Peters, et al., 2017). However, there is some evidence that insects may not have as stringent a demand for cholesterol as a regulator of membrane fluidity in a few isolated cell types (Phalen and Kielian, 1991; Cleverly, et al., 1997). For mosquitoes, this means that the precise storage and regulation of these sterols is vital to the reproductive success of the adult mosquito, as cholesterol both serves a homeostatic function and is a limited resource for steroid hormone production.

1.4.1. The problem

There are a number of challenges to understanding sterol transport within the steroidogenic cells of the mosquito ovary, not the least of which is the fact that very little basic data on sterol quantity or distribution is known for most insects, let alone mosquitoes. Indeed, a series of studies under the direction of Clayton on the cockroach *Eurycotis floridana* is the only exhaustive analysis of cholesterol distribution in any insect (Clayton and Edwards, 1961; Bade and Clayton, 1963; Clayton, et al., 1964; Lasser and Clayton, 1966; Lasser, et al., 1966). A survey of literature reveals one study that examined lipids in the mosquito ovary prior to and following a blood meal, but this study only considered the ovary, and did not delineate anything beyond “sterols” and “free sterols” (Troy, et al., 1975). Similarly, other authors have looked at lipid composition in different strains of *A. aegypti*, but again, this work only provides proportional values for ”free sterols” and ”esterified sterols” in whole bodies (Miller and Novak, 1985). The work presented here lays the foundation for understanding sterol transport related to

ecdysteroidogenesis by determining whether sterols are stored in ovaries, particularly at the cellular level, and what changes occur during steroidogenesis.

While there is some evidence that the ecdysteroidogenic system is flexible enough to accommodate several sterols as starting material for ecdysteroidogenesis (Lavrynenko, et al., 2015). For mosquitoes, it is reasonably presumed that the starting sterol is cholesterol, based on the evidence that mosquito larvae readily convert dietary plant sterols to cholesterol where possible (Svoboda, et al., 1982), and fail to develop on sterols they cannot convert to cholesterol (Golberg and De Meillon, 1948). Because of the hydrophobicity of cholesterol, it cannot diffuse through aqueous environments inside the insect unaided, and must be actively transported among sites of metabolism, storage, and membranes.

1.4.2. Cholesterol transport broadly

Cholesterol, or a related sterol, is a dietary requirement for insects. Absorption of cholesterol varies in location, and is difficult to categorize along taxonomic or ecological lines. Absorption occurs either in the insect foregut in the crop or the midgut in the gastric caeca or ventriculus (Clayton, et al., 1964; Joshi and Agarwal, 1977; Jouni, et al., 2002). Following absorption into the midgut cells, cholesterol must first be transported to the relevant tissue before it can be used for steroidogensis. The transport of cholesterol, and related sterols, in a dietary context has been examined in several insect systems. Generally speaking from the *Drosophila* system, this appears to be regulated by *Drosophila* hormone receptor 96 (DHR96), which alters the transcription of genes that codes for Niemann-Pick C1 b (NPC1b), which is itself involved in cholesterol absorption in the gut (Voght, et al., 2007; Horner, et al., 2009; Sieber and Thummel, 2012; Lemaitre and Miguel-Aliaga, 2013). In *A. aegypti*, it is known that dietary sterols are obtained in the form of plant sterols, and these are converted to cholesterol as the primary sterol

in this species (Svoboda, et al., 1982; Merritt, et al., 1992). Additionally, there is evidence that cholesterol absorbed into gut epithelial cells in *A. aegypti* larvae is transported intracellularly by sterol carrier protein 2 (SCP2), which knockdown experiments suggest functions homeostatically (Krebs and Lan, 2003; Blitzer, et al., 2005). Cholesterol is also obtained by adult mosquitoes through a blood meal, though it is unclear how this additional cholesterol pulse relates to ecdysteroidogenesis.

Redistribution of cholesterol from the site of absorption to the rest of the insect body is accomplished through co-transport with triglycerides in lipoprotein-phospholipid transporters called lipophorins. These lipophorins are loaded and unloaded at the cell membrane surfaces (Canavoso, et al., 2001). In *Drosophila*, the protein component of these transporters is mostly apolipophorins (apoLp) with phospholipid and diacylglycerol, together forming a particle that is a class of high-density lipophorin (HDL) (Arrese, et al., 2001, Van der Horst and Rodenburg, 2010). These lipophorins are bound to receptive tissues for unloading lipids through a lipid transfer particle (LTP)-assisted receptor interaction (Palm, et al., 2012; Rodríguez-Vázquez, et al., 2015). *A. aegypti* and other insects are an exception to this mechanism for lipid uptake, in that oocyte receptor-meditated uptake of lipophorin is endocytotic, thus internalizing the lipid particle (Sun, et al., 2000; Cheon, et al., 2001). In the African malaria mosquito *Anopheles gambiae*, the total cholesterol content of the lipid portion of the lipophorin following a blood meal is about 12% (Atella, et al., 2006).

1.4.3. Cholesterol transport specific for ecdysteroid production

Free cholesterol must be mobilized from stores for the purpose of ecdysteroidogenesis. Whether these stores are in membranes (Venugopal, et al., 2016) or esterified cholesterol in lipid droplets (Kraemer, et al., 2002) is unknown for insects. The mechanisms by which cholesterol

reaches the intracellular site of steroidogenesis is believed to start in the endoplasmic reticulum with the conversion of cholesterol to 7-dehudrocholesterol initially (Brown, et al., 2009; Ou and King-Jones, 2013) and only recently investigated (Danielsen, et al., 2016). If these are intracellular stores, it could be as simple as movement from lipid droplets to the mitochondria. Following activation, there evidence for a feedback loop regulating cholesterol shuttling in *Drosophila* PGs, where ECD triggers the formation autophagosomes for the liberation of cholesterol to maintain ecdysteroidogensis with a fatty acid elongase (Sit) regulating this trafficking (Danielsen, et al., 2016). Intracellular stores of esterified cholesterol in other tissues may also serve as a sterol pool for ecdysteroidogenesis; however, work presented herein suggests that may not be a regulatory factor in mosquito ovarian ecdysteroidogenesis.

The transport of sterols with respect to ecdysteroidogenesis remains relatively unexplored from the perspective of the precursor sterols involved. The fact that many steroidogenic tissues can produce steroid hormones ex vivo, as reported for oenocytes, PGs, ovaries, testes, and the epidermis suggests that at least the initial step of steroid production is accomplished through mobilization of cellular stores (Romer, et al., 1974; Lagueux, et al., 1977; Hagedorn, et al., 1979; Loeb, et al., 1984; Delbecque, et al., 1990). Several proteins have been implicated in cholesterol transport in the PG as relates to ecdysteroidogenesis, including StAR-related lipid transfer domain 1 (Start1), NPC1, and possibly ATP-binding cassette (ABC) transporter complex Mdr49 (Roth, et al., 2004; Fluegel, et al., 2005; Sakudoh, et al., 2005; Deshpande, et al., 2016). Recent studies have implicated the glutathione S-transferase called noppera-bo (nobo) in cholesterol transport tied to ecdysteroidogenesis. Knockout phenotypes in *Drosophila* show developmental arrest (Enya, et al., 2014). However, the knockout mutants could be rescued by feeding cholesterol to the larvae, which casts doubt on whether this new target is directly related to

cholesterol transport. Additionally, the results with *Drosophila* nobo were not replicated when similar knockouts were applied to the silkworm, *Bombyx mori* nobo (Enya, et al., 2015). Regardless of how cholesterol is mobilized in prothoracic glands for steroid hormone production, very little is known for ovarian steroid production in insects broadly, let alone mosquitoes specifically. What is known for *A*. *aegypti* is that Start1 is not transcriptionally responsive to ovary steroid hormone production, suggesting that it does not serve a regulatory role in this context (Sieglaff, et al., 2005).

1.5. Study objectives

There are a wide array of gaps in our knowledge concerning the manner in which cholesterol mobilization and ecdysteroidogenesis are activated in the follicle cells of the mosquito ovary. This work contributes to three important areas of the field of insect molecular endocrinology. First, there is a technical demand for the ability to quantify ECD as a readout for physiological activity related to the hormone's functions. Additioanly, extraction methods for insect ecdysteroids have not been analyzed in the context of current ecdysteroid quantification methods. An EIA was developed and is presented here, along with a simple extraction proceedure, to address this demand in chapter 2. Second, the nature of the signal cascades that regulate ecdysteroidogenesis in ovary follicle cells relative to the better-studied PG of insect larvae is not well understood. This is complicated by the difficulty of seperate post activation regulation of ecdysteroidogenesis from processes involved in intial activation. Calcium as a secondary messenger is explored as a potential intracellular signaling component of ecdysteroidogenesis in follicle cells, which is presented in chapter 3. Third, the spatial relationship of cholesterol to ecdysteroid production in mosquito follicle cells is entirely

unexplored. Cholesterol distribution in relationship to ecdysteroidogenesis in mosquito ovaries is considered in chapter 4.

1.6. References

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CHAPTER 2

EVALUATION OF ECDYSTEROID ANTISERA FOR A COMPETITIVE ENZYME IMMUNOASSAY AND OF EXTRACTION PROCEDURES FOR THE MEASUREMENT OF MOSQUITO ECDYSTEROIDS[1]

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Abstract

Ecdysteroid hormones regulate many aspects of insect development and reproduction. The predominant insect ecdysteroids are ecdysone (ECD) and 20-hydroxyecdysone (20ECD) that are synthesized de novo from ingested sterols. The ability to measure ecdysteroid titers is essential for many studies but few sensitive, low cost options are currently available for doing so. To address this deficiency, we developed a new enzyme-linked immunoassay (EIA). In the first part of the study, we compared the affinity of two new antisera named EAB25 and EAB27 to other available ecdysteroid antibodies. EAB25 had a 27-fold higher affinity for 20ECD than ECD, while EAB27 had a four-fold higher affinity for 20ECD. In the second part of the study, EIA protocols were developed for analyzing ecdysteroids secreted by tissues or extracted from whole body samples of the mosquito *Aedes aegypti*. Results indicated that fourth instar larvae and adult females contain 20ECD and ECD. Methanol extraction in the presence of magnesium from whole body samples altered ecdysteroids and antibody recognition by EIA. However, extraction with 1-butanol and two organic/water phase separations eliminated this problem and improved assay performance using whole body samples. We conclude these new EIAs provide low cost, flexible, and sensitive methods for measuring ECD and 20ECD in insects.

2.1. Introduction

Ecdysteroid hormones regulate many aspects of insect development and reproduction (Brown, et al., 2009; Nijhout and Callier, 2015). The predominant forms circulating in most insects are ecdysone (ECD) and 20-hydroxyecdysone (20ECD). They are usually synthesized de novo from ingested sterols by the prothoracic glands in immature stages or cells associated with the gonads or other tissues in adults (Brown, et al., 2009; Lafont, et al., 2012). Related forms, such as makisterone A, are found in the honey bee *Apis mellifera*, *Drosophila melanogaster*, and the Pentatomomorpha (Hemiptera infraorder) (Tohidi-Esfahani, et al., 2011; Lafont, et al., 2012; Lavrynenko, et al., 2015). In addition, many ecdysteroid derivatives circulate as catabolites that are excreted or stored as conjugates in eggs or other tissues and used later for ecdysteroid production (Lafont et al., 2012). Ecdysteroid hormone secretion is regulated by different neuropeptides and is typically phasic (Brown, et al., 2009; Marchal, et al., 2010; Smith and Rybczynski, 2012), resulting in the activation or repression of specific cellular and physiological processes (Heinrich, 2012; Bonneton and Laudet, 2012).

Understanding of ecdysteroid biosynthesis, function, and signaling requires the ability to accurately measure ecdysteroid levels in whole insect or tissue extracts, hemolymph, or medium after ex vivo tissue culture. Bioassays, together with different chromatography techniques, were initially used to qualitatively assess and measure ecdysteroid titers (Borst and O'Connor, 1974; Wilson, et al., 1981; Lafont, et al., 2012). Subsequent development of immunoassays provided simpler, more sensitive, and more quantitative measurements but depend on antisera that specifically recognize ECD and 20ECD, and not their catabolites or other sterols. For more than four decades, the radioimmunoassay (RIA) was the predominant format, as first developed by Borst and O'Connor (1972) and modified by different laboratories (Porcheron, et al., 1976;

Takeda, et al., 1986; Warren and Gilbert, 1988). The durability of the RIA as the primary approach for measuring ecdysteroid titers was due to several factors, including: 1) a commercial source of radiolabeled ECD with a high specific activity and long half-life, 2) freely available specific antisera, 3) repeatability, 4) flexible protocol, and 5) low sample cost. This situation changed a few years ago when Perkin-Elmer (Waltham, MA) ended production of radiolabeled ECD (23,24-^{3}H(N)] ECD) used in RIAs. Stocks of characterized ecdysteroid antisera also have diminished or are no longer available.

Enzyme-linked immunoassays (EIAs) provide an alternative approach for measuring ecdysteroids that do not require radiolabelled ECD (Kingan, 1989; Porcheron, et al. 1989; Pascual, et al. 1995; Shiotsuki, et al., 2005; Blais, et al., 2010). However, EIAs have been regarded as less reliable than RIAs due to enzyme tracer effects on antigen-antibody interactions and variable well binding due to evaporation and other factors (Shiotsuki, et al., 2005; Skrzipczyk and Verdier, 2013). As a result, EIAs have been less used for measuring ecdysteroids but are now the only option outside of chromatographic and mass spectrometry (MS) approaches. Antisera and secondary tracers for EIAs are currently available from two commercial sources: Bertin Pharma, Montigny-le-Bretonneux, France/Cayman Chemical Company, Ann Arbor, MI (20ECD-thyroglobulin rabbit antiserum and 20ECD-acetylcholinesterase) and Cosmo Bio, Tokyo, Japan (ECD-6-carboxymethyloxime (CMO)-BSA rabbit antiserum and 20-ECD-CMO-horseradish peroxidase). Bertin Pharma/Cayman Chemical Company also distributes a 20ECD EIA kit (single 96-well plate, A05120 /501390, respectively). In contrast, these reagents and kits are only offered in small amounts and single-plate formats, which limit the number of experimental samples that can be examined when combined with controls and standards.

The goal of this study was to improve the ecdysteroid EIA by developing antisera and protocols that can be easily used at low cost. We first report the sensitivity and specificity of two new antisera for the predominant ecdysteroids in insects, and compare their properties to two older antisera and one commercial antiserum in a competitive EIA. Results indicated that one of the new antisera measured ECD, 20ECD, and makisterone mixtures, while the other measured 20ECD. We then assessed the utility of these antisera in studies of the yellow fever mosquito *Aedes aegypti* that compared ecdysteroid titers to previously reported data generated by RIA. Protocols were developed for analyzing ecdysteroids secreted by tissues in ex vivo assays or extracted from whole body samples. Results analyzed by EIAs, high performance liquid chromatography (HPLC) and MS indicated that 20ECD and ECD circulate in both larvae and adult females.

2.2. Materials and methods

2.2.1. Chemicals

ECD, 20ECD, bovine serum albumin (BSA), bovine beta-lactoglobulin (BLG), and other reagents were obtained from Sigma Aldrich. Phosphate buffered saline 1X (PBS; HyClone™, GE Life Sciences) was used to prepare samples and as a rinse solution with 0.05% Tween 20 (PBS-T). Wells were blocked with non-fat dry milk (0.08% in PBS; BioRad, blotting grade, 170-6404) instead of serum or BSA, which was the conjugate antigen for the new antisera. HPLC-grade methanol (Fisher, A452) was used for extraction and HPLC. The buffered saline was made as previously described (Dhara, et al. 2013).

2.2.2. EIAs

2.2.2.1. Ecdysteroid-protein conjugate preparation

We generated ecdysteroid-BSA conjugates to use as antigens, and ecdysteroid-BLG conjugates to coat plate wells. BLG was chosen because it is commercially available as a pure protein, strongly absorbs to polystyrene, and is similar to BSA in conjugate site-to-weight ratio, solubility and globular shape. As a major milk protein, BLG also provided a competitive match for non-specific antibody binding sites with the milk block used in the EIA.

ECD and 20ECD were separately conjugated to BSA or BLG using the two-step process of Kingan (1989). First, aminooxyacetic acid (AOA, 5 mg) was linked to 1 mg of each ecdysteroid at carbon 6 in the B ring by mixing with 0.4 ml of pyridine in a microfuge tube overnight at 30°C. Pyridine was evaporated with an air stream. The AOA-ecdysteroid mixture was solubilized in water (2 ml) and loaded onto a C_{18} solid phase extraction (SPE) cartridge (3 ml, Analytichem International 606303) activated with 100% methanol. The column was then rinsed with 5% methanol (5 ml), followed by 60% methanol (2 ml) to elute the conjugant, which was dried in a vacuum centrifuge. Second, the solubilized AOA-ecdysteroid (2 ml water with 10 µl of 10% HCl to optimize pH for the reaction) was covalently linked to BSA or BLG with 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (approximate ratio 1:2.5 protein) by stirring overnight at room temperature (RT). The AOA-ecdysteroid-protein mixtures were diluted with PBS to 0.4 mg/ml protein, loaded into dialysis tubing (MWCO 3500, Fisher) for 3 changes in PBS overnight at 4°C. Sodium azide (0.02%) was then added to the ecdysteroid-protein conjugant stocks for storage at -80°C.

2.2.2.2. Antisera production and screening

Antisera to ECD-BSA and 20ECD-BSA were produced in rabbits by Pacific Immunology (Ramona, CA). Preimmune sera were collected prior to injecting two rabbits in multiple sites with ECD-BSA and another two with 20ECD-BSA in Freund's complete adjuvant. After three antigen boosts with incomplete adjuvant every two weeks and four production bleeds for sera, the rabbits were exsanguinated to obtain the final sera, which was treated with 0.02% sodium azide and stored at -80°C. Sera that bound to ECD-BLG or 20ECD-BLG were identified by EIA (see below). Combinatorial EIAs were then used over a range of dilutions for the primary antibody, secondary antibody, and ecdysteroid-BLG conjugates to optimize conditions.

2.2.2.3. EIA Protocol

For all steps, 96-well microplates (Corning 3590) were covered and held in closed wet chambers to minimize evaporation between solution changes. The protocol began with adsorption of either ECD-BLG or 20ECD-BLG in PBS (28 ng of protein conjugate/100 µl per well) to wells overnight at 4°C. Plate wells were then cleared, rinsed with PBS-T (100 µl), and cleared again before adding blocking solution (100 µl/well of 0.1% milk powder in PBS-T; Bio-Rad 170-6404; solution filtered to remove particles prior to use) for 1 h at RT. Wells were cleared and rinsed as above with PBS-T, and then 10 µl of PBS was added per well to keep the surface wet. ECD or 20ECD standards (4 to 2000 pg) and samples (50 µl) were added to the wells followed by diluted primary antisera (50 µl/well) for incubation overnight at 4°C. Wells were cleared, rinsed two times, and treated with peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma SAB3700831) diluted 1:15,000 in 100 µl of blocking solution for 3 h at RT. Wells were cleared, rinsed with two changes of PBS-T, and treated with substrate (3,3',5,5'-tetramethylbenzidine, 100 µl/well, KPL 50-76-00) for 20-30 min at 27°C. Addition of 1 M

phosphoric acid (100 µl/well) stopped the reaction so that the absorbance values could be recorded with a plate reader at 450 nm (BioTek MQX200).

The most sensitive and reliable detection of ecdysteroid-BLG conjugates was obtained with antisera from rabbit EAB25, diluted to 1:30,000, and rabbit EAB27, diluted to 1:4,000 (Table 1). These antisera were compared in competitive ECD and 20ECD EIAs to three others: L2 and H22, which had previously been used in ecdysteroid RIAs (Jenkins, et al., 1992; Sieglaff, et al., 2005; Telang, et al., 2007), and Cayman (Table 1). The L2 antiserum was generously provided by J.-P. Delbecque, Université Bordeaux 1, Talence, France and diluted 1:14,000 (Aribi, et al., 1997; Blais, et al., 2010). The H22 antiserum was generously provided by L. I. Gilbert, University of North Carolina, and diluted 1:7,000 (Warren et al., 2009). The Cayman antibody (482202 for 100 determinations) was purchased from the Cayman Chemical Company and dissolved in 6 ml of PBS buffer plus milk block. Cayman final dilution was 1:2 of prepared stock.

2.2.2.4. Specificity assessment

EAB25 and EAB27 were tested for relative specificity by conducting competitive binding assays to 7-dehydrocholesterol, cholesterol, beta-estradiol, corticosterone, makisterone A (Santa Cruz Biotechnology), ponasterone A (Santa Cruz Biotechnology), and testosterone. Each was dissolved in ethanol and then diluted with water to make 40 pg/µl stocks. A standard range of each steroid or sterol (16 to 1000 pg) was then prepared for incubation with EAB25 antiserum (1:30,000) and bound 20ECD-BLG or EAB27 antisera (1;4000) with ECD-BLG for three replicate EIAs as above.

2.2.3. Ex vivo ecdysteroid production by larval tissues and ovaries

UGAL strain *A*. *aegypti* was reared as previously described (Telang, et al., 2007; 2010). Newly molted last (fourth) instar larvae were transferred from laboratory rearing pans to small plastic cups and provided with food. Previous RIA assays indicated that ecdysteroid production by the body wall of last instar larvae peaks at 30 to 36 h post eclosion (Jenkins, et al., 1992; Telang, et al., 2007). During this period, staged larvae were opened laterally in saline using micro-scissors followed by removal of the head and gut. The remaining larval pelt was placed in a polypropylene cap with saline (60 µl) for 6 h at 27°C, after which the saline was collected and frozen. In adult females, ovary production of ecdysteroids peaks 18 to 24 h post blood meal (PBM; Sieglaff, et al., 2005). Ovaries with the last two abdominal segments were dissected from females (3-5 days old) at 24 h PBM and transferred to small caps (2 pairs in 60 µl of saline) for incubation and collection as above. Individual larval pelts or female ovary samples were diluted to 200 µl with ultrapure, washed against 300 µl of chloroform, and centrifuged for 2 min at 20,000 x g. The aqueous fraction was transferred to a new tube, and the chloroform was rewashed with 200 µl of water and centrifuged. The aqueous fractions were pooled and reduced to 80 µl of volume in a vacuum centrifuge (Thermo Electron Corp., UVS400; medium setting, 40°C). Samples so prepared were promptly analyzed by EIA.

2.2.4. Extraction of ecdysteroids from whole larvae and females

Three approaches were used to extract ecdysteroids from mosquitoes. First, last instar larvae or adult females (up to 50 per batch) staged as above were homogenized in methanol (95 or 100%, 1 ml) in 1.5 ml polypropylene tubes using plastic pestles, followed by vortexing and centrifugation for 5 min and 13,000 x *g*. Each supernatant was transferred to another tube, and the pellet processed as above in 500 µl of methanol from which the supernatant was pooled with

the first one. The extracts were dried in a vacuum centrifuge, frozen, and later rehydrated in PBS for EIA or HPLC analysis.

Second, cohorts of 1, 5 or 10 non-blood fed adult females (3 days old) were placed into tubes containing 500 pg of 20ECD followed by extraction in methanol and drying of the supernatants as described above. After rehydrating in 1 ml of 5% methanol, each sample was passed over an activated C_{18} SPE cartridge (Phenomenex), rinsed once with 1 ml of 5% methanol, and retained molecules eluted using 1 ml of 100% methanol. As controls, 20ECD (100 ng) alone or with 30 mM $MgCl_2$ was dissolved in 1 ml of 95% methanol, held on ice for a few minutes, and then loaded and eluted from C_{18} SPE cartridges as above. All eluates were dried and reconstituted in PBS for the ECD EIAs or 30% methanol (15 µl) for mass spectrometry analysis.

Third, cohorts of 1, 5, or 10 last instar larvae or adult females staged as above were homogenized in 300 µl of PBS followed by addition of an equal volume of 1-butanol. Samples were vortexed until uniformly cloudy and then centrifuged at 20,000 x *g* for 10 min. The organic phase (top) was collected into a new tube, and dried in a vacuum centrifuge. Dried material was reconstituted in 200 µl of chloroform, and twice extracted against 200 µl of water. The water phases were pooled, frozen, and lyophilized to remove trace chloroform. Control samples containing 500 pg of ECD or 20ECD extracted in the same manner served as recovery controls. All samples were then reconstituted in PBS for the ECD EIAs.

2.2.5. Identification of immunoreactive ecdysteroids

To identify the ecdysteroids recognized by the EAB25 and EAB27 antisera, ex vivo and whole body samples reconstituted in 200 µl of PBS were diluted in an equal volume of 10% methanol. Each sample (400 µl) was injected onto a Spherisorb ODS1 column (4.6 x 150 mm, Waters PSS830613) and eluted with a gradient (10 – 100% methanol, 60 min, 1 ml/min) on a

Beckman Gold 125s/166 HPLC system. Absorbance of the eluted solvent was monitored at 254 nm. ECD, 20ECD, and makisterone standards were injected and eluted using the same conditions. Fractions (1 ml/min) were collected for the samples and ecdysteroid standards, and aliquots were dried and rehydrated in PBS. Duplicate 50 µl aliquots were then added to plate wells for the EIAs. For a subset of the samples extracted by methanol/$MgCl_2$ treatment, HPLC fractions containing the major immunoreactive ecdysteroids were diffused into a Bruker Esquire 3000 mass spectrophotometer with APCI while running in negative ion mode. This provided soft ionization of ecdysteroids, giving dominant M-1 values for masses of standards.

2.2.6. Quantification and statistical analysis

Standard lines were generated by calculating the percentage absorbance of the known ecdysteroid samples as a percentage of the maximum absorbance control. A linear regression (StatPlus 5.8.2.0) of the standard line was used to calculate the quantity of ecdysteroids in samples. Data from HPLC fraction detection by EIA was prepared in Excel 2011 (Microsoft, 14.7.0). Treatment differences were examined by analysis of variance (ANOVA) followed by a post-hoc Tukey Kramer Honest Significant Difference test at the $p<0.05$ level.

2.3. Results

2.3.1. Comparison of antisera for recognition of ECD and 20ECD

Sera from four rabbits were examined by EIA for binding to ECD-BLG and 20ECD-BLG. EAB25 from a rabbit immunized with 20ECD-BSA exhibited the strongest binding to 20ECD-BLG while EAB27 from a rabbit immunized with ECD-BSA exhibited the strongest binding to ECD-BLG. Binding curves generated over a range of dilutions indicated EAB25 antiserum used at 1:30,000, EAB27 antiserum used at 1:4000, and coating wells with BLG conjugates at 28 ng/100 µl was optimal for competitive EIAs (Table 2.1; Fig. 2.S1). To compare

the sensitivities of EAB25 and EAB27 to the L2, H22, and Cayman antisera, we generated binding curves over a 4 to 2,000 pg range of free ECD or 20ECD standards. EAB25 had the lowest sensitivity for ECD of the antisera tested but showed similar sensitivity for 20ECD as H22 and Cayman (Fig. 2.1A, B). EAB27 had the highest sensitivity for 20ECD, and a similar sensitivity for ECD as H22 and Cayman. For each antiserum, ECD values for 250 pg were divided by 20ECD values for 250 pg to yield an affinity ratio. Under the conditions of this EIA, ECD/20ECD ratios were 0.037 for EAB25, 0.226 for EAB27, 0.498 for L2, 0.649 for H22, and 0.307 for Cayman (Table 2.1). These results indicated that EAB25 had a 27-fold higher affinity for 20ECD than ECD, while EAB27 had a four-fold higher affinity for 20ECD. In contrast, L2 and H22 had a two-fold higher and Cayman a three-fold higher affinity for ECD than 20ECD.

2.3.2. Steroid specificity of the new ecdysteroid antisera

We next examined whether EAB25 and EAB27 recognized precursor sterols for ECD biosynthesis (cholesterol and 7-dehydrocholesterol), related steroids with an unmodified E chain (makisterone and ponasterone, a plant ecdysteroid), or steroids with no E chain (beta-estradiol, corticosterone, and testosterone). Binding curves were generated to each for comparison to ECD and 20ECD over a 16 to 1,000 pg range with: 1) EAB25 and ECD-BLG, and 2) EAB27 and 20ECD-BLG. EAB25 antiserum specifically bound only 20ECD (Fig. 2.2A). EAB27, in contrast, exhibited parallel binding curves with nearly identical high binding to ECD and 20ECD and lower binding to makisterone and ponasterone. Neither antiserum recognized 7-dehydrocholesterol as illustrated by its respective flat lines (Fig. 2.2A, B). Cholesterol, beta-estradiol, corticosterone, and testosterone also were not recognized by either antiserum (data not shown).

2.3.3. Characterization of ecdysteroids produced by larval and female tissues ex vivo

We previously used ex vivo tissue incubations and RIAs to measure titers of secreted ecdysteroids from pelts of staged *A. aegypti* larvae during the last (fourth) instar (Jenkins, et al., 1992; Telang, et al., 2007) and ovaries from 24 h PBM adult females (Hagedorn, et al., 1975; Greenplate, et al., 1985; Borovsky, et al., 1986; Sieglaff, et al., 2005). These previous RIAs could not distinguish between ECD and 20ECD. We repeated these assays using the new EIAs to both compare values to prior results and assess the abundance of ECD versus 20ECD. Results from the EAB25 and EAB27 EIAs indicated that 20ECD was a component of the ecdysteroid mixture present in the incubation medium from both tissue types (Fig. 2.3). This was confirmed by HPLC fractionation, which sufficiently separated 20ECD and ECD as shown for the ecdysteroid standards (Fig. 2.4A, B). Using the EAB25 EIA, the content of fraction 31 from both samples was determined to be 20ECD, as it was the same fraction that the 20ECD standard eluted into under the same HPLC conditions. 20ECD and ECD in fraction 36 (where the ECD standard eluted) were detected in both samples with the EAB27 EIA. No makisterone was detected in fraction 33 or 34 (where the makisterone standard eluted) of either sample after HPLC.

2.3.4. Characterization of ecdysteroids extracted from whole last instar larvae and females

Another method for measuring ecdysteroids is extraction of whole bodies. This is usually done using methanol extraction followed by organic phase separation or SPE over C_{18} cartridges to enrich ecdysteroid content for measurement by immunoassay. We used this approach with two objectives: 1) determine whether ecdysteroid content of last instar larvae and adult females staged as above was similar to previously reported data and 2) assess whether the EIAs in combination with HPLC fractionation recognized known ecdysteroids with minimal or no

interference from other extracted molecules. Extractions of staged larvae and females with methanol followed by SPE yielded higher than expected values from the EAB25 and EAB27 EIAs. We therefore extracted groups of one, five, and ten non-blood fed adult females in methanol alone or spiked with 500 pg of 20ECD to determine the efficiency of its recovery as measured with the EIAs. Results indicated that ecdysteroid values increased non-linearly with samples of five and ten females yielding higher than expected values than extractions from fewer females (Fig. 2.5A, B). Overall, these results suggested that homogenization of mosquitoes in methanol altered the immunoreactivity of the extracted ecdysteroids.

A review of the literature indicated that magnesium ions (Mg^{2+}) are commonly involved in redox and non-redox catalysis in biological systems, and that the combination of methanol and Mg^{2+} is often used to reduce many types of chemical functional groups, including those found on steroids (Lee, et. al. 2004). In all cellular systems, Mg^{2+} is present in the millimolar range, with insect hemolymph exceeding 10 mM (Clark and Craig, 1953) independent of diet (Wyatt, 1961). Thus, we questioned whether homogenizing insect bodies in methanol for the extraction of ecdysteroids might result in the chemical reduction of ecdysteroids and alter their recognition by ecdysteroid antisera. To examine this, 20ECD (500 pg) was processed in methanol or water (500 µl) with and without 10 mM $MgCl_2$. Processing of 20ECD in water or methanol alone had little to no effect on its recovery or immunoreactivity in both EIAs (Fig. 2.6). In contrast, adding $MgCl_2$ to water resulted in both EIAs yielding 20ECD values that were almost two-fold higher, while adding $MgCl_2$ to methanol increased values three-to-four-fold. These inflated ecdysteroid values were similar to the values for the whole body female samples extracted in methanol with a 20ECD spike (see Fig. 2.5). Collectively, these data suggested that methanol/$MgCl_2$ altered the structure of 20ECD, which in turn influenced titer estimates generated by the EIAs. This was

further supported by MS analysis, which showed a 20-fold decrease in the mass intensity of 20ECD in methanol/$MgCl_2$ along with new mass species absent for 20ECD in methanol alone (Sup. Fig. 2.2B, C). The identity and relative immunoreactivity of the new species that formed in methanol/$MgCl_2$ were not determined.

Other organic solvents have been used to extract ecdysteroids from insects that do not affect their structure as determined by MS (Hikiba, et al., 2013). We found that 1-butanol extracted ecdysteroids from whole larvae and females, but an additional step of phase separation was required because the ecdysteroids were not readily rehydrated in this solvent for use in the EIAs. This procedure gave reliable results and scaled ECD and 20ECD recovery for staged last instar larvae (Fig. 2.7A, B) and females (Fig. 2.7C, D). More than 95% of the standards extracted in parallel were also recovered (Fig. 2.7E).

The above 1-butanol-treated ecdysteroid standards and mosquito extract samples were fractionated with HPLC, and the ecdysteroid content of fraction aliquots measured with both EIAs. 20ECD and ECD standards were recovered within 95% of the expected value (600 pg) using the EAB27 EIA (total value for two fractions covering the elution of each standard) (Fig. 2.8A, B). Makisterone A in contrast was 61% of its value, which was consistent with the lower affinity of the EAB27 antiserum for this ecdysteroid. EAB25 detected two-fold more 20ECD than present in the fraction aliquots, but not the other standards. The discrepancy in values may be an effect of the methanol carrier or light on the 20ECD standard during HPLC, since the EAB25 EIA reliably measured 20ECD content in aqueous solutions. HPLC of the mosquito extracts resolved 20ECD as the predominant ecdysteroid (Fig. 2.8C, D), but only 45% of the amount estimated to be in the respective samples was eluted. Notably, similar amounts of 20ECD were measured with both EIAs in the fractions from larval or adult female extracts. No

ECD was detected in the extract from larvae after HPLC (Fig. 2.8C), but it was present in the extract from adult females (Fig. 2.8D). Only negligible amounts of other immunoreactive factors were detected in fractions flanking those shown in Fig. 2.8C and D.

2.4. Discussion

The EIAs reported in this study used new antisera that recognize 20ECD and ECD. We also conclude these EIAs offer several advantages. First, they are not proprietary and do not require specialized equipment or great expense to synthesize the ecdysteroid-BLG used to plate wells. Second, the EIA is flexible because different ecdysteroid antisera can be used with ecdysteroid-BLG as the bound competitor and a peroxidase-conjugated secondary antibody as the reporter. Third, the costs of these EIAs are approximately one hundred-fold lower than currently available ecdysteroid EIA kits (Table 2.S1). All EIA protocols, including these, are similar in that they indirectly quantify ecdysteroids based on an enzymatic colorimetric reaction. We found that using ecdysteroid-BLG as the well coating extends the functional range of ecdysteroid antisera beyond the limit of the well surface area, which allows for an economical use of ecdysteroid antisera. However, it also limits the upper range of detection, as only so much ecdysteroid-conjugate adsorbs to the well. With a checkerboard titration, we determined this maximum to be around 400 ng for 20ECD-BLG with the EAB25 antiserum, or 200 ng ECD-BLG with the EAB27 antiserum (Fig. 2.S1). On the other hand, the quantity of absorbed epitope-protein conjugate does not represent the actual quantity of potential binding sites.

Under the conditions developed for our competitive EIAs, the limit of detection (LOD; defined as 80% maximal binding, I_{80}) for 20ECD with the EAB25 antiserum and 20ECD-BLG is 84.2 pg ($1.8x10^{-13}$ moles), and for ECD with the EAB27 antiserum and ECD-BLG, is 4.5 pg ($9.7x10^{-15}$ moles). The working range for both antisera is about three log-10 units. This is

comparable to the commercially available 20ECD EIA kit (A05120, Bertin Pharma/501390, Cayman Chemical Company), which reports a linear standard range of 39 to 5000 pg/ml, LOD of 31 pg 20ECD (6.5 $x10^{-14}$ moles), and cross reactivity to ECD (100%) and ponasterone A (43%). As used in our EIA, Cayman had an I_{80} of 57.3 pg ($1.2x10^{-13}$ moles) for 20ECD in the presence of 20ECD-BLG, and 17.9 pg ($3.9x10^{-14}$ moles) for ECD in the presence of ECD-BLG.

We used the new antisera and EIAs to quantify total ecdysteroids and 20ECD in mosquito larvae and females at times of peak titer to compare with previously reported results generated by RIA. In addition, the immunoreactive ecdysteroids in these samples were identified by HPLC with EIA screening of the fractions. Previous RIA data for *A*. *aegypti* last instar larvae (30-36 post eclosion) measured 20 to 200 pg of ecdysteroids per individual in hemolymph, 80 to 360 pg of ex vivo-produced ecdysteroids per pelt over 6 h (Jenkins, et al., 1992; Telang, et al., 2007). Approximately 200 pg of ecdysteroids per individual was also measured by EIA after batch extraction of larvae using methanol (Margam, et al., 2006). The values reported in Fig. 2.3 for total ecdysteroids produced ex vivo by pelts are less than two-fold higher than these previous results, while values from extracted larvae are similar. Both ECD and 20ECD were present in the media after ex vivo pelt incubation, but 20ECD appeared to be the predominant form extracted from larvae, as quantified with the EAB25 EIA and confirmed by HPLC (Fig. 2.4A and 2.8C).

Hagedorn, et al. (1975) was the first to quantify ecdysteroids in *A*. *aegypti* females after blood feeding by using a multistep extraction procedure that began with 60% methanol, which was followed by RIA. These data indicated that individual females at 20 h PBM contain approximately 250 pg of ecdysteroid. Using HPLC and gas-chromatography, the authors further showed that ECD was the predominant form secreted ex vivo by ovaries from blood-fed females while 20ECD was the predominant form extracted from whole females, which supported the

conversion of ECD to 20ECD. A subsequent study reported that similarly staged females after a single-step 50% methanol extraction contained ~600 pg of ecdysteroids (Greenplate, et al., 1985). This study also noted substantial variation in values between experiments. A third study, using a different extraction method, also measured ~600 pg in the body of similarly staged *A. aegypti* by RIA, while HPLC and thin layer chromatography identified only ECD in ovaries but two-fold more 20ECD than ECD in whole bodies (Borovsky, et al., 1986). Our own previous studies have focused on ex vivo ecdysteroid production by *A. aegypti* ovaries with results generated by RIA showing that ovaries from females 18-30 h PBM produce 100-120 pg of ecdysteroids when incubated in the same saline used in this study (Sieglaff et al., 2005). With the EAB27 EIA total ecdysteroids produced ex vivo by ovaries were almost six-fold higher, but the amount extracted from a female was within previously reported values (Table 2.2). Results from the current study also indicate that 20ECD is present in both the ovary incubation medium and whole body extracts (Table 2.2, Fig. 2.4B and 2.8D).

Altogether, the differences between ecdysteroid values reported here versus previous studies likely reflect the use of different ecdysteroid antisera and assay formats. The antisera used for RIAs in the earlier studies of mosquito ecdysteroids differed in their relative recognition of ECD and 20ECD, so values were reported as total ecdysteroids. Now with the EIA using the new ecdysteroid antisera, we can measure total ecdysteroids and 20ECD, and the 20ECD value can be subtracted from the total ecdysteroid value to give an ECD value. The relative amount of circulating ECD and 20ECD is important because binding to the ecdysteroid receptor (EcR)-Ultraspiracle (USP) complex likely differs between these ligands (Wang, et al., 2000; Heinrich, 2012).

Homogenization of insects in methanol followed by its evaporation is widely used for the extraction of ecdysteroids from insects including mosquitoes (Wilson, et al., 1990; Margam, et al., 2006). In contrast, our results indicated methanol was not the best choice for ecdysteroid extractions from whole body samples due to interactions with Mg^{2+}. We did not find any reference to this interaction in the insect ecdysteroid literature, or to any prior studies that compared the efficiency of methanol for extraction of endogenous ecdysteroids from whole insect bodies alone or with known amounts of added ecdysteroids. In contrast, directed reduction of insect steroids has been used to enhance identification (Dinan and Rees, 1978; Savchenko and Odinokov, 2012), while prior studies also provide evidence for reductive cleavage of the 14α-hydroxy group on the ecdysteroid under specific conditions (Horn and Bergamasco, 1985; Harmatha, et al., 2002). Our MS data showed the presence of multiple species rather than a single identifiable reduced form after treatment of 20ECD with methanol and $MgCl_2$ (Fig. 2.S2). One possibility is that these multiple species are immunoreactive and account for the anomalous values obtained from the EIAs. Another is that extraction results in the methanol/Mg^{2+} reduction of other endogenous sterols that are recognized by the antisera used in the EIAs.

Regardless of why methanol extraction elevated ECD and 20ECD values, we now avoid its use with mosquitoes and recommend that extractions be done macerating samples in PBS or water, followed by 1-butanol extraction and water/chloroform phase separation to purify ecdysteroids from the other lipids. This procedure did not alter the immunoreactivity of known ecdysteroid standards, and consistent ecdysteroid values were obtained from one to ten last instar larvae or adult female mosquitoes. Extraction of larger numbers of mosquitoes was less efficient. In addition, we suggest that known amounts of ECD and 20ECD be processed with or without mosquitoes to monitor efficiency of recovery and any changes in immunoreactivity. Further

studies will help to resolve whether methanol extraction of insects that have high Mg^{2+} concentrations is a significant problem for the quantification of ecdysteroids by EIAs in other insects. In conclusion, the EIA protocol described here takes advantage of the high specificity of the EAB25 antiserum for 20ECD, which is unique among the available ecdysteroid antisera. We further demonstrate that our EIAs in combination with HPLC provided ecdysteroid-specific quantification.

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Table 2.1. Antisera characteristics used for EIAs with 20 hydroxyecdysone (20ECD) or ecdysone (ECD) conjugated to bovine beta-lactoglobulin. EAB25 and EAB27 antisera data presented herein. L2 antisera was produced and characterized, as described in Pascual et al. 1995. H22 antisera was produced, as described in Horn, et al., 1976, and characterized by Warren and Gilbert, 1986. Information about the Cayman antiserum is referenced as Porcheron, et al., 1989 in the EIA manual from Cayman Chemical Co.

Antiserum	**Source**	**Antigen**	**Dilution**	**ECD/20ECD specificity ratio**	**Makisterone A cross reactivity**
EAB25	Current study	20ECD-6-BSA	1:30,000	0.037	< 0.1%
EAB27	Current study	ECD-6-BSA	1:4,000	0.226	5%
L2	Delbecque	ECD-thyroglobulin	1:14,000	0.498	9%
H22	Gilbert	ECD-22-thyroglobulin	1:7,000	0.649	22%
Cayman	Cayman Chemical Company	20ECD-6-BSA	1:2	0.307	4%

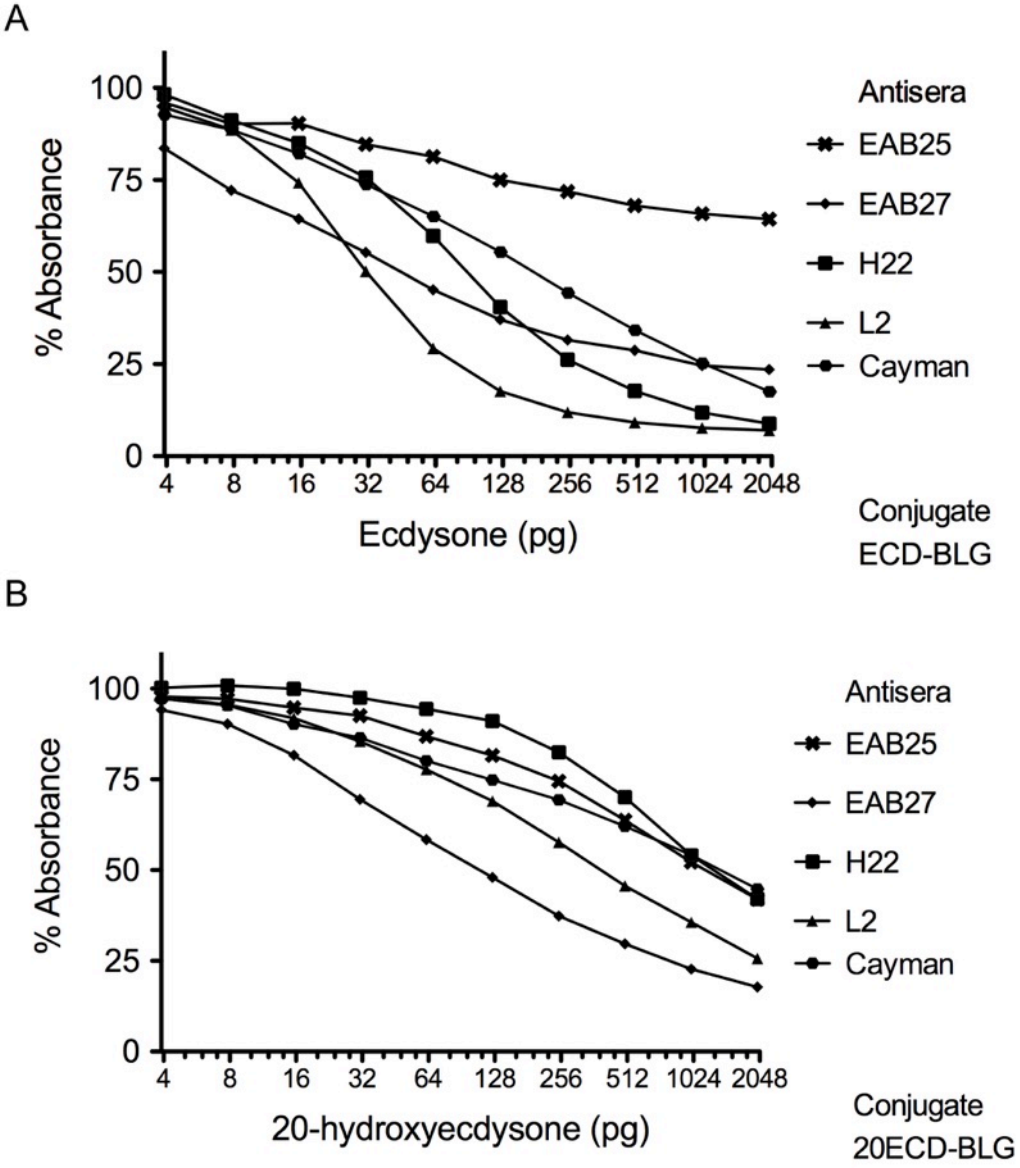
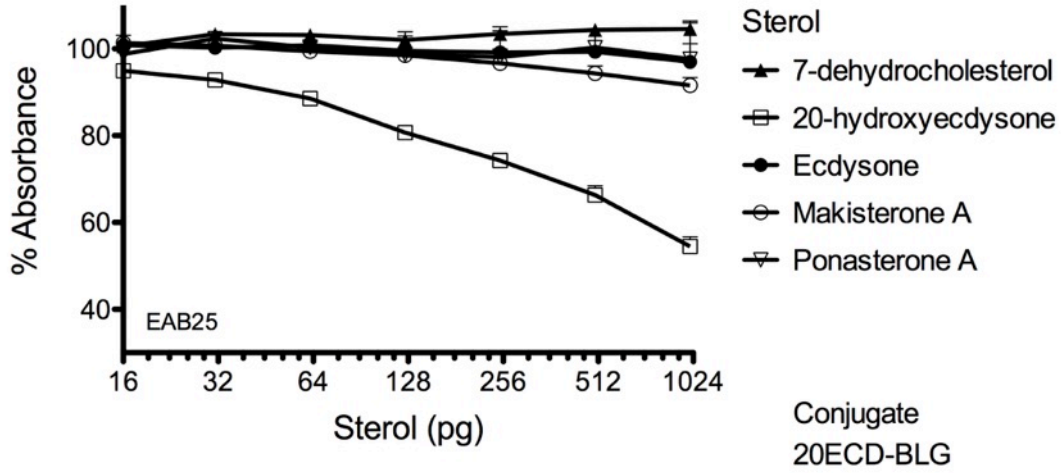

A
100
75
50
25
0
% Absorbance
4
8
16
32
64
128
256
512
1024
2048
Ecdysone (pg)
Antisera
EAB25
EAB27
H22
L2
Cayman
Conjugate
ECD-BLG
B
100
75
50
25
0
% Absorbance
4
8
16
32
64
128
256
512
1024
2048
20-hydroxyecdysone (pg)
Antisera
EAB25
EAB27
H22
L2
Cayman
Conjugate
20ECD-BLG

Figure 2.1. Comparison of EAB25, EAB27, H22, L2, and Cayman antisera recognition of free ecdysone (ECD) and bound ECD-bovine lactoglobulin (BLG) (A) or free 20-hydroxyecdysone (20ECD) and bound 20ECD-BLG (B). The standard range is 4-2000 pg free ECD or 20ECD. Antisera dilution: EAB25, 1:30,000; EAB27, 1:4,000; H22, 1:7,000, L2, 1:14,000, Cayman antibody's recommended concentration (actual concentration undisclosed). Each standard value on the curve represents the mean of six replicates.

A


% Absorbance
100
80
60
40
EAB25
16
32
64
128
256
512
1024
Sterol (pg)
Sterol
7-dehydrocholesterol
20-hydroxyecdysone
Ecdysone
Makisterone A
Ponasterone A
Conjugate
20ECD-BLG

B

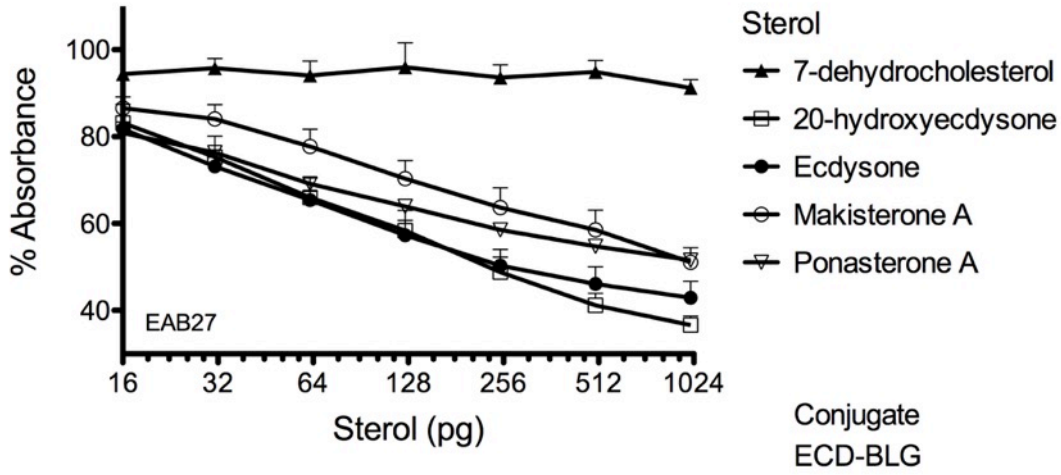

% Absorbance
100
80
60
40
EAB27
16
32
64
128
256
512
1024
Sterol (pg)
Sterol
7-dehydrocholesterol
20-hydroxyecdysone
Ecdysone
Makisterone A
Ponasterone A
Conjugate
ECD-BLG

Figure 2.2. EAB25 and EAB27 antisera binding affinity for five different steroids. (A) EAB25 (1:30,000 with bound 20ECD-BLG) strongly preferentially binds 20ECD. (B) EAB27 (1:4000 with ECD-BLG bound) recognizes 20ECD and ECD equally while also recognizing makisterone and ponasterone. Neither antiserum recognizes 7-dehydrocholesterol. Non-specific binding of EAB25 and EAB27 are 5.2 ± 0.5% and 5.1% ±1.7% of maximum binding respectfully. Each value on the curve represents the mean of three replicates ± S.E over a standard range for each free steroid of 4 to 1000 pg.

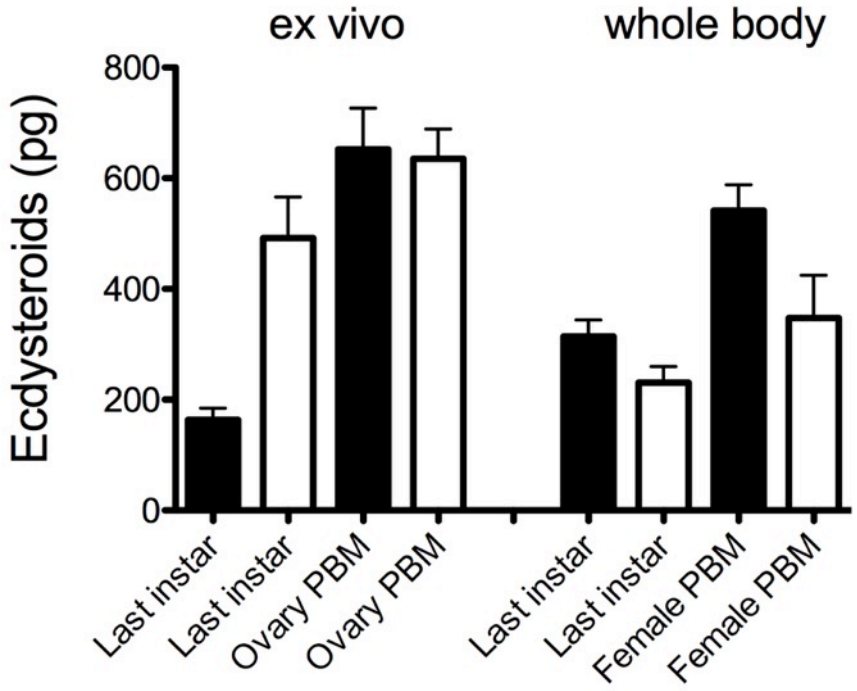


Figure 2.3. 20-hydroxyecdysone and ecdysone are present in tissue ex vivo medium or extracted from whole bodies of *A. aegypti* last instar larvae or females. Last instar pelts (30-36 h post eclosion) and ovaries from females (24 h post blood meal, PBM) were incubated for 6 h in saline. Ex vivo medium samples were phase separated with chloroform. Whole bodies of similarly staged larvae and females were extracted in 1-butanol followed by chloroform/water phase separation. Ecdysteroids in the aqueous samples were diluted in PBS to represent a single individual and then quantified with the EAB25 (black bars) and EAB27 (white bars) EIAs, which recognize 20ECD only or both 20ECD and ECD, respectively. n = 9 for each sample.

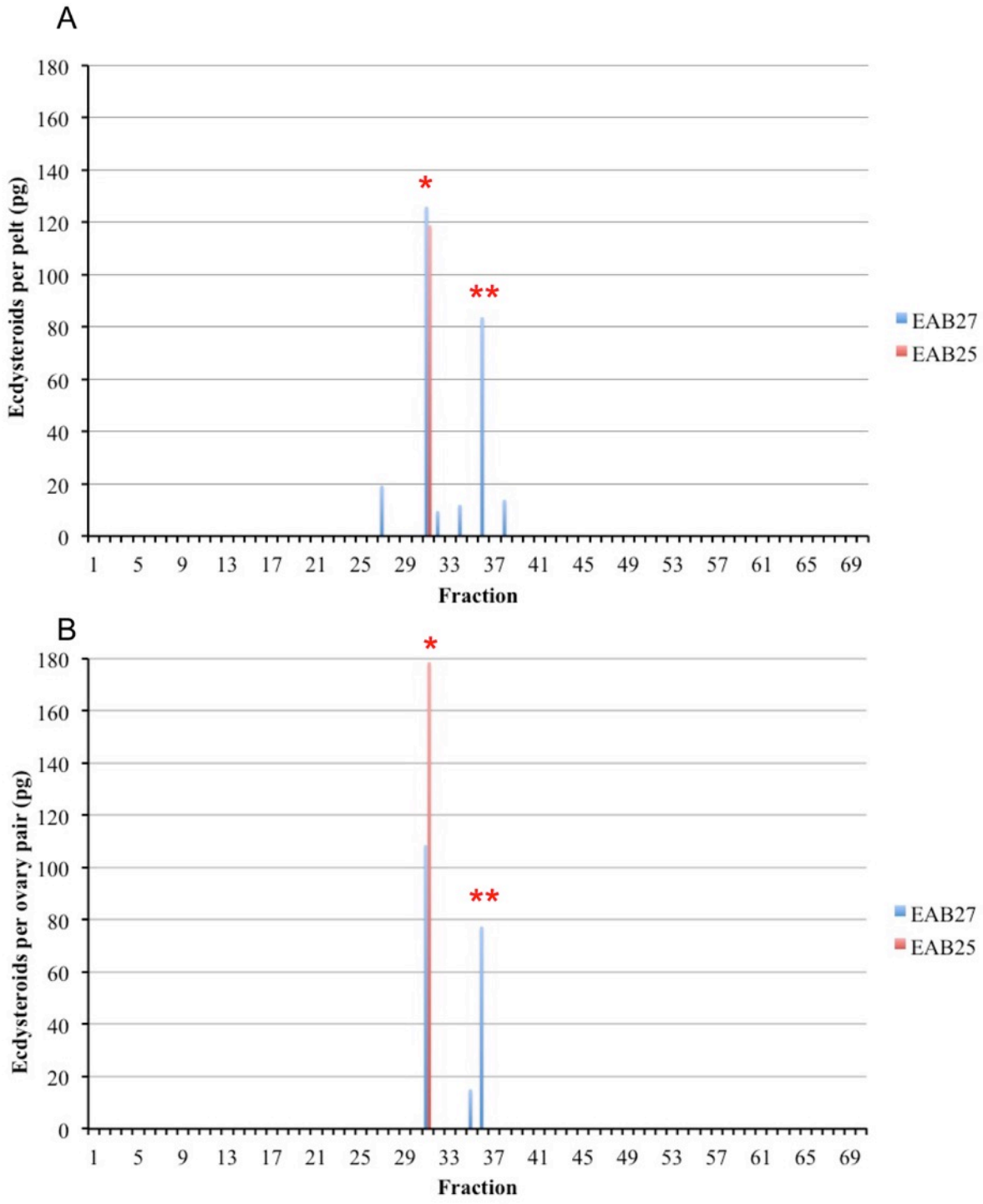
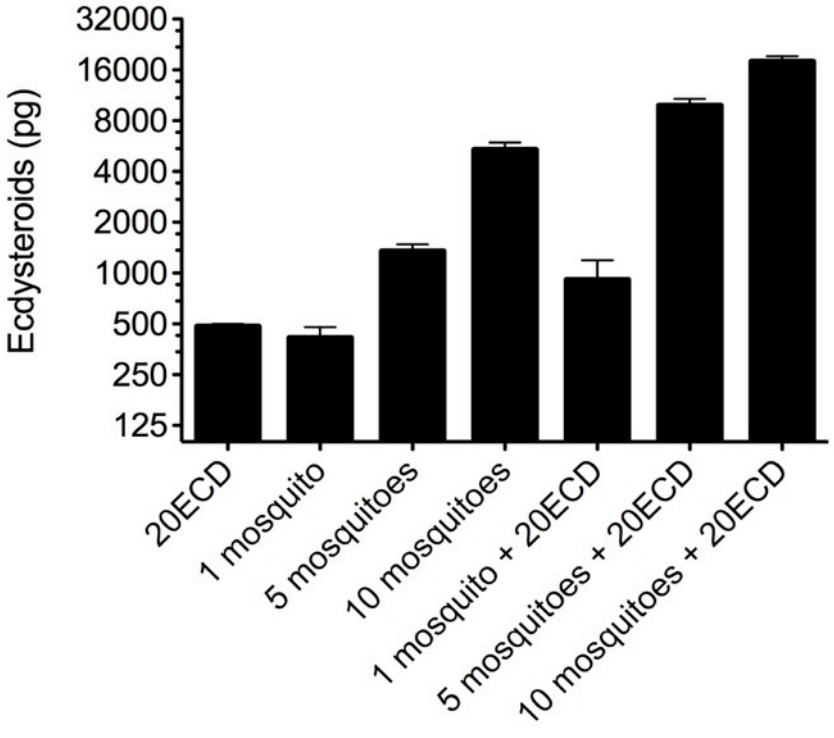

A
Ecdysteroids per pelt (pg)
180
160
140
120
100
80
60
40
20
0
*
**
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69
Fraction
EAB27
EAB25
B
Ecdysteroids per ovary pair (pg)
180
160
140
120
100
80
60
40
20
0
*
**
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69
Fraction
EAB27
EAB25

Figure 2.4. Identification of immunoreactive ecdysteroids in HPLC fractionated samples obtained from *A*. *aegypti* (A) last instar body walls (30 to 36 h post eclosion) and (B) ovaries from females 24 h post blood meal. Tissues were incubated in saline for 6 h. Samples were then pooled and subjected to HPLC. 20-hydroxyecdysone (*) and ecdysone (**) eluted in the designated fractions, which was determined by loading and elution of the same ecdysteroid standards along with makisterone (100 ng each) in a later HPLC run. Ecdysteroid content of the fraction aliquots was determined with the EAB25 and EAB27 EIAs.

A


Ecdysteroids (pg)
32000
16000
8000
4000
2000
1000
500
250
125
20ECD
1 mosquito
5 mosquitoes
10 mosquitoes
1 mosquito + 20ECD
5 mosquitoes + 20ECD
10 mosquitoes + 20ECD

B

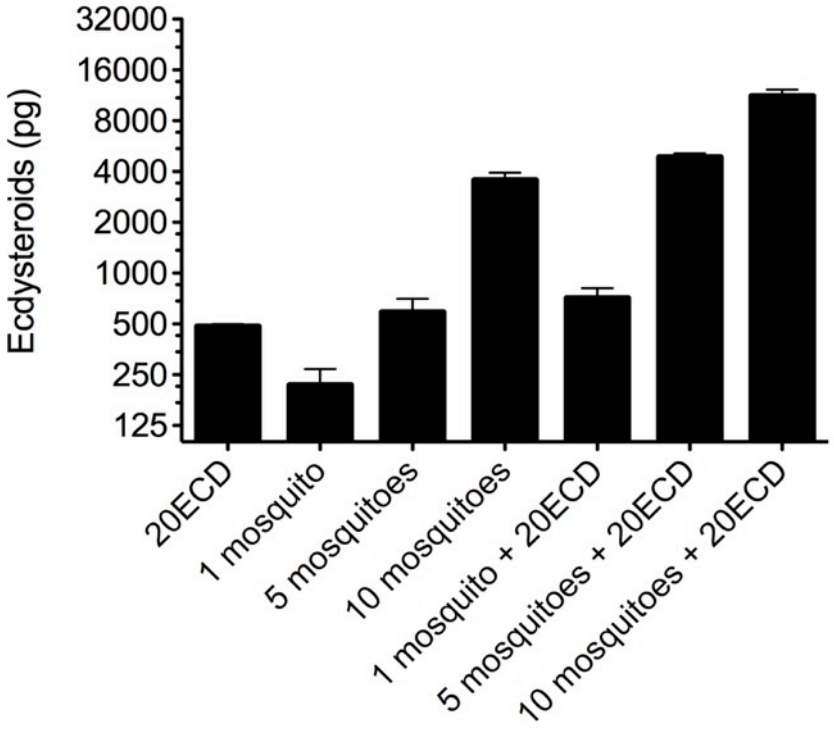

Ecdysteroids (pg)
32000
16000
8000
4000
2000
1000
500
250
125
20ECD
1 mosquito
5 mosquitoes
10 mosquitoes
1 mosquito + 20ECD
5 mosquitoes + 20ECD
10 mosquitoes + 20ECD

Figure 2.5. Ecdysteroid values for the 95% methanol extracts of 1, 5, or 10 non-blood fed *A. aegypti* females alone or spiked with 20-hydroxyecdysone (20E) as measured with the (A) EAB25 or (B) EAB27 EIA. Samples were diluted to remain within the EIA standard range. Y axis in Log(2); n = 8-9.

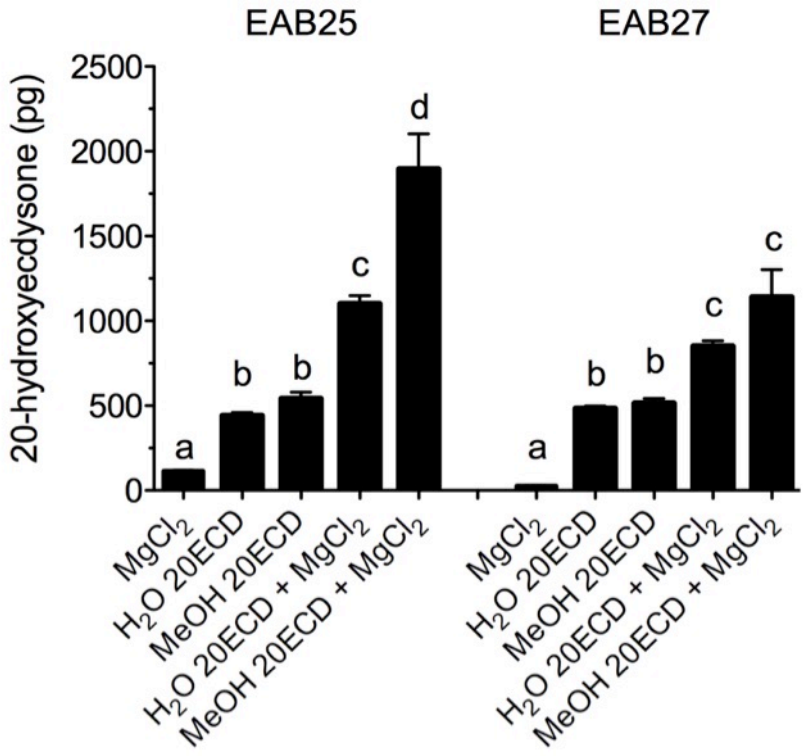


Figure 2.6. Treatment of 20-hydroxyecdysone (20E, 500 pg) in water or 95% methanol (MeOH) with $MgCl_2$ (100 mM) increased the amounts measured with the EAB25 and EAB27 EIAs. Aqueous samples (H_2O) were directly analyzed by EIA, while the methanol samples were dried and rehydrated before analysis. Letters indicate a statistical difference between groups at $p < 0.05$. EAB25 $F_{4, 39} = 51.26$; EAB27, $F_{4, 39} = 31.14$, $P < 0.0001$. $n = 8$.

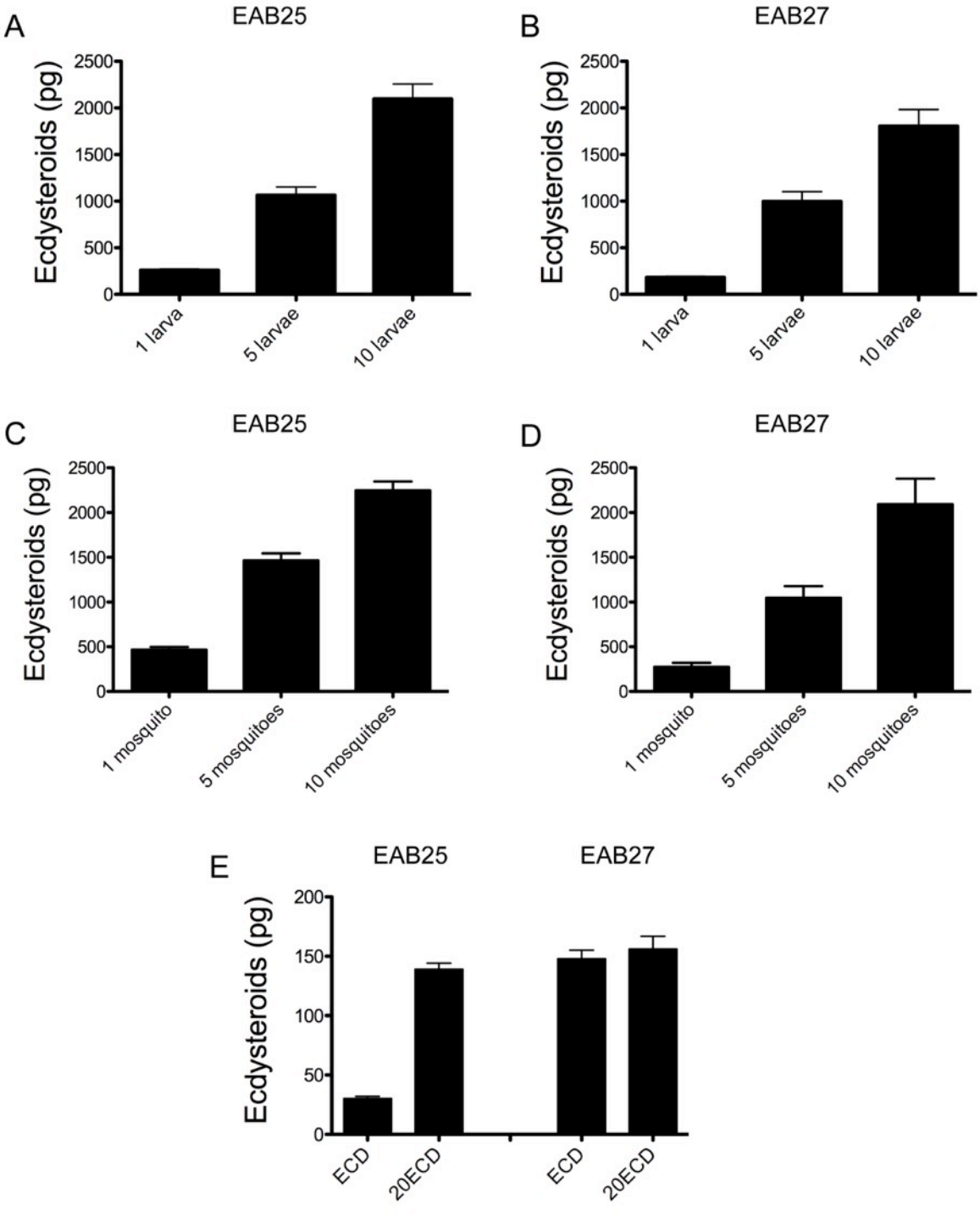

A
EAB25
Ecdysteroids (pg)
2500
2000
1500
1000
500
0
1 larva
5 larvae
10 larvae
B
EAB27
Ecdysteroids (pg)
2500
2000
1500
1000
500
0
1 larva
5 larvae
10 larvae
C
EAB25
Ecdysteroids (pg)
2500
2000
1500
1000
500
0
1 mosquito
5 mosquitoes
10 mosquitoes
D
EAB27
Ecdysteroids (pg)
2500
2000
1500
1000
500
0
1 mosquito
5 mosquitoes
10 mosquitoes
E
EAB25
EAB27
Ecdysteroids (pg)
200
150
100
50
0
ECD
20ECD
ECD
20ECD

Figure 2.7. Total ecdysteroids extracted with 1-butanol and water/chloroform phase separation from 1, 5, and 10 (A,B) last instar larvae 30-36 h post eclosion, (C,D) female 24 h post blood meal, and (E) ecdysone (ECD) and 20-hydroxyecdysone (20E) standards (145 pg), as measured with the EAB25 and EAB27 EIAs. Samples were diluted to remain within the EIA standard range. n = 9.

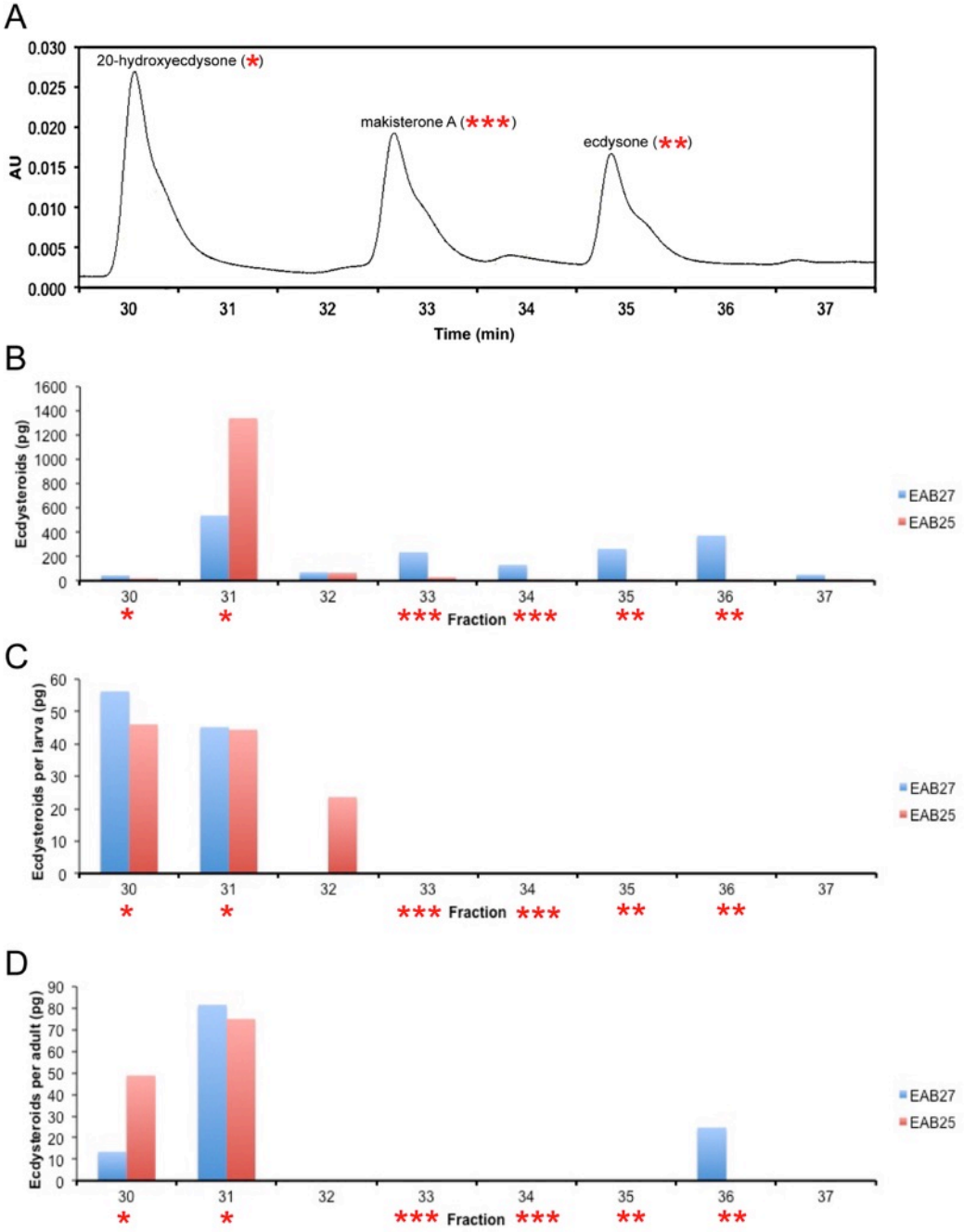
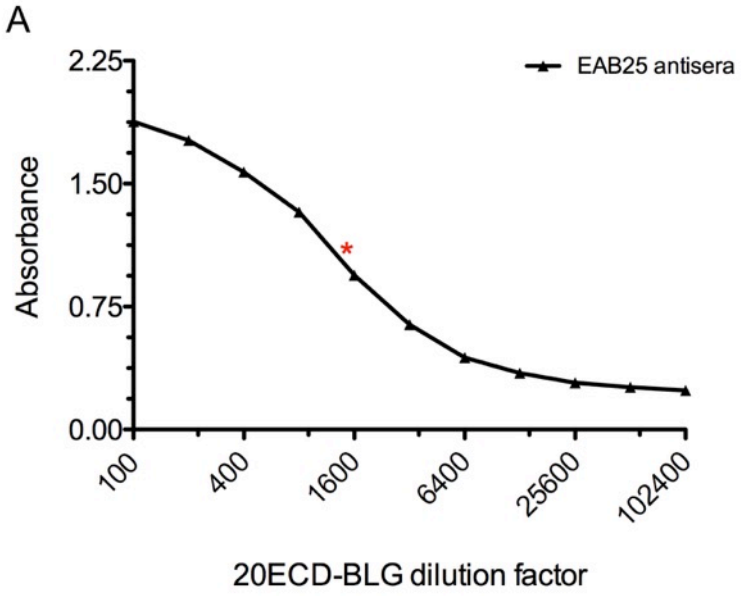

A
20-hydroxyecdysone (*)
makisterone A (***)
ecdysone (**)
AU
Time (min)
B
Ecdysteroids (pg)
EAB27
EAB25
Fraction
C
Ecdysteroids per larva (pg)
EAB27
EAB25
Fraction
D
Ecdysteroids per adult (pg)
EAB27
EAB25
Fraction

Figure 2.8. HPLC fractionation of ecdysteroid standards and whole body extracts from *A. aegypti* followed by quantification using the EAB27 or EAB25 EIA. (A) Absorbance profile (254 nm) for the elution of 20-hydroxyecdysone (*), makisterone (***) and ecdysone (**) standards (100 ng each). (B) Quantity of ecdysteroids detected in each fraction from A as measured by the EAB27 or EAB25 EIA. (C) HPLC fractionation and quantification of ecdysteroids from whole body extracts of *A. aegypti* fourth instars. (D) HPLC fractionation and quantification of ecdysteroids from whole body extracts of *A. aegypti* females (24 h PBM). Samples were extracted as in Fig. 2.6.

Table 2.S1. Material costs for the EIA described herein and for the EIA from Cayman Chemical Company (list prices from www.caymanchem.com). Costs include conjugate synthesis components from Sigma-Aldrich (ECD-BLG & ECD-BSA primary antisera production costs at current consumer prices (2017), and consumables purchased from Fisher Scientific (www.fishersci.com).

Cost per well	**EIA**	**Cayman EIA components**	**Cayman EIA Kit**
Conjugate	$0.00	$1.29	
Plate	$0.04	$0.22	
Primary antiserum	$0.00	$0.43	
Goat anti-rabbit IgG-HRP	$0.00	n/a	
Substrate	$0.00	$0.10	
Rinse buffer	$0.01	$0.38	
Block buffer	$0.00	$0.01	
Total	$0.05	$2.44	$5.16
Cost per plate	$4.89	$233.92	$495
Total samples per plate (singlet)	72	72	68
Total manufacturers recommended samples	36	36	34

Low cost per sample (singlet)	$0.07	$3.25	$7.28
High cost per sample (duplicate)	$0.14	$6.50	$14.56


A
EAB25 antisera
2.25
1.50
0.75
0.00
Absorbance
*
100
400
1600
6400
25600
102400
20ECD-BLG dilution factor

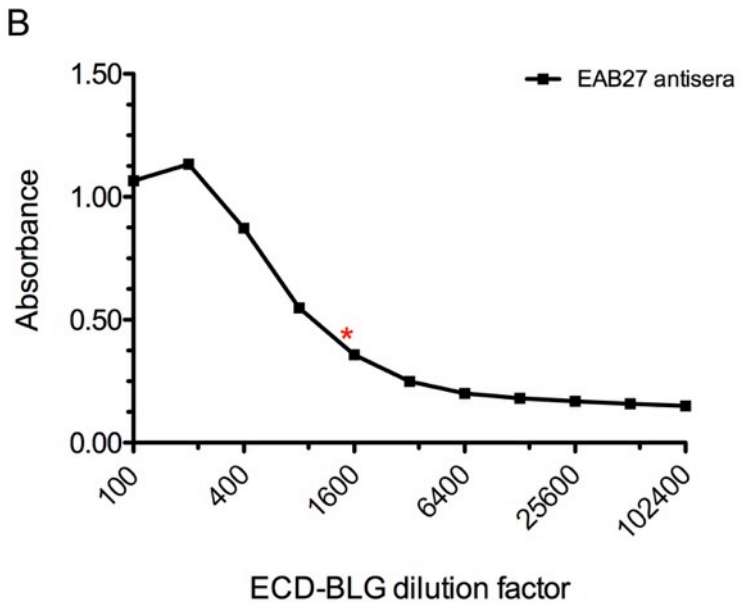

B
EAB27 antisera
1.50
1.00
0.50
0.00
Absorbance
*
100
400
1600
6400
25600
102400
ECD-BLG dilution factor

Figure 2.S1. Binding curves to determine functional dilution of (A) bound 20-hydroxyecdysone (20-ECD)-bovine lactoglobulin (BLG) to use with EAB25 antiserum (1:30,000) and (B) ecdysone (ECD)-BLG with EAB27 antiserum (1:4,000). The dilution of 1:1430 (red asterisk) represents 28 ng/100 µl of protein conjugate delivered to each well for the EIA and was chosen because it fell within the linear range of response while maintaining sensitivity to lower quantities of ecdysteroids. n = 1-7.

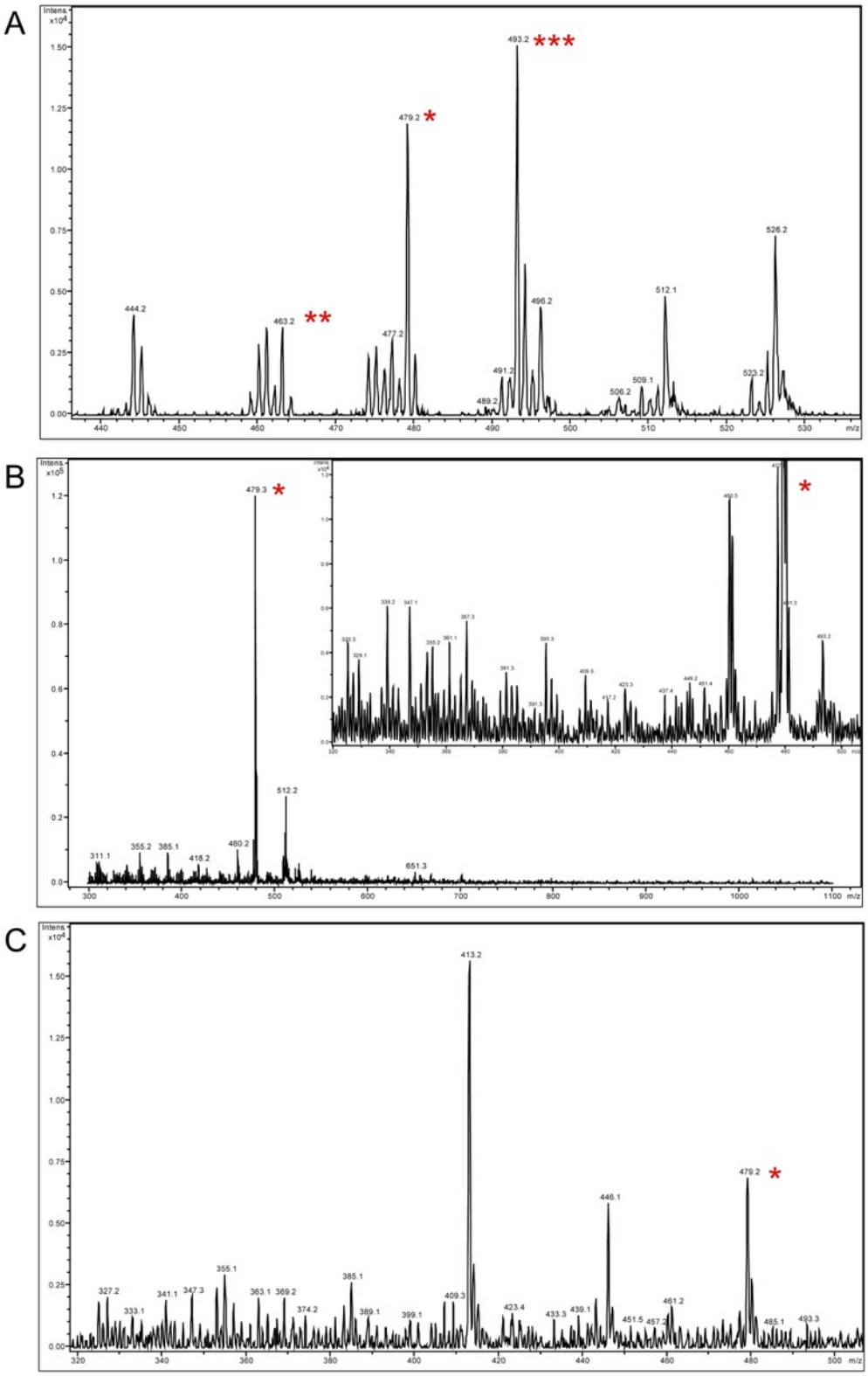

A
Intens.
x10^4
493.2 ***
479.2 *
526.2
444.2
463.2 **
477.2
496.2
512.1
491.2
489.2
506.2
509.1
523.2
m/z
B
Intens.
x10^5
479.3 *
512.2
311.1
355.2
385.1
418.2
460.2
651.3
m/z
C
Intens.
x10^4
413.2
479.2 *
446.1
355.1
327.2
341.1
347.3
333.1
363.1
369.2
374.2
385.1
389.1
399.1
409.3
423.4
433.3
439.1
451.5
457.2
461.2
485.1
493.3
m/z

Figure 2.S2. Mass spectrometry of ecdysteroid standards. A) 20-hydroxyecdysone (20ECD) (*), ecdysone (**), and makisterone (***), 12 ng each. B) 20ECD (25 ng) in 95% methanol or C) treated with $MgCl_2$ (30 mM). Inset in B shows spectra at equivalent scale for comparison with C.

CHAPTER 3

CALCIUM INFLUX ENHANCES NEUROPEPTIDE ACTIVATION OF ECDYSTEROID HORMONE PRODUCTION BY MOSQUITO OVARIES[2]

[2] McKinney, D.A., Eum, J.H., Dhara, A., Strand, M.R. and Brown, M.R., 2016. Calcium influx enhances neuropeptide activation of ecdysteroid hormone production by mosquito ovaries. Insect Biochem. Mol. Biol. 70, 160-169.
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Abstract

A critical step in mosquito reproduction is the ingestion of a blood meal from a vertebrate host. In mosquitoes like *Aedes aegypti*, blood feeding stimulates the release of ovary ecdysteroidogenic hormone (OEH) and insulin-like peptide 3 (ILP3). This induces the ovaries to produce ecdysteroid hormone (ECD), which then drives egg maturation. In many immature insects, prothoracicotropic hormone (PTTH) stimulates the prothoracic glands to produce ECD that directs molting and metamorphosis. The receptors for OEH, ILP3 and PTTH are different receptor tyrosine kinases with OEH and ILP3 signaling converging downstream in the insulin pathway and PTTH activating the mitogen-activated protein kinase pathway. Calcium (Ca^{2+}) flux and cAMP have also been implicated in PTTH signaling, but the role of Ca^{2+} in OEH, ILP3, and cAMP signaling in ovaries is unknown. Here, we assessed whether Ca2+ flux affects OEH, ILP3, and cAMP activity in *A*. *aegypti* ovaries and also asked whether PTTH stimulated ovaries to produce ECD. Results indicated that Ca^{2+} flux enhanced but was not essential for OEH or ILP3 activity, whereas cAMP signaling was dependent on Ca^{2+} flux. Recombinant PTTH from *Bombyx mori* fully activated ECD production by *B*. *mori* PTGs, but exhibited no activity toward *A*. *aegypti* ovaries. Recombinant PTTH from *A*. *aegypti* also failed to stimulate either *B*. *mori* PTGs or *A*. *aegypti* ovaries to produce ECD. We discuss the implications of these results in the context of mosquito reproduction and ECD biosynthesis by insects generally.

3.1. Introduction

For most species, female mosquitoes must consume blood from a human or other vertebrate host to mature a clutch of eggs. This nutritional requirement can also result in the acquisition and transmission of disease-causing pathogens to other hosts during subsequent bouts of blood feeding and oviposition. Blood digestion provides amino acids to the fat body for the production of yolk proteins, which are then packaged into primary oocytes that develop into mature eggs. Studies conducted primarily with *Aedes aegypti* indicate that ecdysteroid hormone (ECD) produced by the ovaries is the primary factor that activates the fat body to synthesize yolk proteins (Attardo, et al., 2005; Baldridge and Feyereisen, 1986; Gulia-Nuss, et al., 2012; Pondeville, et al., 2008; 2013; Roy, et al., 2015). Prior studies further show that ovaries produce ECD in response to insulin-like peptides (ILPs) and ovary ecdysteroidogenic hormone (OEH), which are released from neurosecretory cells in the brain within a few hours after females consume a blood meal (Riehle and Brown, 1999; Riehle and Brown, 2002; Gulia-Nuss, et al., 2011; Dhara, et al., 2013).

A. *aegypti* encodes eight ILPs (Brown, et al., 2008). ILP3 directly stimulates the ovaries to produce ECD by binding to the insulin receptor (IR), which is a receptor tyrosine kinase (RTK) that activates the insulin signaling pathway (Riehle and Brown, 1999; 2002; Brown, et al., 2008; Wen, et al., 2010; Gulia-Nuss, et al., 2011; Dhara, et al., 2013). OEH also directly stimulates ovaries to produce ECD but does so by binding to the OEH receptor (OEHR), which is an RTK that is closely related to the IR (Vogel et al. 2013; 2015). Recent studies further show that OEH binding to the OEHR activates components of the insulin signaling pathway such as Akt (Dhara, et al., 2013; Vogel, et al., 2015).

In immature insects, ECD directs molting and metamorphosis (Smith and Rybczynski, 2012), and the prothoracic glands (PTG) are the primary source in most insects examined to date but not in larval mosquitoes (Jenkins, et al., 1992, Telang, et al., 2007). The neuropeptide prothoracicotropic hormone (PTTH) is the key factor that stimulates PTGs to produce ECD in lepidopteran larvae and presumably other insects (De Loof, et al., 2015; Marchal, et al., 2010; Yamanaka, et al., 2013). This large peptide hormone was shown to interact with Torso, an RTK first identified in *Drosophila melanogaster* (McBrayer, et al., 2007; Rewitz, et al., 2009). Phylogenetic data indicate that Torso is structurally distinct from the IR and OEHR (Vogel, et al., 2013), while genetic studies indicate that Torso signaling transduces through the mitogen-activated protein kinase (MAPK) pathway (Rewitz, et al., 2009).

Although strong evidence supports the function of OEH, ILPs, and PTTH, several lines of study suggest diverse signal pathways are involved in the activation of ECD biosynthesis. In the case of PTGs, experiments with Lepidoptera link PTTH activity to elevated calcium (Ca^{2+}) flux and cAMP levels (Smith, et al., 1985; Gu, et al. 2000; Fellner, et al., 2005), while showing that cAMP analogs stimulate ECD production in the absence of PTTH (Smith, et al., 1984). More recent studies in *D. melanogaster* and the lepidopteran *Bombyx mori* also implicate insulin and target of rapamycin (TOR) pathway signaling in regulating PTG function (Ishizaki and Suzuki, 1994; Mizoguchi and Okamoto, 2013; Columbani, et al., 2005; Walkiewicz and Stern, 2009; Ohhara, et al., 2015; Gu, et al., 2011; 2012; 2015). In the case of mosquito ovaries, an early study of *A. aegypti* reported that a cAMP analog stimulates ECD production (Shapiro, 1983), while more recent data show that TOR signaling enhances ECD production activated by ILP3 or OEH (Gulia-Nuss, et al., 2011; Dhara, et al., 2013). Other results indicate that blood

feeding stimulates expression of the *ptth* gene in mosquitoes (Marinotti, et al., 2005; Zhang and Denlinger, 2011) and a *torso* ortholog in *A*. *aegypti* ovaries (Akbari, et al., 2013).

Overall, these findings suggest activation of ECD production by ovaries and PTGs through ILP, OEH, or PTTH signaling may share more features than generally recognized. A deficiency in the mosquito literature, however, is that the role of Ca^{2+} flux, cAMP, or PTTH in stimulation of ovaries has not been examined in relation to ILP3 and OEH. Here, we address these issues by conducting studies with *A*. *aegypti* ovaries using similar methodology to the PTG literature. Our results show that ECD production by ovaries treated with ILP3 and OEH was enhanced but not dependent on Ca^{2+} flux, while stimulation by a cAMP analog was Ca^{2+} dependent. In contrast, recombinant PTTH from *A*. *aegypti* and *B*. *mori* had no effect on ECD production.

3.2. Materials and Methods

3.2.1. Mosquitoes

The UGAL strain of *A*. *aegypti* was reared as described (Dhara, et al., 2013). Adults were provided water continuously but fed a 5% sucrose solution (weight/volume) on the second day after eclosion. The p20 strain of the silkmoth, *Bombyx mori*, was reared on artificial diet (Coastal Exotics) at 27° C and a 16 h light: 8 h dark photoperiod (Clark and Strand, 2013). Larvae used in experiments were monitored so that timing of the molt to the fifth instar was known. Larvae were then collected on day 6 for use in experiments.

3.2.2. Neuropeptides

A. aegypti ILP3 was synthesized by CPC Scientific, Inc., (90% purity, Sunnyvale, CA) and exhibited bioactivity identical to previous reports (Brown, et al., 2008; Gulia-Nuss, et al., 2015). Recombinant long OEH from *A*. *aegypti* was produced in *Escherichia coli* and purified as

described (Gulia-Nuss, et al., 2012). Recombinant PTTHs from *A*. *aegypti* (AaPTTH) and *B*. *mori* (BmPTTH) were also produced in *E*. *coli*. Total RNA was isolated from *A*. *aegypti* and *B*. *mori* larvae using TRIzol (Life Technologies, U.S.A.) followed by first-strand cDNA synthesis using the Transcriptor High Fidelity cDNA synthesis Kit (Roche). PTTH is expressed as a preproprotein, which is processed at a dibasic (Arg-Lys) cut site to its mature form (Kawakami et al., 1990). Amplicons corresponding to mature *B*. *mori* and *A*. *aegypti* PTTH were PCR amplified using the above cDNA pools as templates and specific primers designed for mature *B*. *mori* PTTH (5'-ATCGTTCAGTTGAGTTATCCAGCATTC-3' and 5'-AATTCGATTCGGAAC AAATCATCA G-3') (Kawakami, et al., 1990) and mature *A*. *aegypti* PTTH derived from an expressed sequence tag (EST) (reverse complement of GenBank: DV370510.1) (5'-ATGAAGTTAGTGTTCATATTAATATGTGCCATC-3' and 5'-CTATATCGAACA TTGGCAAGCGGC-3').

The above products were cloned into pCR2.1 TA (Invitrogen) followed by subcloning into pET30 Ek/LIC (Novagen) using primer sets with Ek/LIC ovarhangs (*B*. *mori*, 5'-GACGACGACAAGATGGGAAACATTCAAGTT-3' and 5'-GAGGAGAAGCCCGGT TTATTATTATTATTATATCGTAGTTGGTAGTC-3': *A*. *aegypti*, 5'-GACGACGACAAGA TGAACGACAAGCATGGCGATCT-5' and 5'-GAGGAGAAGCCCGGTATCGAACAT TGGCAAGCGG-3'). After sequencing to confirm both constructs were correct, plasmids were transformed into *E*. *coli* BL21 (DE3) cells, followed by culture in SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose), supplemented with 10 μg/ml of kanamycin. Bacteria were grown to an optical density of 1.0 at 37°C, induced with isopropyl-β-d-thiogalactopyranoside (IPTG) up to a final concentration of 0.1 mM, and grown as 800 ml cultures for 16 h at 16°C. Bacterial cells were

then harvested by centrifugation at 5000 x *g* for 10 min followed by storage at -20°C. Bacterial pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) on ice for 1 h followed by addition of lysozyme (1 mg/ml) in 50 mM Tris buffer (pH 8.0) and sonication. After centrifugation of the lysate at 10,000 x *g* for 25 min, supernatants were bound to a Ni-NTA matrix (5 Prime) pre-equilibrated with lysis buffer. Bound proteins were washed 5x with wash buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM imidazole, 10 mM Tris-Cl (pH 5.9) followed by elution with three column volumes of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 40-300 mM imidazole). The eluted proteins from wash fractions were loaded onto 10% SDS-PAGE gels and visualized by coomassie blue staining. Proteins eluted using 300 mM imadizole were desalted and concentrated by Centricon 10 (Millipore, USA). Approximately 125 µg of soluble rAaPTTH and rBmPTTH were produced, which had N-terminal cleavable 6xHis- and S-tags, and a C-terminal His-tag.

3.2.3. RT-PCR

Total RNA was isolated from *A*. *aegypti* 48 h old pupae, newly emerged adult females (6 h post eclosion), or 3 day old females at 24 h post blood ingestion using TRIzol reagent (Thermo Fisher). Samples were then quantified using a Nanodrop spectrometer (Thermo Fisher). First-strand cDNA synthesis reactions were performed using 100 ng of total RNA, oligo dT primer, and Superscript III (Invitrogen). Reverse transcription (RT) PCR reactions were then run using a BioRad thermocycler and 25 µl volumes containing 1 µl of cDNA and 2.5 µM of primers specific for *A*. *aegypti ptth* (5'-ATGT CTGCCGGTCCAGTGCT-3' and 5'-CTAAGCTGACTCGCTACTGT AGTAA-3'), *torso* (AAEL002404) (5'-TGAGCACTTTGTACCTTCT-3' and 5'-TCTGT TCCAGTTCCTTGAAT-3'), or *ribosomal S7* (5'-ACCGCCGTCTACGAT-'3 and 5'- ATGGTGGTCTGCTGGTTCTT-3'), which served as a

loading control. Reactions were carried out using AccuPower RT premix and AccuPower HotStart PCR premix (Bioneer) with the following conditions: RT at 50°C for 60 min and denaturation at 95°C for 5 min; HotStart activation cycle for 6 m at 94°C; and 35 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 68°C. Following a final extension step for 5 min at 68°C, products were run on 1% agarose gels and visualized using ethidium bromide.

3.2.4. Salines

Ovary ECD assays were conducted in four saline solutions referred to as standard saline, nominally Ca^{2+} free saline, Ca^{2+} free saline, and high Ca^{2+} saline. Previous studies established that ovaries dissected from *A. aegypti* females sustain ECD production in vitro over multiple hours when cultured in standard saline (1.7 mM $CaCl_2$, 1 mM $MgCl_2$, 1.8 mM $NaHCO_3$, 3.4 mM KCl, 5 mM trehalose, 25 mM HEPES, 150 mM NaCl) (Riehle and Brown, 1999). Nominally Ca^{2+} free saline was standard saline without $CaCl_2$. Ca^{2+} free saline lacked $CaCl_2$ and contained EGTA (2 mM) while high Ca^{2+} saline contained 8.5 mM Ca^{2+}. Other modifications to standard saline included the addition of 10 or 100 nM gadolinium chloride ($GdCl_3$) (Sigma G7532), 2 or 20 µM ionomycin (Sigma 10634), or 10 µM thapsigargin (Calbiochem 586005). Stocks of thapsigargin and ionomycin (2 mM) were prepared in ethanol while $GdCl_3$ (1 mM) was prepared in water. $NaHCO_3$ was replaced in standard saline with additional NaCl to 151.8 mM to avoid formation of insoluble $Gd_2(CO_3)_3$ when $GdCl_3$ was used at a working concentration of 100 nM. Preliminary experiments showed this modification in the absence of $GdCl_3$ had no effect on ovary ECD production. 8-pCPT-cAMP (Sigma C3912) was made as a 10 mM stock in water and added to saline solutions at 100 uM.

3.2.5. Labeled calcium visualization in vitro

A stock of Fluo-3, AM (F-23915, ThermoFisher Scientific) was prepared fresh in 1:1 DMSO (D-12345) and Pluronic F-127 (P-3000MP), according to instructions. Ovaries from 3-5 day-old *A. aegypti* were dissected into Ca^{2+}-free saline (2 mM EGTA) with Fluo-3, AM (15 uM final) and Hoechst stain (H3570; 1:3000 of the 10 mg/ml stock) for 1 h incubation at 27° C. Other ovaries were treated with OEH (10 pmol), ILP3 (20 pmol), or ionomycin (2.5 uM). Thereafter, ovaries were transferred to slides with a pool of Ca^{2+}-free saline (2 mM EGTA), mechanically dispersed, and then an equal volume of saline with 10 mM CaCl was added. After applying cover slips, digital images of ovaries before and within 3 min after the addition of the Ca^{2+} saline were captured using a Leica SP5 microscope (Georgia Electron Microscopy core facility) in confocal mode as illuminated with a 405 nm UV laser and an Argon laser set to 488 nm. Three or more ovaries treated as above with each reagent were processed, and images were captured for 6 or more ovarioles. Capture settings were the same for all images taken simultaneously in two channels: 415-480 nm for the Hoechst stain and 505-550 nm for the Fluo-3. Images were processed in Adobe Photoshop CS5 (v12.1), and adjustments to brightness level and sharpness filters were applied equally to all images.

3.2.6. ECD assays

In vitro ECD production assays with *A. aegypti* ovaries were conducted by dissecting non-blood fed females (3–5 days post-eclosion) in standard saline and removing the paired ovaries attached to the last two abdominal segments. Ovaries from two females were transferred to a small polypropylene cap containing 60 µl of one of the salines described above and incubated in a humidified chamber for 6 h at 27°C. OEH (10 pmol= 330 nM) or ILP3 (20 pmol= 400 nM) was added at the start of an incubation. For the PTTH assays, ovaries were placed in 60

µl standard saline, Sf-900 II medium (Life Technologies), or TC-100 medium (Sigma) followed by addition of AaPTTH or BmPTTH. At the end of the incubation period, medium from each sample was collected and stored at -80°C. In vitro ECD assays with PTGs were conducted by dissecting day 6 fifth instar *B. mori* in phosphate buffered saline, followed by incubation of single glands in 50 µl of TC-100 medium alone or medium plus 100 nM of BmPTTH or AaPTTH for 3 h at 25° C (Yokoyama et al., 1996; Pruijssers et al., 2009). All treatments were setup as triplicate samples of ovaries or PTGs and performed a minimum of three times with different cohorts of insects.

Secreted ECD was measured in some assays using a well-established radioimmunoassay (RIA) (Sieglaff et al., 2005). Briefly, sample medium or ecdysone standards (50 µl; 2 to 2,000 pg) were incubated overnight at 4°C with ECD antiserum (50 µl; 1:21,000 final dilution; L2 rabbit serum provided by J.-Paul Delbecque, Université Bordeaux 1, Talence, France) and [23, 24-^{3}H(N)]-ecdysone (^{3}H-ECD; 50 µl; ~12,000 cpm; PerkinElmer). Antibody-bound and free ^{3}H-ECD in the sample and standard solutions were separated by the ammonium sulfate method. Pelleted bound ^{3}H-ECD was dispersed in scintillation fluid (10% water) and counted using a scintillation counter (Beckman).

To complete the study, however, we had to develop an alternative approach for measuring ECD because commercial production of ^{3}H-ECD was discontinued. We therefore established an enzyme immunoassay (EIA) based on methods originally outlined by Kingan (1989). Plate wells (Costar 3590 96 well plates) were coated with an ecdysone-lactoglobulin conjugate. Sample medium (50 µl) or ecdysone standards (4 – 2000 pg/50 µl) were added to the wells, followed by ECD antiserum (50 µl, 1:30,000 final dilution). After washing, horseradish peroxidase-labeled secondary antibody (Sigma-Aldrich SAB3700949) was added to each well

followed by the substrate 3,3',5,5'-tetramethylbenzidine using the Microwell Peroxidase Substrate System (KPL). Absorbance values were used to calculate percentage absorbance, and ECD content in each sample was calculated from a standard regression line (typically 4 – 250 pg/well). Medium from the *B*. *mori* PTG samples was diluted 1:10 and up to 1:1000 for those incubated with *B*. *mori* PTTH to fall in the linear range of the immunoassays. Sample values were reported as pg of ECD because the L2 antiserum detects ecdysone and 20-hydroxyecdsone equally over the same range (Sieglaff, et al., 2005). While lepidopteran PTGs are known to secrete 3-dehydroecdysone, it is rapidly converted to ecdysone, which is the main form of ECD produced by mosquito ovaries (Hagedorn, et al., 1975; Lafont, et al., 2012). Ecdysone is also known to be converted to 20-hydroxyecdysone by enzymes from tissues or cells associated with lepidopteran PTGs or mosquito ovaries in primary cultures. ECD per sample was determined by RIA or EIA with each sample internally replicated in triplicate. Sample replicates were then averaged to generate ECD values for the data points. Treatment effects were analyzed by ANOVA followed by a post-hoc Tukey-Kramer honest significant difference (HSD) test using Graphpad Prism (5.0). Graphs were also generated using Graphpad Prism followed by labeling in Adobe Illustrator.

3.3. Results

3.3.1. Extracellular Ca^{2+} enhances OEH and ILP3 activity

Each *A*. *aegypti* ovary consists of 60-70 ovarioles, and each ovariole contains a primary follicle comprised of an oocyte and nurse cells plus enveloping follicle cells (Fig. 3.3A). These cells are the site of hormone signaling and ECD biosynthesis (Riehle and Brown, 2002), which requires cholesterol and shuttling of sterol metabolites between the endoplasmic reticulum and biosynthetic enzymes in the mitochondria, as in PTG cells (Brown, et al., 2009). Prior studies

also indicate that ovaries produce ECD in standard saline up to 8 h in vitro when stimulated by ILP3 or OEH (Riehle and Brown, 1999; Brown, et al. 1998; Brown, et al. 2008; Dhara, et al., 2013). We began this study by testing whether extracellular Ca^{2+} promoted ECD production by *A*. *aegypti* ovaries as previously reported for PTGs from *B*. *mori* and *Manduca sexta* (Smith, et al., 1985; Gu, et al., 1998). Assays conducted in nominally Ca^{2+} free (0 mM), standard (1.7 mM Ca^{2+}), or high Ca^{2+} (8.5 mM) saline indicated that Ca^{2+} alone had no effect on ECD production (Fig. 3.1A). Adding ILP3 or OEH to each treatment, however, showed that ECD production dose-dependently increased with Ca^{2+} concentration (Fig. 3.1A). Ovaries produced more ECD when OEH or ILP3 was present in nominally Ca^{2+} free saline than in high Ca^{2+} saline without each hormone (Fig. 3.1A). We also noted that the effects of OEH or ILP3 on ECD production did not differ between nominally Ca^{2+} free saline and Ca^{2+} free saline containing the chelator EGTA (Fig. 3.1B).

3.3.2. Increased Ca^{2+} flux also enhances OEH and ILP3 activity

Pharmacological agents have long been used to study the effects of Ca^{2+} flux on cellular functions, including the response of PTGs to PTTH (Birkenbeil and Dedos, 2002; Fellner, et al., 2005). The literature also indicates that resting cells normally maintain low cytoplasmic Ca^{2+} levels (nM range) in the presence of excess of extracellular Ca^{2+} (mM range), with the endoplasmic reticulum (ER) serving as an intracellular store of Ca^{2+} (μM range) (Berridge, et al., 2000). In contrast, activation of cell surface receptors by neurohormones can result in second messenger signaling that initially promotes Ca^{2+} release from the ER followed by activation signals that promote the influx of extracellular Ca^{2+} via the plasma membrane in a process known as store-operated Ca^{2+} entry (SOCE) (Putney, 2010). We therefore compared the effects of gadolinium chloride ($GdCl_3$), a lanthanide that blocks influx of extracellular Ca^{2+}, to the

ionophore ionomycin, which promotes extracellular Ca^{2+} movement through the plasma membrane (Adding, et al., 2001; Putney, 2010). Each was added to standard saline containing OEH or ILP3. Ten and 100 nM $GdCl_3$ reduced ECD production to levels that were intermediate between treatments containing OEH or ILP3 but no $GdCl_3$ (positive control) and treatments containing no OEH or ILP3 (negative control) (Fig. 3.2A). Reciprocally, 2 μM ionomycin increased ECD production but 20 μM ionomycin reduced ECD production to levels below our positive control (Fig. 3.2B).

We then examined the effects of thapsigargin, which increases intracellular Ca^{2+} by inhibiting sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA) that deplete Ca^{2+} while secondarily activating a SOCE response via Orai plasma membrane Ca^{2+} channels (Stathopulos et al., 2013). For these experiments, we used the same concentration of thapsigargin (10 μM) previously used to enhance ECD production by lepidopteran PTGs in response to PTTH (Gu et al., 1998) in saline with increasing Ca^{2+} concentration. In the absence of OEH and ILP3, thapsigargin in high Ca^{2+} saline increased ECD production to similar levels as OEH or ILP3 in nominally Ca^{2+} free saline (Fig. 3.2C; see Fig. 3.1). In contrast, thapsigargin substantially increased ECD production when OEH or ILP3 was added to standard or high Ca^{2+} saline (Fig. 3.2C).

The above results suggested OEH and ILP3 signaling promoted Ca^{2+} flux into the follicle cells, and to observe this process directly, we treated ovaries with the Ca^{2+} indicator, Fluo-3, AM, to visualize its fluorescent complex with extracellular Ca^{2+}, in response to treatment with OEH, ILP3, or ionomycin in Ca^{2+}-free saline. Within a few minutes after adding Ca^{2+}, a strong fluorescent signal was present in the cytoplasm of some follicle cells in ovaries treated with OEH (Fig. 3.3B) and ILP3 (Fig. 3.3C), but faded within 10 min of observation. As expected, many

such cells were seen in the ovaries treated with ionomycin (Fig. 3.3D). No fluorescent cells were observed in the control ovaries (Fig. 3.3E) or prior to the addition of Ca^{2+} (Fig. 3.3F).

3.3.3. cAMP modestly enhances ECD production in the presence of extracellular Ca^{2+}

cAMP is the second intracellular signaling component implicated in activating ECD production by lepidopteran PTGs (Smith and Rybczynski, 2012). The membrane permeable analogue 8-pCPT-cAMP had no effect on ECD production by *A*. *aegypti* ovaries in nominally Ca^{2+} free saline, but did increase ECD production in standard or high Ca^{2+} saline (Fig. 3.4). However, this stimulatory effect was approximately half the level induced by OEH or ILP3 in high Ca^{2+} saline (see Fig. 3.1).

3.3.4. *A. aegypti* and *B. mori* PTTHs do not stimulate ECD production by ovaries

Comparative genomic and transcriptome data indicate that mosquitoes encode a single *ptth* gene (Predel, et al., 2010), which in *A*. *aegypti* would yield a predicted 217 amino acid preproprotein. This preproprotein contains a tribasic cleavage site as seen for prepro-PTTHs of Lepidoptera like *B*. *mori* to yield a 111 amino acid mature peptide from the C-terminus (Fig. 3.S1). Alignment of predicted mature PTTHs for *A*. *aegypti* (AaPTTH) and *B*. *mori* (BmPTTH) (Kawakami, et al., 1990) showed a similar spacing pattern for the seven cysteines that form predicted intramonomeric bonds (Rybczynski, 2005), but overall identity was only 28% (Fig. 3.5A). RT-PCR assays detected expression of *A*. *aegypti ptth* in pupae, newly emerged adults, and 24 h post-blood meal adult females (Fig. 3.S2A). RT-PCR assays also detected expression of *A*. *aegypti torso,* which consistent with transciptome data for ovaries following blood feeding (Akbari et al., 2013), was seemingly was stronger in 24 h post-blood meal females than in newly emeged adult females or pupae (Fig. 3.S2A).

We therefore asked whether PTTH stimulated ovaries to produce ECD. The approach we took was to produce rAaPTTH in *E. coli* because prior results with *B. mori* and *M. sexta* indicated that rPTTHs produced in *E. coli* had similar activity to purified PTTH or enriched brain extracts containing PTTH (Kataoka, et al., 1991; Shionoya, et al., 2003). Since no studies of a mosquito PTTH had previously been conducted, we also produced rBmPTTH by identical methods. rAaPTTH and rBmPTTH were present in the soluble fraction from lysed *E. coli*. Ni-NTA chromatography followed by elution with imidazole buffer, and SDS-PAGE showed that high imidazole eluted proteins corresponding to the predicted molecular masses of rAaPTTH and rBmPTTH with N- and C-terminal epitope tags (Fig. 3.5B; Fig. 3.S2B). Fractions from high imidazole buffer were then transferred to Centricon filters for buffer exchange into distilled water and concentration. We first conducted in vitro ECD assays with *B. mori* PTGs using a 100 nM dose of each rPTTH. These experiments showed that rBmPTTH strongly stimulated PTGs to produce ECD, whereas rAaPTTH did not (Fig. 3.5C). We then conducted in vitro ECD assays with *A. aegypti* ovaries using a single concentration of ILP3 as a positive control and two concentrations of rAaPTTH and rBmPTTH. ILP3 stimulated ovaries to produce ECD but neither rPTTH did (Fig. 3.5D).

3.4. Discussion

ILP3 and OEH stimulate *A. aegypti* ovaries to produce ECD by binding to different receptors that activate the insulin signaling pathway (Brown, et al., 2008; Wen, et al., 2010; Dhara, et al., 2013; Vogel, et al., 2015). In the first part of this study, we examined the effects of Ca^{2+} flux on ILP3 and OEH activity as previously examined for PTTH and PTGs from lepidopteran larvae (Fellner, et al., 2005; Rybczynski and Gilbert, 2006; Gu, et al., 2010; Smith and Rybczynski, 2012). Our results overall indicate the influx of extracellular Ca^{2+} into follicle

cells enhance OEH and ILP activity as measured by increased production of ECD. However, they also show that ILP3 and OEH stimulate ovaries to produce ECD in saline without Ca^{2+}, which indicates that neither hormone fully depends on extracellular Ca^{2+} for function.

Additional experiments with reagents that selectively alter the flux of extracellular or intracellular Ca^{2+} supported the positive interaction with ILP3 and OEH signaling. That $GdCl_3$ reduces activity but thapsigargin increases activity suggests a role for SOCE in amplifying hormonal signaling, ECD biosynthesis, or both. Interpreting the effects of ionomycin is less clear. Our finding that 2 μM ionomycin enhances ILP3 and OEH activity supports a role for the influx of extracellular Ca^{2+} in ECD production by ovaries. However, the decreased activity of ILP3 and OEH in the presence of 20 μM ionomycin suggests excess free cytoplasmic Ca^{2+} either disrupts signaling or ECD biosynthesis.

In total, the above results indicated OEH, ILP3, and Ca^{2+} signaling are linked and together play a physiological role in the activation and maintenance of ECD production by ovaries. We further showed that the influx of extracellular Ca^{2+}, as indicated its fluorescent complex with Fluo-3, occurs specifically in the follicle cells of ovaries treated with OEH, ILP3, and ionomycin. The number of fluorescent follicle cells was much greater in ovaries treated with ionomycin, which is consistent with its action as an ionophore. Although fewer fluorescent cells were evident in the ovaries treated with OEH and ILP3, this response nevertheless indicates OEH and ILP signaling potentiate Ca^{2+} influx, which in turn enhances their activation of ECD production. Interestingly, the observed ~10 min duration of cellular Ca^{2+} fluorescence in OEH and ILP treated ovaries is consistent with activation of cellular processes that occur up to 1-2 h, such as gene expression in mammalian cells and egg fertilization (Uhlén and Fritz, 2010), which typifies ECD production by mosquito ovaries. No rapid oscillations (< 1 min) in fluorescence

were observed that are indicative of short-term cellular responses in muscle cells (Uhlén and Fritz, 2010) and PTG cells (Fellner, et al., 2005). The positive effects of Ca^{2+} flux through SOCE channels on ILP3 and OEH activity is interesting in light of studies from the insect and mammalian literature, which supports a connection between Ca^{2+} flux, inositol triphosphate signaling, and potentiation of insulin signaling (Worrall and Olefsky, 2002; Fellner, et al., 2005; Lanner, et al., 2008; Krüger, et al., 2008).

Beyond signaling, Ca^{2+} flux may play a role regulating the rate of ECD production rather than its activation, which is consistent with data showing that steroid biosynthesis and release in mammalian adrenal cortical cells is Ca^{2+} dependent (Rossier, 2006). In particular, shuttling of steroid intermediates may be affected by SOCE given that ovaries produced more ECD in the presence of thapsigargin and increased Ca^{2+} concentrations even in the absence of ILP3 or OEH. This could be through Ca^{2+} flux into the mitochondria, which play a primary role in steroid hormone synthesis in both mammalian and insect cells (Cherradi, et al., 1998). Another aspect long suspected is that the release of ECD by PTG cells following synthesis is a Ca^{2+}-dependent event (Birkenbeil, 1983; Hanton, et al., 1993), and recently demonstrated for *D. melanogaster* (Yamanaka, et al., 2015). If this is a conversed mechanism for ECD release by steroidogenic cells in insects, it may explain in part the Ca^{2+}enhancement of ovarian ECD production stimulated by OEH and ILP3 in vitro. However, it fails to account for stimulation of ECD production by ionomycin and thapsigargin forced Ca^{2+} entry, or ECD production in the absence of extracellular Ca^{2+}.

As previously noted, Shapiro (1983) reported that cAMP levels increase in *A. aegypti* ovaries after a blood meal and a cell permeable cAMP analog stimulated ovaries to produce ECD. This work preceded identification of ILP3 and OEH as the neurohormones that stimulate

A. *aegypti* ovaries to produce ECD. Later work showed that PTTH elevates cAMP levels in lepidopteran PTG cells in the presence of extracellular Ca^{2+}, but in the absence of Ca^{2+}, PTTH has no effect on cAMP levels, phosphorylation of MAPK (also called extracellular signal-regulated kinases (ERK)), and ECD biosynthesis (Smith and Rybczynski, 2012). This led to the suggestion PTTH increases Ca^{2+} flux via activation of phospholipase C and phosphokinase C, and may elevate cAMP levels through a Ca^{2+}/calmodulin dependent adenylyl cyclase (summarized in Smith and Rybczynski, 2012). Yet studies also report that a cAMP analog activates ECD production by lepidopteran PTGs in the absence of a Ca^{2+}, which indicates a role for cAMP that is independent of PTTH mediated alterations in Ca^{2+} flux (Smith, et al., 1984; Smith and Rybczynski, 2012).

Our experiments corroborate the findings of Shapiro (1983) by showing that 8-pCPT-cAMP stimulates ovaries to produce ECD in the absence of ILP3 and OEH, but differ from studies of PTGs because this effect only occurred in the presence of extracellular Ca^{2+}. Yet the highest amount of ECD produced by *A*. *aegypti* ovaries in response to 8-pCPT-cAMP was similar to the response elicited ILP3 and OEH in the absence of Ca^{2+}. Thus, our data support a role for cAMP in ECD production by *A*. *aegypti* ovaries, but the ability of ILP3 and OEH to simulate ovaries without extracellular Ca^{2+} indicates ILP3 and OEH activity are not cAMP dependent. We also note parallels between the findings of this study and work conducted in the blowfly, *Phormia regina,* whose ovaries also produce ECD required for egg maturation following consumption of a protein meal (Manière et al., 2000). Like *A*. *aegypti*, *P*. *regina* ovaries exhibit an increase in intracellular cAMP after feeding and produce ECD when incubated in vitro with bovine insulin, but this response is not cAMP dependent (Manière, et al., 2004). These data together with the findings of Shapiro (1983) thus suggest cAMP may function as a

second messenger for another, currently unknown, peptide hormone that stimulates ovaries to produce ECD in dipterans requiring a protein meal for egg maturation.

We considered that PTTH could be a candidate for this other peptide hormone given the above data suggesting lepidopteran PTTH activity depends on Ca^{2+} and cAMP, although how this dependency is related to Torso and MAPK signaling is unclear (Smith and Rybczynski, 2012). Prior studies had also shown that PTTH is transcribed in the heads of *A*. *aegypti* larvae (Telang, et al., 2010), while other results suggest PTTH regulates processes associated with mosquito reproduction given: 1) elevated *ptth* gene expression in *Anopheles gambiae* and *Culex pipiens* following blood feeding (Marinotti, et al., 2005; Zhang and Denlinger, 2011) and 2) elevated expression of *torso* in ovaries of blood fed *A*. *aegypti* (Akbari, et al., 2013). Our RT-PCR assays support these previous results by suggesting transcript abundance of PTTH and Torso is also higher in adult female *A*. *aegypti* following consumption of a blood meal. However, our bioassays detect no increase in ECD production by *A*. *aegypti* ovaries in response to rAaPTTH or rBmPTTH. Given the rBmPTTH we produced strongly stimulated *B*. *mori* PTGs to produce ECD, we conclude it was biologically active in *B*. *mori* and that the lack of activity against *A*. *aegypti* ovaries was not due to improper folding or dimerization. On the other hand, we cannot conclude with certainty the basis for the lack of activity of rAaPTTH. Although produced and isolated identically to *B*. *mori* rPTTH, it is possible improper folding underlies its lack of activity in the bioassays we conduced. Alternatively, the lack of activity of rAaPTTH against *B*. *mori* PTGs could reflect cross-species barriers, given that amino acid identity relative to BmPTTH is only 28% and studies in the literature showing that PTTHs or brain extracts from phylogenetically distant insects often exhibit greatly reduced or no activity in cross-species experiments (summarized by Smith and Rybczynski, 2012). An earlier study using brain

extracts from *M*. *sexta* larvae also showed no activity in stimulating mosquito ovaries to produce ECD (Kelly, et al.,1986). The third option, which our data overall most strongly support, is that PTTH does not stimulate *A*. *aegypti* ovaries to produce ECD.

Other peptide hormones that could potentially play a role in stimulating mosquito ovaries to produce ECD via cAMP as a second messenger are unclear. However, the most likely candidates would be neuropeptides that activate G protein coupled receptors, which often function through Ca^{2+}/cAMP signaling pathways (Vogel, et al., 2013). Many such neuropeptides exist in mosquitoes (Predel, et al., 2010; Vogel, et al., 2013), including orthologs of pigment dispersing factor, which was recently reported to stimulate PTGs from *B*. *mori* to produce ECD (Iga, et al., 2014). Several other peptide hormones in *B*. *mori* have also been suggested to play roles in PTG activation (Marchal, et al., 2010).

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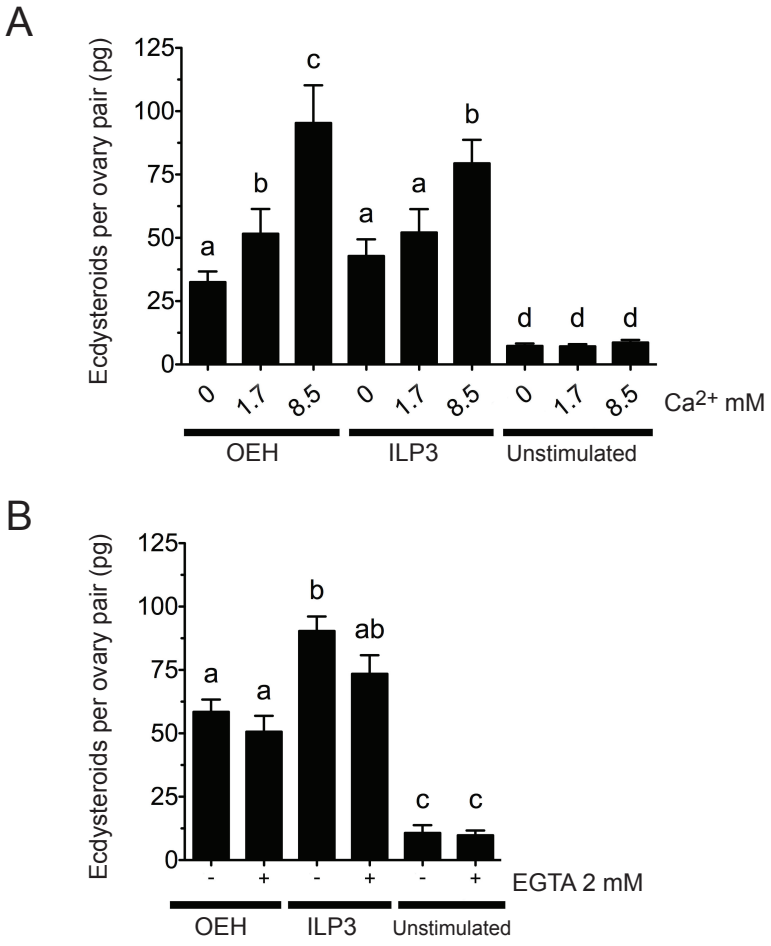
A
Ecdysteroids per ovary pair (pg)
125
100
75
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25
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1.7
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1.7
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1.7
8.5
Ca2+ mM
OEH
ILP3
Unstimulated
B
Ecdysteroids per ovary pair (pg)
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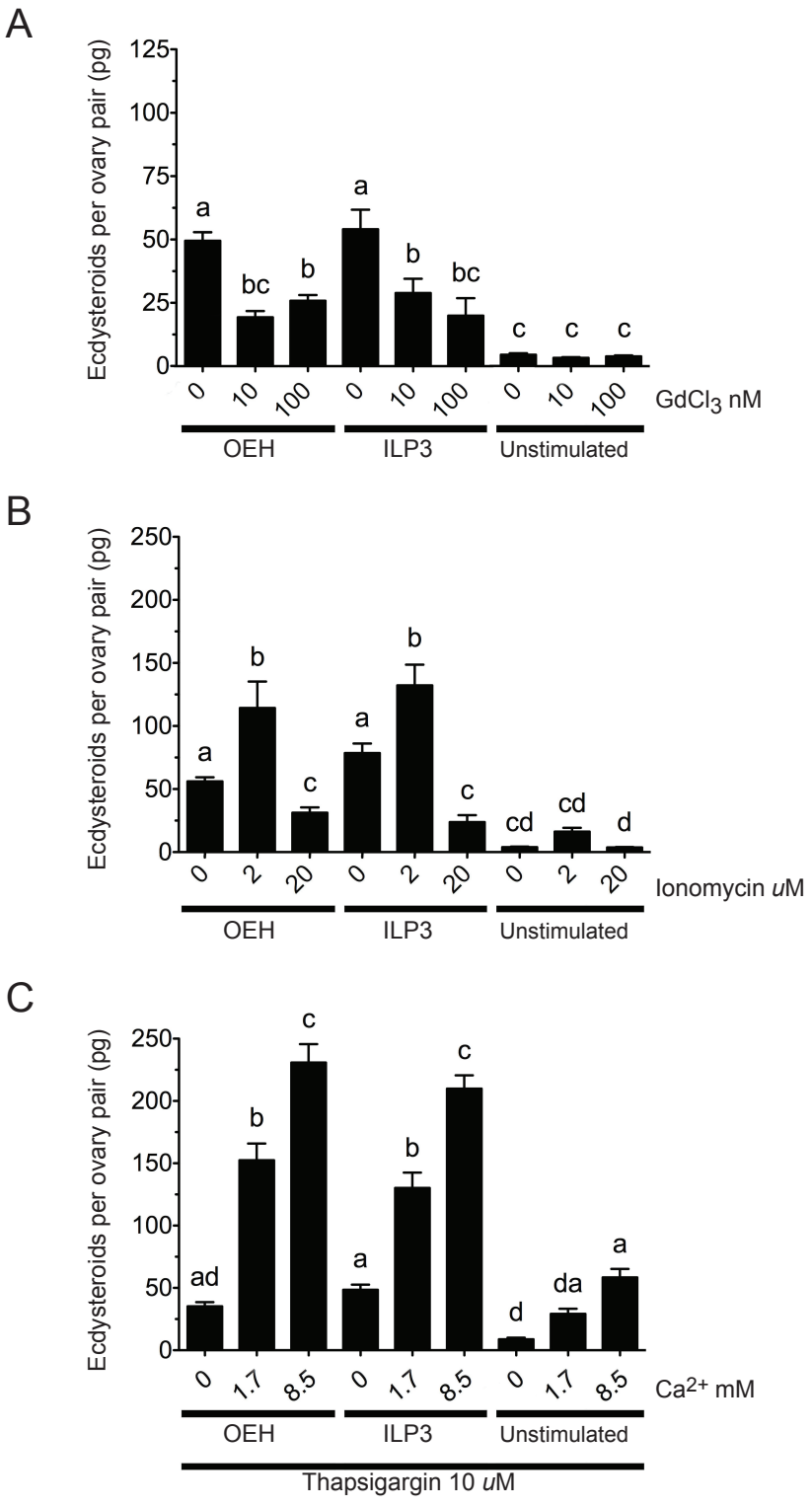
+
-
+
EGTA 2 mM
OEH
ILP3
Unstimulated

Figure 3.1. Extracellular calcium (Ca^{+2}) enhances ecdysteroid hormone (ECD) production by ovaries after treatment with OEH (330 nM) or ILP3 (400 nM). (A) Ovaries were placed into saline containing 0, 1.7 or 8.5 mM Ca^{+2} plus OEH, ILP3, or no hormone (unstimulated) followed by measurement of ECD content in the medium after a 6 h incubation. A one-way ANOVA indicated that treatments overall differed significantly from one another ($F_{8, 230} = 30.95$, $P < 0.0001$). Bars with different letters indicate treatments significantly differed as determined by a post-hoc Tukey-Kramer HSD test ($p \leq 0.05$). (B) Ovaries were placed into standard saline containing 1.7 mM Ca^{+2} with or without 2 mM EGTA followed by addition of OEH, ILP3 or no hormone. ECD in the medium was then determined and statistically analyzed as in (A) ($F_{5, 114} = 40.06$, $P < 0.0001$). Bars in each graph show the mean ± standard error (SE) for each treatment.


A
Ecdysteroids per ovary pair (pg)
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100
GdCl3 nM
OEH
ILP3
Unstimulated
B
Ecdysteroids per ovary pair (pg)
250
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100
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Ionomycin uM
OEH
ILP3
Unstimulated
C
Ecdysteroids per ovary pair (pg)
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Ca2+ mM
OEH
ILP3
Unstimulated
Thapsigargin 10 uM

Figure 3.2. The extracellular Ca^{+2} channel blocker, gadolinium chloride ($GdCl_3$) (A), ionophore ionomycin (B), and SERCA inhibitor thapsigargin (C) differentially affect ECD production by ovaries following treatment with OEH or ILP3. In (A), ovaries were placed into standard saline containing 1.7 mM Ca^{+2} without or with $GdCl_3$ (100 nM) followed by addition of OEH (330 nM), ILP3 (400 nM) or no hormone. ($F_{8, 146} = 21.57$, $P < 0.0001$). In (B), ovaries were placed in standard saline without or with ionomycin (2 and 20 μM) followed by addition of OEH, ILP3 or no hormone ($F_{8, 210} = 41.80$, $P < 0.0001$). In (C), ovaries were placed in saline containing 0-8.5 mM Ca^{+2} plus thapsigargin (10 μM) by addition of OEH, ILP3 or no hormone ($F_{8, 102} = 93.97$, $P < 0.0001$). Determination of ECD in the medium, data presentation, and statistical analyses for each experiment were performed as in Fig. 1.

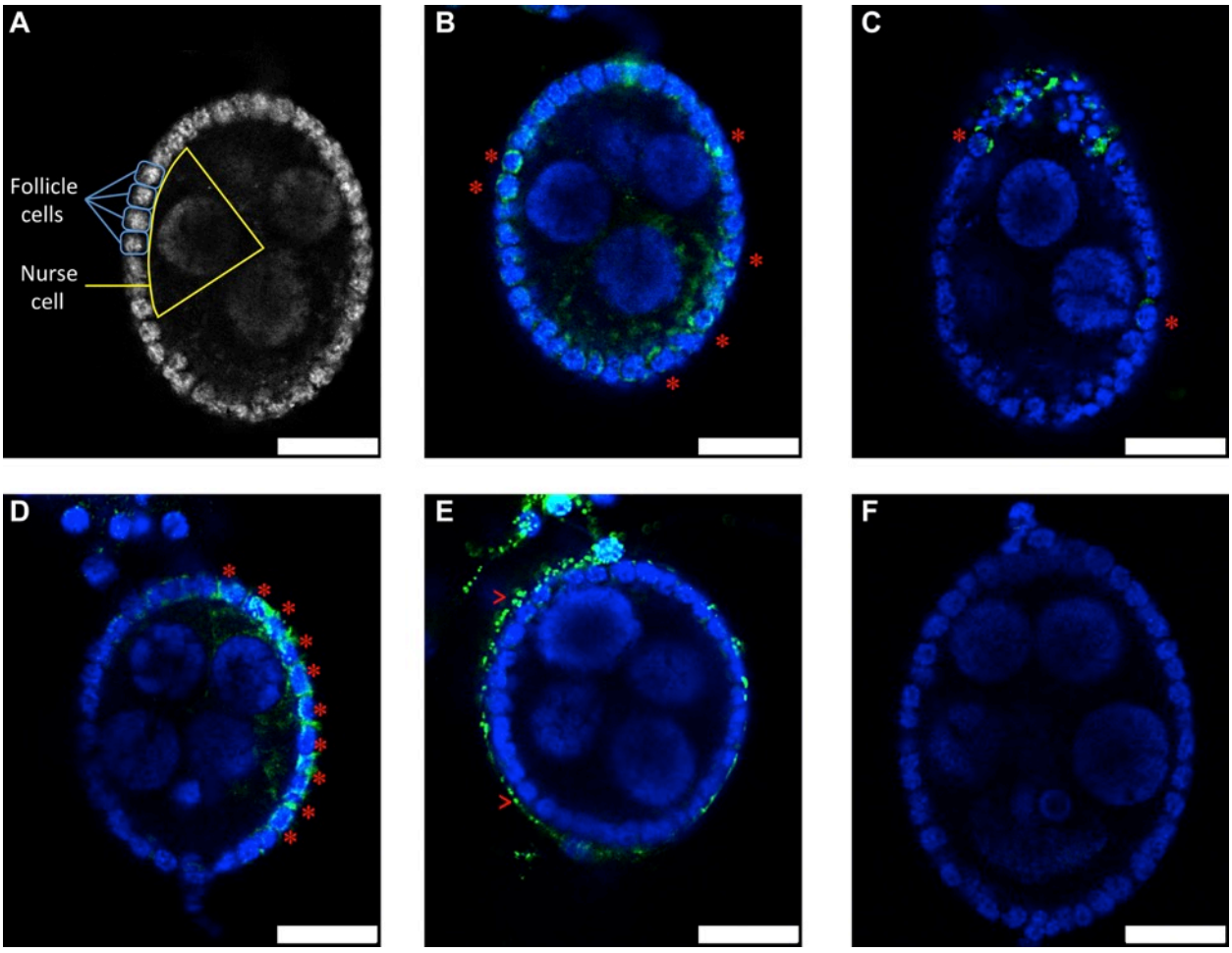


Figure 3.3. Follicle cells exhibit extracellular Ca^{+2}Fluo-3 staining in response to OEH and ILP3 treatment. Representative confocal images of ovarioles post treatment show nuclear staining (blue) and cytoplasmic Fluo-3- Ca^{+2} staining of follicle cells (*) and muscle cells (>). (A) Cellular components of an ovariole. (B-F) Ovaries of non-blood fed *A*. *aegypti* were incubated with Fluo-3, AM alone or with the following reagents in Ca^{+2}-free saline for 1h (F, control pre-Ca^{+2} addition) followed by the addition of Ca^{+2} saline and confocal microscopy: (B) OEH 10 pmol, (C) ILP3 20 pmol, D) ionomycin 2.5 µM, and (E) no reagent control. Scale bar = 25 µm.

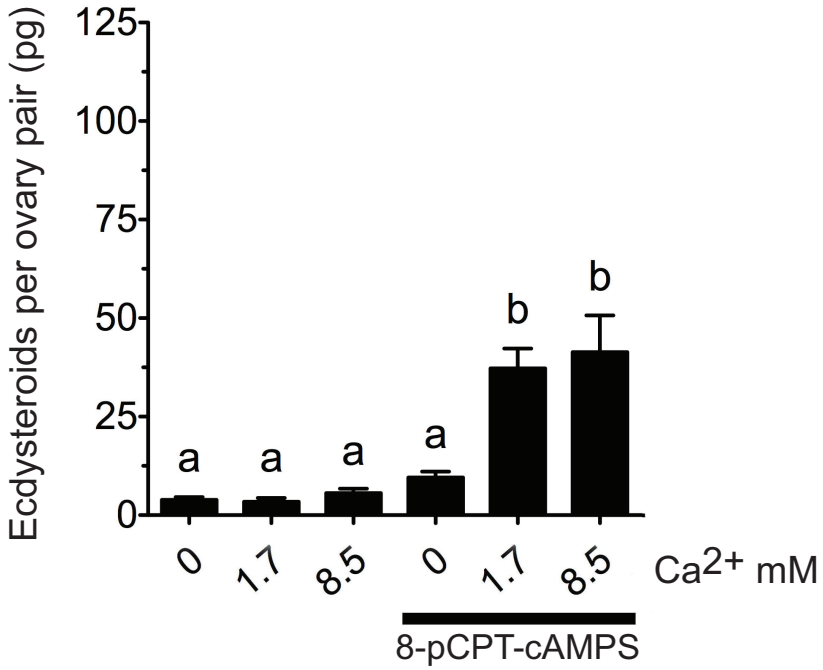
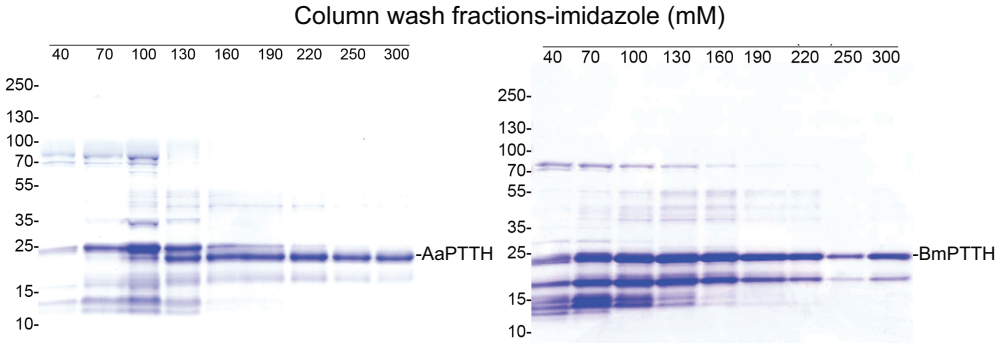


Figure 3.4. cAMP analog 8-pCPT-cAMP stimulates ovaries to produce ECD in the presence of extracellular Ca^{+2}. Ovaries were placed into saline containing 0, 1.7, or 8.5 mM Ca^{+2} without or with 8-pCPT-cAMP (100 uM) followed by determination of ECD in the medium and statistical analysis as described in Fig. 1 ($F_{6, 54}$= 17.6, P < 0.0001). Determination of ECD in the medium, data presentation, and statistical analyses for each experiment were performed as in Fig. 1.

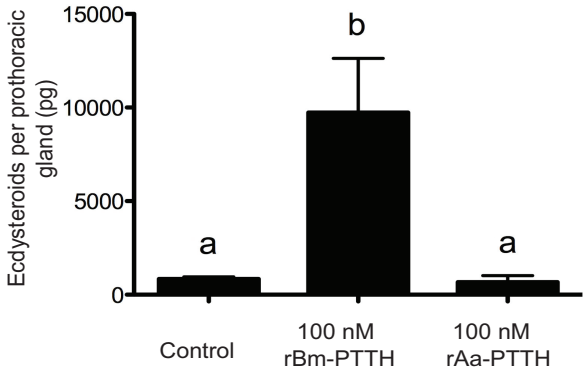
A


AaPTTH NDKHGDLSADLFSQYPSSCACQTKYELLDLGYTHFPRYIVNAICQNRNINSRKCWRGSRCKE
BmPTTH ----GNIQVENQAIPDPPCTCKYKKEIEDLGENSVPRFIETRNCN--KTQQPTCRPPYICKE
AaPTTH IPYKVRVLTQRAEGESIEQDHHSTLLPEPLRDIWRFKTVTVAAACQCSI
BmPTTH SLYSITILKRR-E----TKSQESLEIPNELKYRWVAESHPVSVACLCTRDYQLRYNNNK

B


Column wash fractions-imidazole (mM)
40 70 100 130 160 190 220 250 300
250-
130-
100-
70-
55-
35-
25-
15-
10-
-AaPTTH
-BmPTTH

C


Ecdysteroids per prothoracic gland (pg)
15000
10000
5000
0
a
b
a
Control
100 nM rBm-PTTH
100 nM rAa-PTTH

D

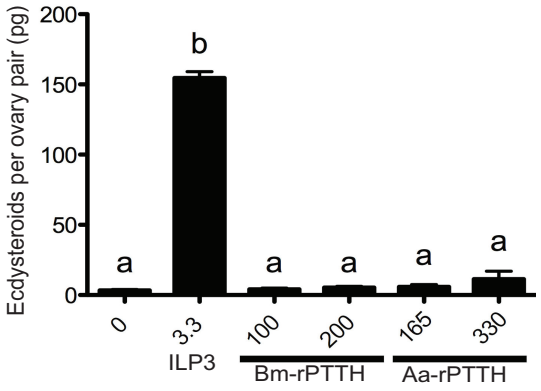

Ecdysteroids per ovary pair (pg)
200
150
100
50
0
a
b
a
a
a
a
0
3.3
ILP3
100
200
Bm-rPTTH
165
330
Aa-rPTTH

Figure 3.5. PTTH has no stimulatory effect on ovaries. (A) Alignment of mature PTTH from *A*. *aegypti* (AaPTTH) and *B*. *mori* (BmPTTH). Identical amino acids including 7 conserved cysteines are shaded in grey. (B) For each PTTH expression construct, the soluble exclusion body of *E. coli* was collected after induction with IPTG and the corresponding recombinant protein purified by Ni-NTA chromatography. The soluble lysate and corresponding eluates after Ni-NTA chromatography and elution using increasing concentrations of imidazole (mM) were subjected to SDS-PAGE under reducing conditions followed by staining with Coomassie Brilliant blue. Each lane shows the elution products at a given imidazole concentration with >220 mM imidazole primarily eluting proteins corresponding to the predicted masses of AaPTTH (left gel) and BmPTTH (right gel). Molecular mass markers are indicated to the left of each gel. (C) ECD production by one *B*. *mori* PTG after a 3 h incubation in TC100 medium alone (control) or with 100 nM of rBmPTTH or rAaPTTH. rBmPTTH stimulated a significance increase in ECD in the medium relative to the control ($F_{2,26}$=14.4; P<0.0001). (D) ECD production by one *A*. *aegypti* ovary pair after a 6 h incubation in Sf-900 medium alone, medium plus 3.3 nM ILP3 or two concentrations of rBmPTTH (100 or 200 nM) or rAaPTTH (165 and 330 nM) ($F_{7, 59}$= 69.2, P < 0.0001). Determination of ECD in the medium, data presentation and statistical analyses in (C) and (D) were performed as in Fig. 1.

A

Aa-prepro-PTTH, pI 5.9, MW 25005

```
   1 ATGAAGTTAGTGTTCATATTAATATGTGCCATCTACTCGCTGACATACCAAGTGCATGGC   60
   1  M  K  L  V  F  I  L  I  C  A  I  Y  S  L  T  Y  Q  V  H  G   20

  61 AAAATAAAATTCGGTTCATCACCTCTGTTCGAGAATGTGTTCCTATCATCTGATTTCGAG  120
  21  K  I  K  F  G  S  S  P  L  F  E  N  V  F  L  S  S  D  F  E   40

 121 CAGAAAAACCTGGCAGGATTAACGGAATCGGAATCTCTCAACTGCTTTCCACCAGCGGAT  180
  41  Q  K  N  L  A  G  L  T  E  S  E  S  L  N  C  F  P  P  A  D   60

 181 CGACACCACAGATATGAATGGGCCGGTGGATTCAACCAAAAGCATATTCTGGACGAACTG  240
  61  R  H  H  R  Y  E  W  A  G  G  F  N  Q  K  H  I  L  D  E  L   80

 241 GATAAAACTAACGAGGAAACGAACGATGAAAGTGATTCAGAAAGTGAGGGGTTCGACGAA  300
  81  D  K  T  N  E  E  T  N  D  E  S  D  S  E  S  E  G  F  D  E  100

 301 AATATTCTGAAGAAACGGAACGACAAGCATGGCGATCTATCGGCGGATTTATTCTCTCAA  360
 101  N  I  L  K  K  R  N  D  K  H  G  D  L  S  A  D  L  F  S  Q  120

 361 TATCCATCGTCTTGTGCTTGCCAAACCAAGTACGAGCTGTTGGACTTGGGATATACACAT  420
 121  Y  P  S  S  C  A  C  Q  T  K  Y  E  L  L  D  L  G  Y  T  H  140

 421 TTTCCTCGCTATATAGTAAACGCCATTTGCCAGAATCGTAACATAAACAGCCGCAAATGC  480
 141  F  P  R  Y  I  V  N  A  I  C  Q  N  R  N  I  N  S  R  K  C  160

 481 TGGCGCGGATCACGCTGCAAAGAGATTCCGTACAAAGTACGCGTTCTGACTCAACGTGCA  540
 161  W  R  G  S  R  C  K  E  I  P  Y  K  V  R  V  L  T  Q  R  A  180

 541 GAAGGAGAATCAATTGAGCAGGACCATCATTCTACGCTCCTTCCGGAACCGTTGCGAGAC  600
 181  E  G  E  S  I  E  Q  D  H  H  S  T  L  L  P  E  P  L  R  D  200

 601 ATATGGCGATTCAAAACTGTCACCATAGCAGCCGCTTGCCAATGTTCGATATAG        654
 201  I  W  R  F  K  T  V  T  I  A  A  A  C  Q  C  S  I  *        217
```

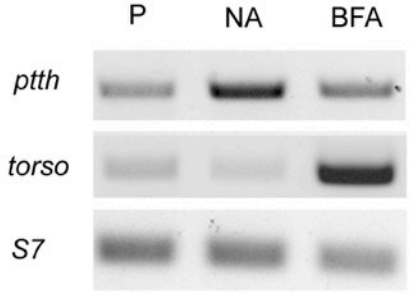
B

Bm-prepro-PTTH, pI 8.6, MW 26,028

```
   1 atgattactcgaccgattatattagtcattttgtgttacgctattcttatgatagtgcag   60
   1  M  I  T  R  P  I  I  L  V  I  L  C  Y  A  I  L  M  I  V  Q   20

  61 tcattcgtgcctaaagcggtagcgctgaaaagaaaaccagacgtgggtggtttttatggta  120
  21  S  F  V  P  K  A  V  A  L  K  R  K  P  D  V  G  G  F  M  V   40

 121 gaagaccaacgcacacataaaagtcacaactacatgatgaaaagagcaagaaatgacgtt  180
  41  E  D  Q  R  T  H  K  S  H  N  Y  M  M  K  R  A  R  N  D  V   60

 181 ttgggagataaagaaaacgtcaggccgaatccttactacacggagccttttgacccagac  240
  61  L  G  D  K  E  N  V  R  P  N  P  Y  Y  T  E  P  F  D  P  D   80

 241 acgagcccagaagaattgtccgctttaatagttgattacgccaatatgattaggaacgat  300
  81  T  S  P  E  E  L  S  A  L  I  V  D  Y  A  N  M  I  R  N  D  100

 301 gttattctgttggataattccgttgaaacgagaactcgaaaaaggggaaacattcaagtt  360
 101  V  I  L  L  D  N  S  V  E  T  R  T  R  K  R  G  N  I  Q  V  120

 361 gaaaaccaagctattccggatccaccttgcacttgcaaatacaagaaagaaatagaagac  420
 121  E  N  Q  A  I  P  D  P  P  C  T  C  K  Y  K  K  E  I  E  D  140

 421 ttgggcgaaaactctgttccacgcttcattgaaaccagaaactgtaataaaacacaacag  480
 141  L  G  E  N  S  V  P  R  F  I  E  T  R  N  C  N  K  T  Q  Q  160

 481 ccgacttgtcgacccccctacatttgcaaagaaagtttatacagtataactattttaaaa  540
 161  P  T  C  R  P  P  Y  I  C  K  E  S  L  Y  S  I  T  I  L  K  180

 541 agaagggaaactaaatcgcaggagtctctcgagataccgaatgaattgaaatatcgatgg  600
 181  R  R  E  T  K  S  Q  E  S  L  E  I  P  N  E  L  K  Y  R  W  200

 601 gtggcggaatctcaccccgtcagcgtggcgtgtttgtgtacaagagactaccaactacga  660
 201  V  A  E  S  H  P  V  S  V  A  C  L  C  T  R  D  Y  Q  L  R  220

 661 tataataataattaa                                               235
 221  Y  N  N  N  *                                                224
```

Figure 3.S1. Nucleotide and amino acid sequences for predicted prepro-PTTH from *A*. *aegypti* (A) and the known sequence for prepro-PTTH for *B*. *mori* (B). Gray highlights the predicted sequence for mature AaPTTH and BmPTTH.

A



B

rAaPTTH

```
   1 ATGCACCATCATCATCATCATTCTTCTGGTCTGGTGCCACGCGGTTCTGGTATGAAAGAA   60
   1  M  H  H  H  H  H  H  S  S  G  L  V  P  R  G  S  G  M  K  E   20
  61 ACCGCTGCTGCTAAATTCGAACGCCAGCACATGGACAGCCCAGATCTGGGTACCGATGAC  120
  21  T  A  A  A  K  F  E  R  Q  H  M  D  S  P  D  L  G  T  D  D   40
 121 GACGACAAGATGAACGACAAGCATGGCGATCTATCGGCGGATTTATTCTCTCAATATCCG  180
  41  D  D  K  M  N  D  K  H  G  D  L  S  A  D  L  F  S  Q  Y  P   60
 181 TCGTCTTGTGCTTGCCAAACAAAGTACGAGCTGTTGGACTTGGGATATACACATTTTCCT  240
  61  S  S  C  A  C  Q  T  K  Y  E  L  L  D  L  G  Y  T  H  F  P   80
 241 CGCTATATAGTGAATGCCATTTGCCAGAATCGTAACATCAATAGCCGTAAATGCTGGCGC  300
  81  R  Y  I  V  N  A  I  C  Q  N  R  N  I  N  S  R  K  C  W  R  100
 301 GGATCACGCTGCAAAGAAATTCCGTACAAAGTACGCGTTCTGACTCAACGTGCAGAAGGA  360
 101  G  S  R  C  K  E  I  P  Y  K  V  R  V  L  T  Q  R  A  E  G  120
 361 GAATCAATTGAGCAGGACCATCATTCTACGCTCCTCCCGGAACCGTTGCGAGACATATGG  420
 121  E  S  I  E  Q  D  H  H  S  T  L  L  P  E  P  L  R  D  I  W  140
 421 CGATTCAAAACCGTCACCGTAGCAGCCGCTTGCCAATGTTCGATACCGGGCTTCTCCTCA  480
 141  R  F  K  T  V  T  V  A  A  A  C  Q  C  S  I  P  G  F  S  S  160
 481 ACCATGGCGATATCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAG  540
 161  T  M  A  I  S  D  P  N  S  S  S  V  D  K  L  A  A  A  L  E  180
 541 CACCACCACCACCACCACTGA                                        561
 181  H  H  H  H  H  H  *                                         186
```

rBmPTTH

```
   1 ATGCACCATCATCATCATCATTCTTCTGGTCTGGTGCCACGCGGTTCTGGTATGAAAGAA   60
   1  M  H  H  H  H  H  H  S  S  G  L  V  P  R  G  S  G  M  K  E   20
  61 ACCGCTGCTGCTAAATTCGAACGCCAGCACATGGACAGCCCAGATCTGGGTACCGATGAC  120
  21  T  A  A  A  K  F  E  R  Q  H  M  D  S  P  D  L  G  T  D  D   40
 121 GACGACAAGATGAACGACAAGCATGGCGATCTATCGGCGGATTTATTCTCTCAATATCCG  180
  41  D  D  K  M  N  D  K  H  G  D  L  S  A  D  L  F  S  Q  Y  P   60
 181 TCGTCTTGTGCTTGCCAAACAAAGTACGAGCTGTTGGACTTGGGATATACACATTTTCCT  240
  61  S  S  C  A  C  Q  T  K  Y  E  L  L  D  L  G  Y  T  H  F  P   80
 241 CGCTATATAGTGAATGCCATTTGCCAGAATCGTAACATCAATAGCCGTAAATGCTGGCGC  300
  81  R  Y  I  V  N  A  I  C  Q  N  R  N  I  N  S  R  K  C  W  R  100
 301 GGATCACGCTGCAAAGAAATTCCGTACAAAGTACGCGTTCTGACTCAACGTGCAGAAGGA  360
 101  G  S  R  C  K  E  I  P  Y  K  V  R  V  L  T  Q  R  A  E  G  120
 361 GAATCAATTGAGCAGGACCATCATTCTACGCTCCTCCCGGAACCGTTGCGAGACATATGG  420
 121  E  S  I  E  Q  D  H  H  S  T  L  L  P  E  P  L  R  D  I  W  140
 421 CGATTCAAAACCGTCACCGTAGCAGCCGCTTGCCAATGTTCGATACCGGGCTTCTCCTCA  480
 141  R  F  K  T  V  T  V  A  A  A  C  Q  C  S  I  P  G  F  S  S  160
 481 ACCATGGCGATATCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAG  540
 161  T  M  A  I  S  D  P  N  S  S  S  V  D  K  L  A  A  A  L  E  180
 541 CACCACCACCACCACCACTGA                                        561
 181  H  H  H  H  H  H  *                                         186
```

Figure 3.S2. (A) RT-PCR of *A*. *aegypti ptth* and *torso* (right) in whole body samples of pupae (P), newly emerged adult females (NA) and 4 day old females blood fed 24 h earlier (BFA). Amplification of *A*. *aegypti* S7 ribosomal protein (*S7*) served as a loading control. The image shown is from one replicate but this assay was repeated three times using independently collected samples, which each time yielded the same qualitative patterns shown. (B) Nucleotide and amino acid sequences for rAaPTTH and rBmPTTH produced in *E*. *coli*. Note that each recombinant PTTH used in bioassays consisted of the mature peptide plus N- and C-terminal His tags.

CHAPTER 4

LOCALIZATION AND DISTRIBUTION OF CHOLESTEROL IN *AEDES AEGYPTI* IN RELATIONSHIP TO ECDYSONE PRODUCTION IN THE ADULT[3]

[3] McKinney, D. A., Strand, M. R., and Brown, M.R. To be submitted to Insect Biochemistry and Molecular Biolology.

Abstract

All insects are cholesterol auxotrophs, meaning that they require cholesterol or a related sterol from a dietary or microbial source. Female mosquitoes use cholesterol to produce the steroid hormone ecdysone (ECD) from their ovaries following a blood meal, which is necessary for vitelligenesis. The distribution of the cholesterol relative to ovarian ecdysteroidogenesis is unknown in the mosquito. We investigated the distribution, quantification, and mobilization in the adult female yellow fever mosquito, *Aedes aegypti*. Free cholesterol is unevenly distributed in the mosquito body, being concentrated in the ovaries, and increases during ECD production. Over the length of a gonadotropic cycle, the distribution of free cholesterol in follicle cells followed the pattern of ECD production. Quantification of cholesterol in the mosquito prior to and after a blood meal showed a dramatic increase in both esterified and free cholesterol. The ex vivo depletion of free cholesterol in ovaries post blood meal (PBM), with methyl-beta-cyclodextrin (MBCD), leads to a reduction in ECD production. Colchicine treament ex vivo of PBM ovaries indicated that intracellular vesicular transport is neccessary for ECD production. Depletion of cholesterol also completely blocked ECD production in ovaries stimulated ex vivo by the neuropeptides ovary ecdysteroidogenic hormone (OEH) and insulin-like peptide 3 (ILP3). However, this block was conditional on depletion prior to activation. Labeled cholesterol revealed that ex vivo neuropeptide activation mobilized cholesterol to specific intracellular locations in both the follicle cells and the nurse cells of the ovary. As a whole, this work demonstrates that cholesterol is stored in the ovaries prior to a blood meal, and that neuropeptide activation is dependent on free cholesterol in the plasma membranes. This establishes for the first time the distribution and movement of cholesterol in steroidogenic mosquito ovaries.

4.1. Introduction

Insects, which are obligatory sterol auxotrophs (Svoboda and Feldlaufer, 1991; Vinci, et al., 2008), utilize sterols for a diverse array of physiological tasks, like the conversion of cholesterol, or its analogues, to the ecdysteroid hormones involved in development and reproduction. In development, the prothoracic gland in immature insects utilizes cholesterol as a substrate for the synthesis of ecdysone (ECD), beginning with the conversion of cholesterol to 7-dehydrocholesterol (7dC) by the Rieske-domain oxygenase designated as neverland in Drosophila melanogaster (Yoshiyama, et al., 2006). A number of other steps accomplished by cytochrome monooxygenases produce ecdysone, which is then released from the gland into the hemolymph. Ecdysone is converted to its active form, 20-hydroxyecdysone (20ECD), by the target tissues (Lafont, et al., 2012). Beyond serving as a substrate for ECD synthesis, cholesterol itself is important for regulating cell membrane fluidity, and is a principal component of lipid rafts in membranes (Simons and Toomre, 2000), which have been demonstrted to modulate plasma membrane channels in insects (Peters, et al., in press). Because of the single hydroxyl group on carbon three, free cholesterol cannot be stored in lipid droplets. Therefore, it is linked to a fatty acid by a cholesterol esterase for storage in lipid droplets. In insect reproduction, the mechanisms involved in the mobilization of cholesterol in ecdysone synthesis, or even its source prior to mobilization, are unknown.

The yellow fever mosquito, *Aedes aegypti*, is an important disease vector, and ECD is crucial to its reproduction. Following a blood meal, neuropeptides are released from the brain that activate ecdysteroidogensis in the mosquito ovaries. The best studied of these neuropeptides are ovary ecdysteroidogenic hormone (OEH) and insulin-like peptide 3 (ILP3), which directly stimulate ECD production (Brown, et al., 1998; Brown, et al., 2009). ECD is synthesized from

cholesterol by the follicle cells of the ovary by a conserved pathway (Sieglaff, et al., 2005), and this ECD then serves to induce vitellogenesis, yolk protein production, in the fatbody (Hagedorn, et al., 1975; Borovsky, et al., 1985).

Despite the long history of this mosquito being used to study ecdysone production by the ovaries, almost nothing is known about the relationship of its ecdysteroidogenesis to cholesterol. Lipophorin serves as a cholesterol transporter in the hemolymph in Lepidoptera, and presumably other insects (Jouni, et al., 2002). One study of *A*. *aegypti* looked at the transporter lipophorin in relationship to oocyte development (Cheon, et al., 2001). Other studies investigated sterol carrier protein 2 (SCP2) in *A*. *aegypti*, implicating it in cholesterol uptake in the larvae with reductions in adult survival when SCP2 was knocked down (Krebs and Lan, 2003; Blitzer, et al., 2005). What is known about cholesterol is that mosquitoes obtain their sterols initially from their diet as larvae, primarily as plant sterols (Merritt, et al., 1992). These sterols are converted to cholesterol and retained into adulthood (Svoboda, et al., 1982). Indirect evidence from ex vivo activation of isolated mosquito ovaries (Graf, et al., 1997), and the capacity of autogenous mosquitoes to complete reproduction without further sterol acquisition (Masler, et al., 1980), suggests that cholesterol is available in sufficient quantities prior to a blood meal for ECD production. The ex vivo experiments also imply that the cholesterol is already contained within the ovaries, making hemolymph transport of cholesterol an unlikely mechanism in initial ecdysteroidogenesis. Obviously a substantial quantity of cholesterol is obtained in a blood meal, and certainly some of this is moved to the oocyte during egg maturation following the blood meal (Troy, et al., 1975). In other insect systems, it has been shown that labeled cholesterol bound to hemolymph lipoproteins is taken up and converted to ECD by the ovaries of the locust, *Locusta migratoria* (Lagueux, et al., 1977).

However, in regard to the relationship of cholesterol to ecdysteroidogenesis, there are questions that remain uninvestigated, including the cholesterol content of the ovaries in mosquitoes before and after a blood meal, the movement of cholesterol within the ovaries, and the relationship between the activation of steroidogenesis and cholesterol transport. Here we establish the cellular localization of free cholesterol in the mosquito ovaries before and after a blood meal, and identify several aspects of its movement in the ovary in relationship to ecdysteroidogenesis.

4.2. Materials and Methods

4.2.1. Reagents

Reagents were purchased from Sigma-Aldrich unless otherwise noted. Incubation saline for ovary incubations was prepared as previously reported (1.7 mM CaCl2, 1 mM MgCl2, 1.8 mM NaHCO3, 3.4 mM KCl, 5 mM trehalose, 25 mM HEPES, 150 mM NaCl) (Riehle and Brown, 1999). Methyl-beta-cyclodextrin (332615), herein MBCD, was prepared as a stock and diluted to 1-5 mM working solution as needed. Working solutions of cholesterol, 7-dehydrocholesterol (7dC), and TopFluor cholesterol (23-(dipyrromethene boron difluoride)-24-norcholesterol: Avantes, 810255P) were made fresh immediately prior to use, and protected from light. Phosphate-buffered saline (PBS) was prepared as a 10x stock and diluted to 1x for the experiments conducted here (NaCl 137 mM, KCl 2.7 mM, Na2HPO4 10 mM, KH2PO4 1.8 mM). PBS-T was made by adding 0.05% Tween-20 to PBS.

4.2.2. Mosquitoes and ex vivo incubations

The UGAL strain of *A. aegypti* was reared as described. Adults were provided water continuously but fed a 5% sucrose solution only for the second day after eclosion. For incubations, two ovary pairs cleaned of extraneous tissues were dissected from females (3–5

days post-eclosion) not blood fed (NBF) or 18-24 hours post blood meal (PBM) and transferred to a small polypropylene cap with saline and incubated in a humidified chamber for 6 h at 27°C. Afterwards, saline and ovaries were collected from each sample and stored in sets of five at -80°C for further analysis. For colchicine experiments, ovaries were incubated with or without colchicine (0.5-50 mM) in SF900 III insect cell medium (ThermoFisher 12658019) for 6 hrs, and then transferred to fresh medium supplemented with 7dC (0.25 mM) complexed with MBCD, free of colchicine, and incubated for another 6 hours.

Saline or medium from ex vivo ovary incubations was assayed directly in radioimmunoassay (RIA). For enzyme-linked immunoassay (EIA), NBF ovaries incubations were assayed directly, but PBM ovary were partitioned against organic solvent. Briefly, the incubation saline was brought to 250 ul with ultrapure water and washed 1:1 with chloroform, and the aqueous phase was retained, with trace chloroform removed by reducing volume to about 100 ul in a vacuum concentrator at RT.

4.2.3. Ecdysone and cholesterol quantification

For ECD quantification, either an established RIA (Sieglaff, et al., 2005) or EIA (McKinney, et al., in review) were used to analyze samples. For the RIA, samples or ecdysone standards (50 µl; 2 to 2,000 pg) were incubated overnight at 4°C with ECD antiserum (50 µl; 1:21,000 final dilution; L2 rabbit serum provided by J.-Paul Delbecque, Université Bordeaux 1, Talence, France) and [23, 24-3H(N)]-ecdysone (3H-ECD; 50 µl; ~12,000 cpm; PerkinElmer). Antibody-bound and free 3H-ECD in the sample and standard solutions were separated by the ammonium sulfate method. Pelleted-bound 3H-ECD was dispersed in scintillation fluid (10% water) and counted using a scintillation counter (Beckman). For the EIA, wells (Costar 3590 96-well plates) were coated with ecdysone-bovine beta lactoglobulin conjugate. Samples, ecdysone

standards, and quality controls (50 µl, same range as above) were added to the wells, followed by ECD antibody EAB27 (50 µl, 1:30,000 final dilution) for overnight incubation at 4°C. After washing, horseradish peroxidase-labeled secondary antibody (Sigma SAB3700949) and then 3,3',5,5'-tetramethylbenzidine substrate (TMB Microwell Peroxidase Substrate System, KPL 507600) were added to the wells. Absorbance values were used to calculate percentage absorbance and ECD content in the samples from the standard regression line. Sample values are reported as "ecdysteroid pg," because the antibody used detects ecdysone and 20-hydroxyecdysone equally.

Cholesterol was quantified using a commercially available Amplex red cholesterol assay (Invitrogen A12216) following the protocol of Tennessen, et al. (2014). Briefly, females (2 NBF, or 1 24 hrs PBM) or 5 ovary pairs dissected free from other tissues were macerated with a plastic pestle in a 1.5 ml microfuge tube in 250 µl cold PBS-T on ice. A further 650 ul of cold PBS-T was added, and homogenates were kept on wet ice and sonicated with a tip sonicator on medium power for three 30-second pulses. Each sample was divided between two tubes, and cholesterol esterase added to one tube. Samples were incubated at 37°C overnight with inversion. Following incubation, saline was partitioned against 900 µl chloroform:methanol (2:1), and the organic layer transferred to a new tube and dried in a vacuum concentrator (ThermoFisher) at room temperature. Samples were rehydrated with 100 µl of PBS-T and sonicated briefly to resuspend extracted lipids. Samples were promptly quantified in Amplex red assay in parallel with standards.

4.2.4. Filipin staining and image capture

Filipin staining kit (Cayman, 10009867) was prepared according to manufacturer directions, and mosquito tissues were dissected from fresh adults or taken after incubation ex

vivo, fixed, stained, and rinsed according to the kit's instructions. Tissues were mounted in glycerol, and examined under minimal fluorescence illumination to maximize the half-life of the filipin. Filipin was visualized with a DAPI filter cube (ex 360 nm, em 358 nm) on an epiflorescent Olympus microscope.

4.2.5. Cholesterol manipulation with methyl-beta-cyclodextrin (MBCD)

MBCD has been established as a nondestructive molecular means of rapidly removing cholesterol from the plasma membranes of cells when incubated with cells alone, or exchanging sterols in membrane with supplemented sterols when MBCD is incubated with sterols (Ohtani, et al., 1989). MBCD was used ex vivo. MBCD was utilized primarily at 1 mM in 50 to 70 µl volumes to to manipulate sterols in ovaries ex vivo. For neuropeptide activation experiments, MBCD was added at a final concentration of 1 mM to ovary incubations just prior to activation, and at 30 or 60 minutes following the addition of OEH or ILP3 at 10 pmol. Sterols were added to mosquito tissues using a molar ratio of sterol to MBCD of 1:5 for cholesterol, and 1:10 for 7dC. Smaller ratios resulted in variable precipitation of the relevant sterols depending on temperature. Addition of TopFluor cholesterol was accomplished using a 1:5 molar ratio of MBCD to TopFluor cholesterol at a final concentration of 0.25 mM TopFluor cholesterol. Ovaries were incubated, protected from light, until they were transferred to glycerol and visualized with the same microscope as before with GFP filters (ex 488 nm, em 510 nm). Additionally, similarly processed activated ovaries were incubated with Hoechst stain (ThermoFisher 33342; 1:3000 of the 10 mg/ml stock) for 1 hr prior to being imaged with a Zeiss LSM 510 META Confocal Microscope (UGA Biomedical Microscopy Core) using two channels (505-530 nm, 420-480 nm) with Argon laser illumination (488 nm) for TF-cholesterol and UV Diode illumination (405 nm)

for Hoechst stain. Images were uniformly adjusted for brightness and contrast, followed by final figure assembly using Adobe Photoshop Cs5 (v12.1).

4.2.6. Data analysis

Data from the experiments were analyzed by ANOVA, and the means separated by the Tukey-Kramer HSD test (Prism 5.0f, GraphPad Software Inc.).

4.3. Results

4.3.1. Cholesterol localization

We first asked what is the tissue distribution of cholesterol in females, and how this distribution changes over a gonadotropic cycle. Free cholesterol in females following a blood meal through a full gonadotropic cycle was visualized via filipin staining. Ovaries are a prominent repository of free cholesterol in mosquitoes prior to a blood meal (Fig. 1A). Other tissues with strong staining include the nerve cord, the alimentary canal (not shown), and groups of cells along the ventral line of the abdomen. While these cells were not directly identified, their staining, location, and arrangement is consistent with oenocytes, being located in the parietal fatbody in adult mosquitoes (Martins, et al., 2011; Martins and Ramalho-Ortigão, 2012). Within the ovaries, much of the filipin staining is in the follicular epithelium (Fig. 1A red arrows). At 12 hrs PBM, free cholesterol increased in follicles, both in follicle cells (red arrows) and in the germarium, and accumulated in droplets in the nurse cells (Fig. 1B, 12 hrs PBM). By 24 hrs PBM, the secondary follicle had separated from the germarium, and increased in size with a greater accumulation of cholesterol (Fig. 1B, 24 hrs PBM). Interestingly, a subset of follicle cells directly above the nurse cells was more stained than the rest of the follicle cells (demarcation line at green arrow) which may reflect the transition of follicle cells over the oocyte from ECD production to choriogenesis. At 48 hrs PBM oocytes were filled with yolk, and the follicular cell

layer was not stained (not shown). Following oviposition of the mature eggs, the new follicles returned to a relatively uniform staining of follicle cells and germarium (Fig. 1B, 72 and 96 hrs PBM). Granules of free cholesterol in the nurse cells were visible again during these time points.

4.3.2. Cholesterol quantification

Given the obvious presence of cholesterol in the ovaries, we next asked how the actual quantity of cholesterol changes in females before and after a blood meal. Using a standard Amplex red assay, free and esterified cholesterol in whole body and ovaries of females NBF or PBM were quantified. Non-blood fed females had a mean of 320 ng of free cholesterol per mosquito, with the ovaries possessing 17 ng, or about 5% of the total free cholesterol (Fig. 2 A-B). Surprisingly, little esterified cholesterol was detected in the NBF females, with 62% of the esterified cholesterol being found in the ovaries alone. Based on normal blood-cholesterol levels in rats used to blood feed our females (Wolford, et al., 1987), a mosquito fed to repletion (about 4 ul) was predicted to have an increase in cholesterol ranging from 1500 ng to 3500 ng, with an ideal mean of 2500 ng, assuming no metabolic loss. With the cholesterol assay whole body females at 24 hrs PBM contained an average of 2714 ng total cholesterol per female, an increase of 2365 ng, or 7.8-fold (Fig. 2 C). Most of the additional cholesterol in the whole mosquito is stored in esterified form, and the increase in free cholesterol is only 2.7-fold. Cholesterol levels in ovaries alone from females 24 hrs PBM, during peak ECD production, also increased, but disproportionally to the whole animal. In particular, free cholesterol in ovaries increased 8-fold.

4.3.3. Depletion of free cholesterol inhibits ecdysteroidogenesis

Having established a baseline for free and esterified cholesterol in ovaries of NBF and PBM females, we predicted that cholesterol must be mobilized within the ovaries for ECD production, and asked if the depletion of free cholesterol inhibited ECD production. First, we

established that cholesterol was greatly reduced in ovaries incubated with MBCD as visualized with filipin (Fig. 3A). This shows that MBCD acted as expected, as well as demonstrating that filipin staining is sterol-specific in the ovary. In the presence of MBCD, ECD production in ovaries from females 24 hrs PBM was partially inhibited (Fig. 3B).

4.3.4. Ovary ecdysteroidogensis requires microtubule-dependent shuttling

Given that cholesterol is mobilized for ECD production, we anticipated that this mobilization required vesicular transport of cholesterol, to the site of ecdysteroidogenesis, apart from a simple homeostatic response to cholesterol depletion via ECD production. Colchicine, a microtubule depolymerizing agent, was used to investigate the role of vesicular transport in ecdysteroidogenesis. This pharmacological agent has been used in vertebrate steroid synthesis studies (Sewer and Li, 2008), and does not affect homeostatic cholesterol movement (Liscum and Underwood, 1995). At higher concentrations, colchicine rapidly inhibited ECD production by ovaries taken from females at 18 hrs PBM (Fig. 4A). Ovaries from these treatments were transferred to colchicine-free incubation medium supplemented with 7dC, solubilized by MBCD at a 1:10 molar ratio, to ensure a sufficient supply of sterol, and incubated for a further 6 hrs. 7dC carried by MBCD could not rescue ecdysteroidogenesis in ovaries previously treated with colchicine at the highest concentration (Fig. 4B), which suggests vesicular transport is a necessary component of ECD production.

4.3.5. Neuropeptide activation dependent on plasma membrane cholesterol

While depletion of free cholesterol interfered with ECD production by ovaries activated by a blood meal, the mechanism of interference was uncertain. We addressed two related questions. First, does neuropeptide stimulation of mosquito ovaries ex vivo result in the

mobilization of free cholesterol from ovary cholesterol stores? Second, does depletion of free cholesterol inhibit the production of ex vivo ecdysteroidogenesis?

To address the first question, the mobilization of esterified cholesterol to free cholesterol was investigated with the Amplex red cholesterol assay. Using ovaries from NBF females, ECD production was activated ex vivo by incubation in saline with the neuropeptide activators OEH and ILP3. Increased free cholesterol via mobilization is supported by an increase in free cholesterol following activation of ovaries by either peptide ex vivo from non-blood fed mosquitoes (Fig. 5A).

The second question was addressed by using MBCD to deplete ovaries of free cholesterol, and then stimulating the ovaries with OEH and ILP3. The initial result was a complete block for ECD production (data not shown). While this confirmed the importance of free cholesterol in ecdysone production, there remained the question of whether this ex vivo inhibition was direct, due to the lack of free cholesterol as a substrate for hormone production or indirectly due to cholesterol withdrawal from the ovary membranes. MBCD depletion of cholesterol from plasma membranes can disrupt cholesterol-enriched lipid rafts, which in turn can inactivate receptors embedded in rafts (Zidovetzki and Levitan, 2007). Cholesterol depletion as a disruptor of neuropeptide receptors was investigated by varying the timing of cholesterol depletion before and after ovary stimulation ex vivo at 0, 30, and 60 mins following the addition of OEH or ILP3. The concentration of MBCD was reduced to avoid overestimation in ecdysone quantification in the EIA (Supplement 2). The results show that MBCD added in concert with neuropeptides results in a dramatic reduction in ECD activation, but if added after activation, there is no negative impact of MBCD on ecdysteroidogenesis for either neuropeptide (Fig. 5B-

C). This suggests that once activated, cholesterol mobilization for ecdysteroidogenesis is not dependent on further neuropeptide signaling.

4.3.6. Sterol supplementation with MBCD

Having looked at cholesterol depletion, we next asked if sterol supplementation might enhance neuropeptide-stimulated ECD production. MBCD serves as a sterol sink when used alone, but can also serve as an exchanger when mixed proportionally with a sterol. MBCD was preloaded with either cholesterol (Chol) or 7-dehydrocholesterol (7dC) at 1:5 or 1:10 molar ratios respectively and added to incubations from NBF females' ovaries with or without OEH or ILP3. Supplementation with cholesterol had little effect on peptide stimulation of ovaries, with a slight drop in activation likely attributable to the MBCD not being saturated with cholesterol (Fig. 6A). 7dC was chosen as a secondary sterol because it is the first conversion step in ecdysteroidogenesis. The addition of 7dC to ovaries via MBCD resulted in a substantial increase in ECD production in all treatments (Fig. 6B). The previous experiments with 7dC (section 3.4) indicated that 7dC supplied to 18 hr PBM ovaries ex vivo did not rescued them from colchicine inhibition. This means that the effect of 7dC is likely not due to directly entering the ecdysteroidogenic pathway, but rather by stimulation of ECD production.

4.3.7. Movement of TopFluor cholesterol following activation of ovaries ex vivo

With cholesterol supplementation via MBCD not affecting ecdysteroidogenesis by ovaries activated ex vivo, it became experimentally viable to supplement ovaries with fluorescently labeled cholesterol and ask how the mobilization of cholesterol changes following ovary activation by neuropeptides. Given that there was no difference in cholesterol mobilization between ILP3 and OEH, ILP3 was chosen as a representative neuropeptide. TopFluor cholesterol (TF-cholesterol) is the only labeled cholesterol known to function similarly to cholesterol in

terms of membrane dynamics and shuttling (Hölttä-Vuori, et al., 2008; Maxfield and Wüstner, 2012). Like cholesterol-loaded MBCD, TF-cholesterol-loaded MBCD did not greatly perturb ovary ecdysteroidogenesis (Supplement 3). Ovaries from NBF females were incubated in the presence of MBCD loaded with TF-cholesterol (0.25 mM) with or without ILP3 for 6 hrs and visualized. Unstimulated ovaries showed a distribution of TF-cholesterol similar to what was observed with filipin staining (Fig. 7 A), while ILP3-activated ovaries showed a condensation of TF-cholesterol into puncta within the follicle cells and the nurse cells (Fig. 7B-C, arrows). These punctate patterns are correlated to the formation of metabolic centers where the mitochondria and endoplasmic reticulum associate closely, as is seen in mammalian steroidogensis (Issop, et al., 2015).

4.4. Discussion

Cholesterol is necessary for life and development in *A*. *aegypti*, and here we examined it in the context of female mosquitoes. Free cholesterol was distributed throughout the body, with proportionally greater amounts in the ovaries and nervous system than in the fatbody or abdominal body wall. During a gonadotropic cycle, free cholesterol became concentrated primarily in the follicle cells and the germarium, with somewhat lower accumulation in the nurse cells. At the ECD peak, 24 hrs PBM, the germarium and the developing secondary follicle had proportionally more free cholesterol than the rest of the ovary. At 48 hrs, the follicle cells have ceased ecdysteroid production, deposited the chorion, and undergone cell death (Clements, 1992). After oviposition, the distribution of free cholesterol returned to NBF conditions as the new follicles mature. The measurement of free cholesterol in the NBF ovaries, and in all instances sampled, was remarkably consistent between mosquitoes within a given treatment, which seems to suggest that there is an optimal membrane cholesterol concentration. Additional

cholesterol acquired in the blood meal is shunted to the ovaries PBM based on the mean total cholesterol in the PMB ovaries exceeded mean whole body cholesterol in NBF mosquitoes. Part of the change in free cholesterol in the ovaries is related to steroid hormone synthesis. Taken together, the cholesterol quantification supports the cholesterol staining observations.

Depletion of ovary cholesterol with MBCD supported the presence and importance of cholesterol in the plasma membranes of follicle cells for ecdysteroidogensis. This suggests that free cholesterol in mosquito ovaries stimulated by a blood meal may play a direct role in ecdysteroidogenesis, as seen in mammals in acute steroidogenesis in Leydig cells (Venugopal, et al., 2016). However, given that the nurse cells and oocytes sequester cholesterol following activation, as suggested by the TF-cholesterol experiments (cf Fig. 7 A vs B), it is reasonable to expect a more mature follicle to serve as a sink for cholesterol, with MBCD serving as an impromptu transporter. Inhibition and recovery of vesicular transport using colchicine demonstrates the importance of vesicular transport for ecdysone production by the ovary. This is consistent with other examples of steroid hormone production, where intracellular vesicular shuttling of the hormone precursors to the site of synthesis is necessary (Issop, et al., 2013).

Neuropeptide stimulation of ecdysone production revealed that mobilization of free cholesterol is a downstream effect of OEH and ILP3. Depletion of free cholesterol prior to neuropeptide stimulation blocked ECD production, but depletion after stimulation did not effect it. This suggests the importance of cholesterol in plasma membranes for receptor activation. However, it may also be that cholesterol in plasma membranes has already been internalized by the 30 min time point as in the mouse Leydig cell model (Venugopal, et al., 2016), though this work bypassed receptor activation with a membrane permeable cyclic adenosine monophosphate (cAMP) analog. However, the saline used in the ovary incubations contains no additional

cholesterol, and it seems unlikely that the follicle cells would sequester all the needed cholesterol in 30 mins. Additionally, OEH activation was consistently blocked by a 30 min post activation addition of MBCD (data not shown), which favors receptor activation over transport when considering signal transduction is typically a fast cellular event. While receptor inhibition is favored by the data, it is possible that both mechanism are occuring. The data supports a conserved regulatory aspect of receptor tyrosine kinases (RTKs), which have been shown to be sensitive to lipid raft composition in vertebrates (Huo, et al., 2003; Romanelli, et al., 2009; Fukui, et al., 2015). Both of the known mosquito ovary receptors are RTKs, with OEH binding a venus fly-trap receptor and ILP3 a insulin receptor (Graf, et al., 1997; Vogel, et al., 2015). The complete block of ECD production by depletion of cholesterol is the first experimental evidence, in that insect RTKs share this aspect of regulation. The supplementation of additional cholesterol to the plasma membranes of ovary cells did little to alter ECD production under any condition, which means that either the cholesterol utilized for initial ECD production is drawn from intracellular stores, or the rate of uptake of free cholesterol from this source is not concentration-dependent. That the addition of 7dC to ovary cells induced high levels of ECD production is rather surprising. This effect may have been due to the entrance of 7dC into the steroidogenesis pathway. Alternatively, the accumulation of 7dC in lipid rafts has been previously shown to induce a rapid and substantial increase in the sensitivity of AKT to phosphorylation (Kovarova, et al., 2006). The latter seems more likely, given that AKT phosphorylation is a central part of the signaling cascade involved in the activation of ecdysteroidogenesis in mosquito ovaries by OEH and ILP3 (Wen, et al., 2010; Vogel, et al., 2015). Furthermore, experiments with colchicine showed that 7dC supplementation did not rescue fully impaired shuttling in ovaries PBM. However, some caution is necessary when interpreting these results, as work in *D. melanogaster*

has demonstrated that ecdysteroid secretion in larval prothoracic glands is tied to exocytotic steroid vesicles, which are presumably dependent on microtubules for transport to the plasma membrane (Yamanaka, et al., 2015). Regardless of this caveat, the results suggest that homeostatic mechanisms do not connect 7dC to the necessary steroidogenic machinery in ovaries from unstimulated females implying that activation is neccessary.

Distribution of TF-cholesterol in unstimulated ovaries mimicked the pattern of filipin-stained cholesterol, implying that the ovary cells responded to it as though it were unlabeled cholesterol. Neuropeptide-stimulated ovaries treated with TF-cholesterol revealed a dramatic change in cholesterol distribution. This was especially evident in changes in the follicle cells that formed bright punctate dots that are presumably areas of high-level cholesterol metabolism. Similar morphological changes occurred in the nurse cells. Metabolically, it is known that the oocyte must be provisioned with cholesterol and the ecdysteroids made from it. In locusts and cockroaches, the latter is accomplished by the follicle cells (Goltzene, et al., 1978; Zhu, et al., 1983), which seems to be true for mosquitoes as well. The fluorescently labeled lipid droplets seen in the oocytes suggest that the TF-cholesterol can be esterified, and arguably links the nurse cells' metabolic activity to at least cholesterol esterification. This suggests that, at least early in mosquito ovary activation, the nurse cells process the cholesterol before it is transported to the developing oocyte for storage. This is further supported by the nurse cells not staining with filipin, indicating that the cholesterol metabolism in the nurse cells is related to esterified cholesterol and not free cholesterol. Beyond this, it is not impossible that the nurse cells may also play a role in ecdysteroidogenesis by providing cholesterol for ECD production.

Taken as a whole, the work here provides a solid platform for further probing questions about cholesterol transport as it relates to ECD production. The distribution of cholesterol is

deliberately biased toward the role of the ovaries as a steroidogenic tissue, and its importance is far beyond simply serving as a precursor for steroidogenesis, but also serves in receptor stabilization and presumably isolation of the oocyte from the milieu of the hemolymph. Cholesterol is mobilized in concert with the induction of ecdysteroidogenesis, and the alteration of free cholesterol in plasma membranes does not alter ECD production rate, implying that steroid hormone production in mosquito ovaries is dependent on internal stores of cholesterol.

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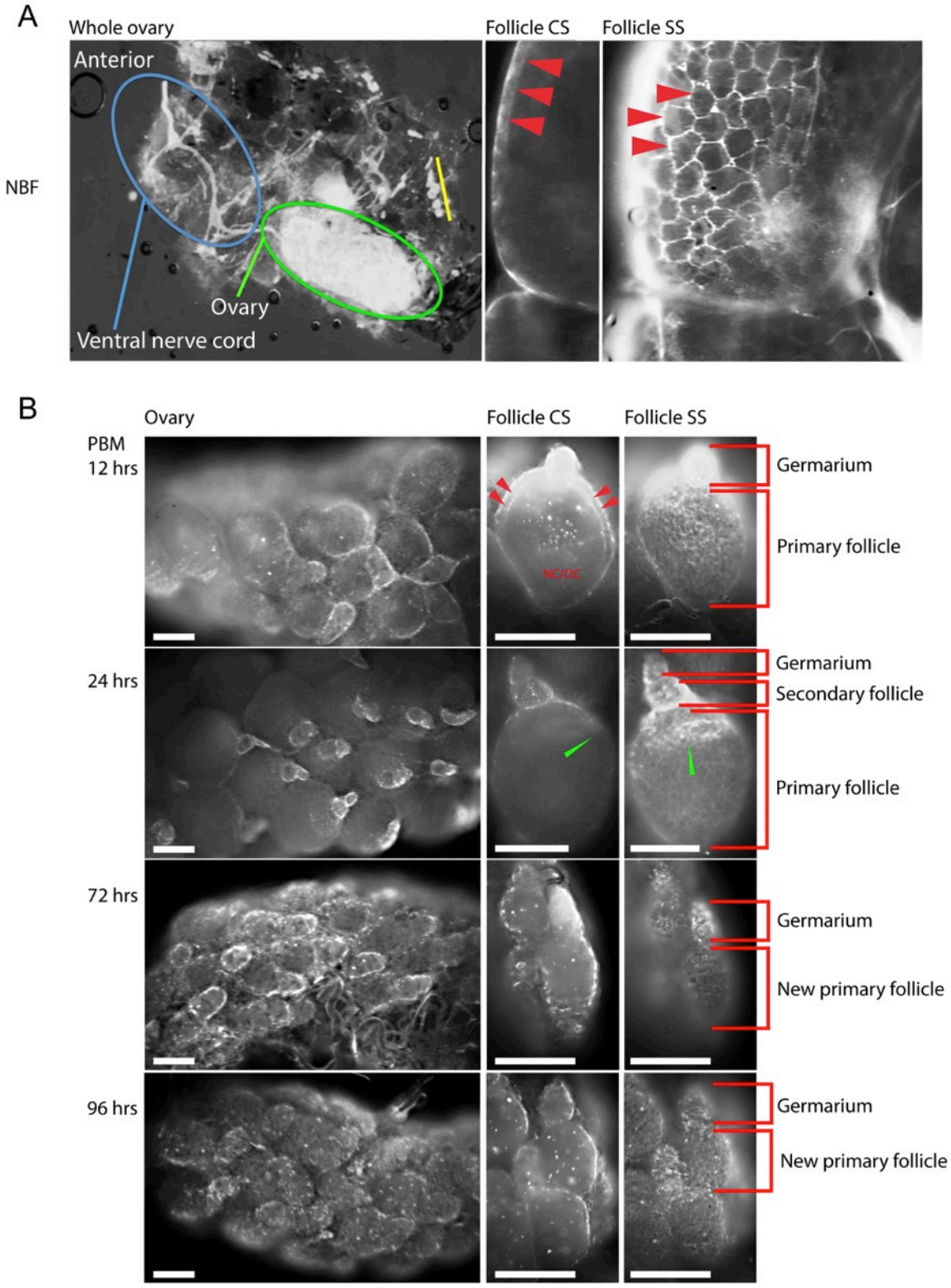

A
Whole ovary
Follicle CS
Follicle SS
Anterior
NBF
Ovary
Ventral nerve cord
B
Ovary
Follicle CS
Follicle SS
PBM
12 hrs
Germarium
Primary follicle
NC/OC
24 hrs
Germarium
Secondary follicle
Primary follicle
72 hrs
Germarium
New primary follicle
96 hrs
Germarium
New primary follicle

Figure 4.1. Filipin staining showing the distribution of free cholesterol in mosquito ovaries (A) prior to, and (B) following a blood meal. Scale bars: 100 μm. (A) Whole non-blood fed female mosquito abdomen (sans alimentary canal) flayed open laterally at 40x magnification, and individual ovariole from similarly prepared material at 600x magnification. (B) Filipin stained ovaries (200X) and follicles (400x) in at 12, 24, 72, and 96 hrs PBM. Ventral nerve cord indicated by blue circle, ovary indicated by green circle, probable oenocytes indicated by yellow line, red triangles indicate subset of representative follicle cells and green arrow shows transition point of follicle cells over nurse cells vs. follicle cells over the oocyte. Cross section (CS), nurse cells (NC), oocyte (OC), post blood meal (PBM), surface section (SS).

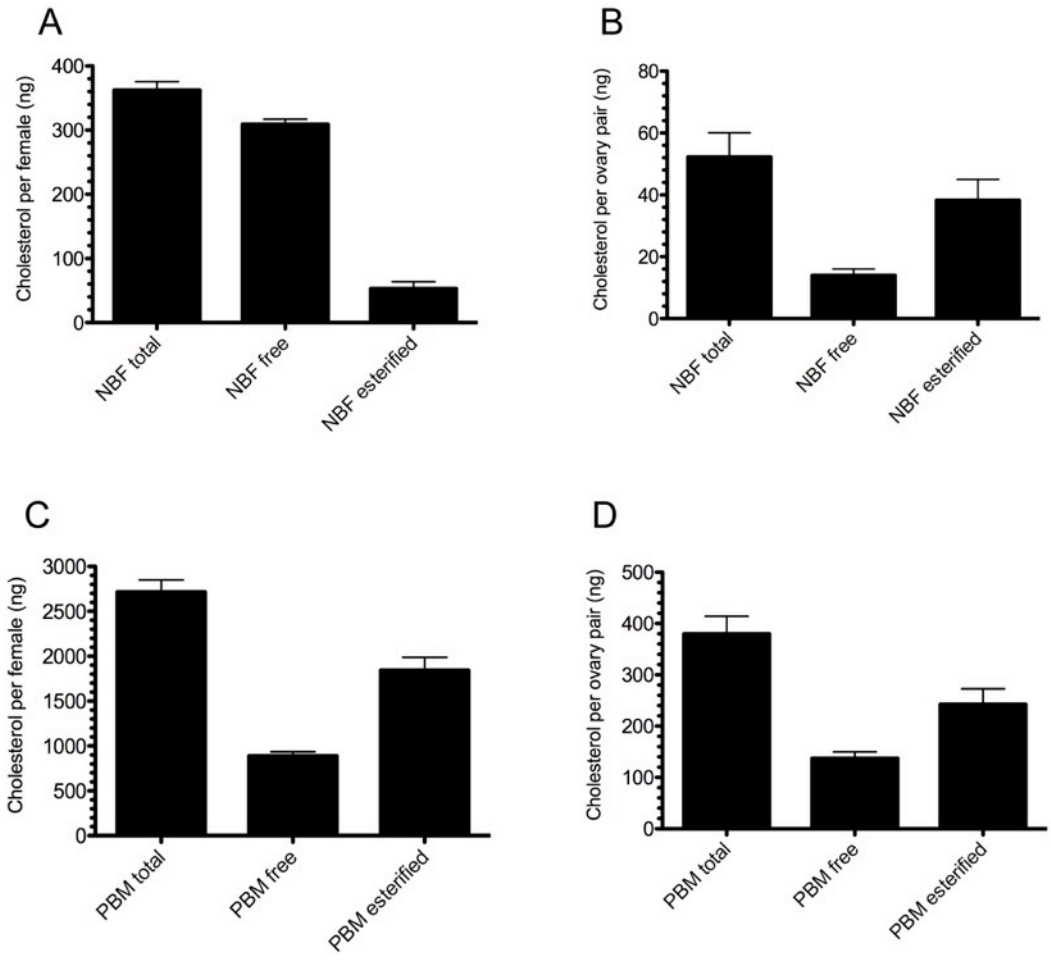
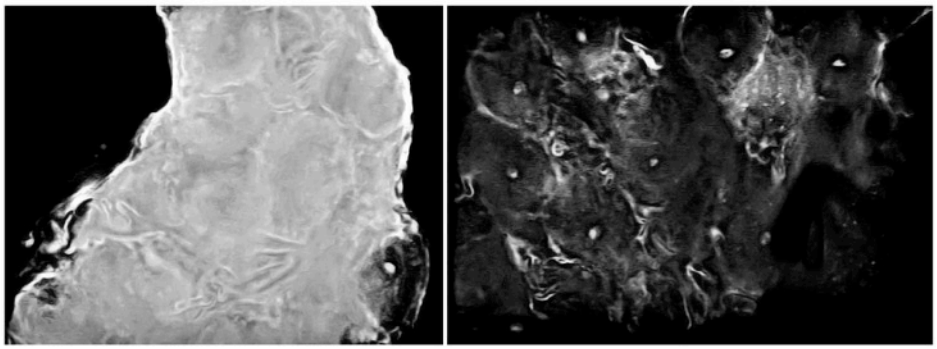

A
Cholesterol per female (ng)
400
300
200
100
0
NBF total
NBF free
NBF esterified
B
Cholesterol per ovary pair (ng)
80
60
40
20
0
NBF total
NBF free
NBF esterified
C
Cholesterol per female (ng)
3000
2500
2000
1500
1000
500
0
PBM total
PBM free
PBM esterified
D
Cholesterol per ovary pair (ng)
500
400
300
200
100
0
PBM total
PBM free
PBM esterified

Figure 4.2. Cholesterol quantification in mosquitoes using a standard Amplex red assay. (A) Cholesterol in whole body non-blood fed (NBF) female mosquitoes, (B) NBF ovaries alone, (C) 24 hour post blood meal (PBM) whole bodies, (D) PBM ovaries. Esterified cholesterol values calculated by the subtraction of free cholesterol values from total cholesterol values in paired sample sets.

A



Untreated MBCD (5 mM)

B

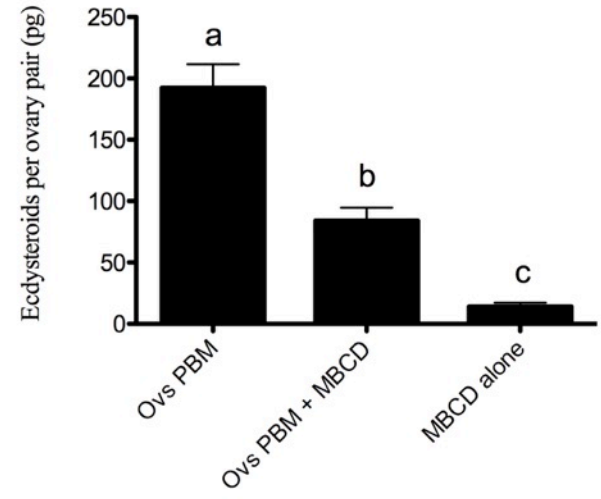


Figure 4.3. The effects of free cholesterol depletion in mosquito ovaries ex vivo by methyl-beta-cyclodextrin (MBCD). (A) Representative images of ovaries showing depletion of free cholesterol by 30 min exposure to 5 mM MBCD as suggested by filipin staining, (B) effects of 1 mM MBCD on ECD production by ovaries 24 hrs PBM with MBCD alone as a background control. Letters indicate a statistical difference between groups at $p < 0.05$, $F_{2, 35} = 48.59$, $P < 0.0001$. $n = 9$.

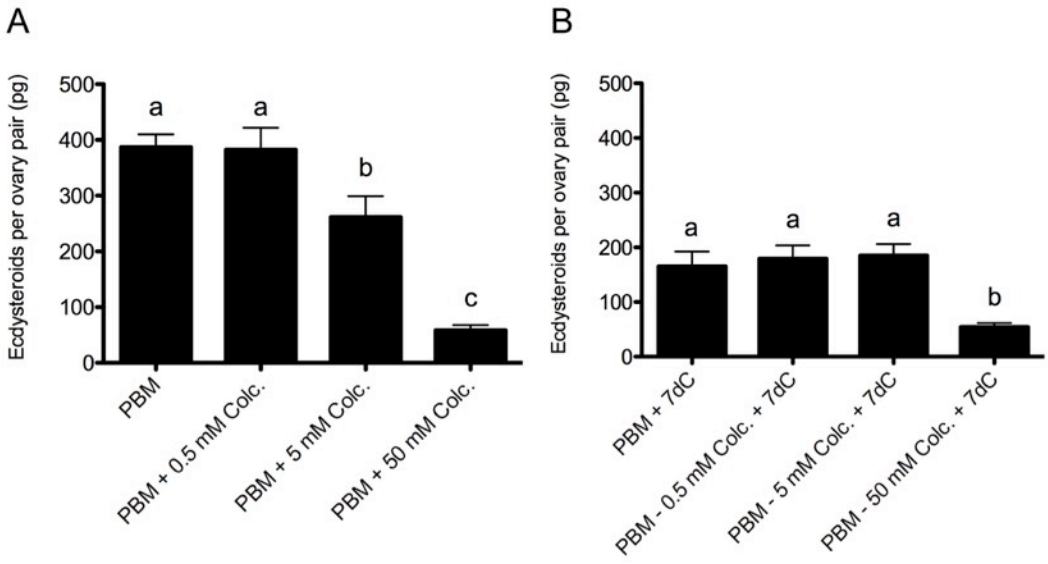


Figure 4.4. Shuttling inhibition of ovaries from PBM mosquitoes (A) incubated with colchicine for 6 hours, and (B) then the same ovaries transferred to colchicine-free media containing 7-dehydrocholesterol complexed with MBCD (1.25 MBCD:0.25 mM 7-dehydrocholesterol). Letters indicate a statistical difference between groups at $p < 0.05$. A) $F_{3, 35} = 26.49$, $P < 0.0001$; B) $F_{3, 33} = 8.14$, $P < 0.0001$. (A) n = 15, (B) n = 8-9.

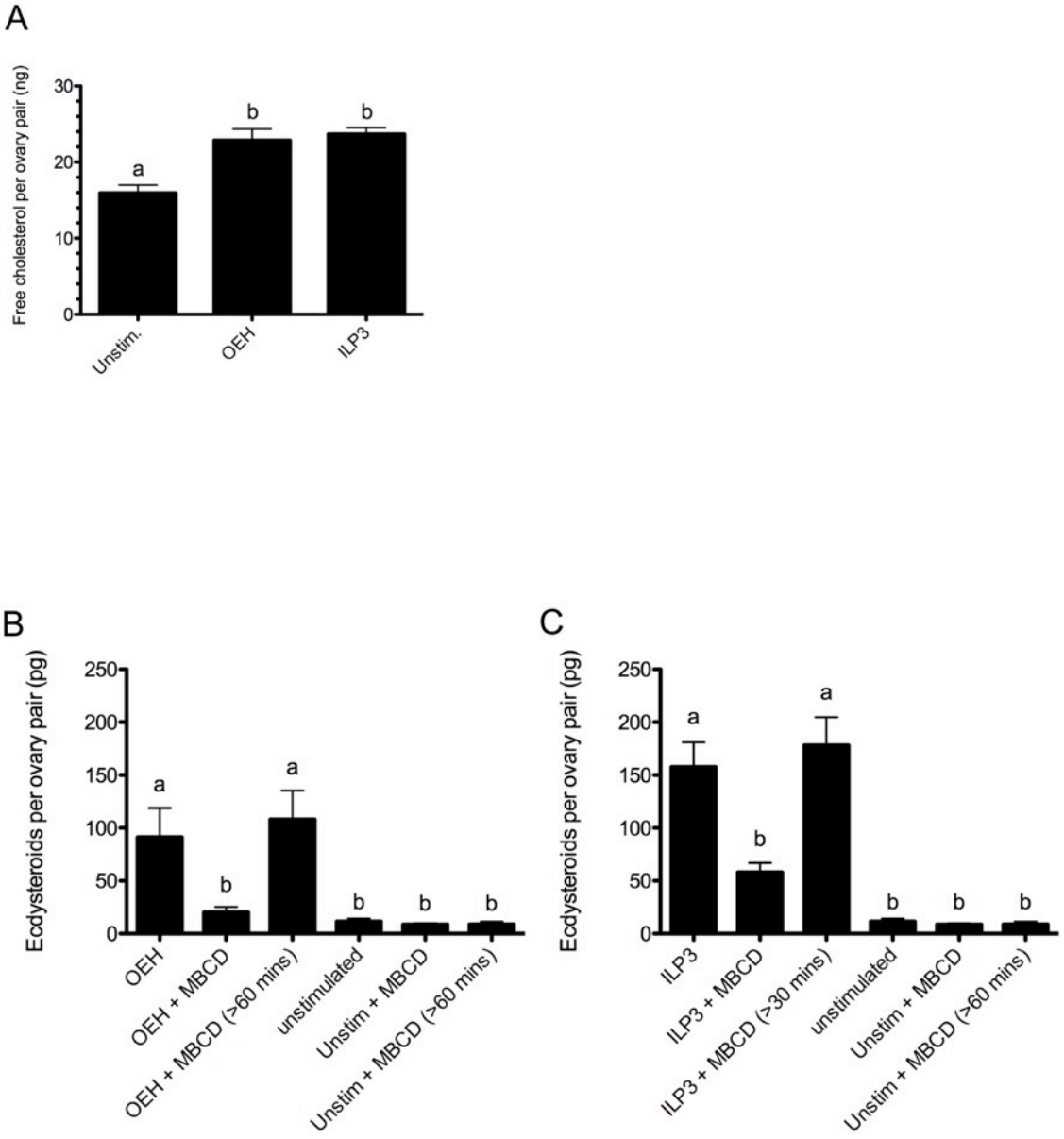

A
Free cholesterol per ovary pair (ng)
30
20
10
0
a
b
b
Unstim.
OEH
ILP3
B
Ecdysteroids per ovary pair (pg)
250
200
150
100
50
0
a
b
a
b
b
b
OEH
OEH + MBCD
OEH + MBCD (>60 mins)
unstimulated
Unstim + MBCD
Unstim + MBCD (>60 mins)
C
Ecdysteroids per ovary pair (pg)
250
200
150
100
50
0
a
b
a
b
b
b
ILP3
ILP3 + MBCD
ILP3 + MBCD (>30 mins)
unstimulated
Unstim + MBCD
Unstim + MBCD (>60 mins)

Figure 4.5. Neuropeptide-stimulated ECD production of mosquito ovaries ex vivo with or without the addition of MBCD at differing time intervals post activation. (A) Quantification of free cholesterol in neuropeptide stimulated ovaries (OEH and ILP3 at 10 pmol each), (B) ovary ecdysteroidogenic hormone (10 pmol) with 1 mM MBCD, and (C) insulin-like peptide 3 (20 pmol) with 1 mM MBCD. Letters indicate a statistical difference between groups at $p < 0.05$. A) $p < 0.01$, $F_{2, 23} = 13.69$, $P < 0.0001$, n = 7-12. B) $F_{5, 55} = 8.54$, $P < 0.0001$; C) $F_{5, 55} = 29.09$, $P < 0.0001$, n = 9-11.

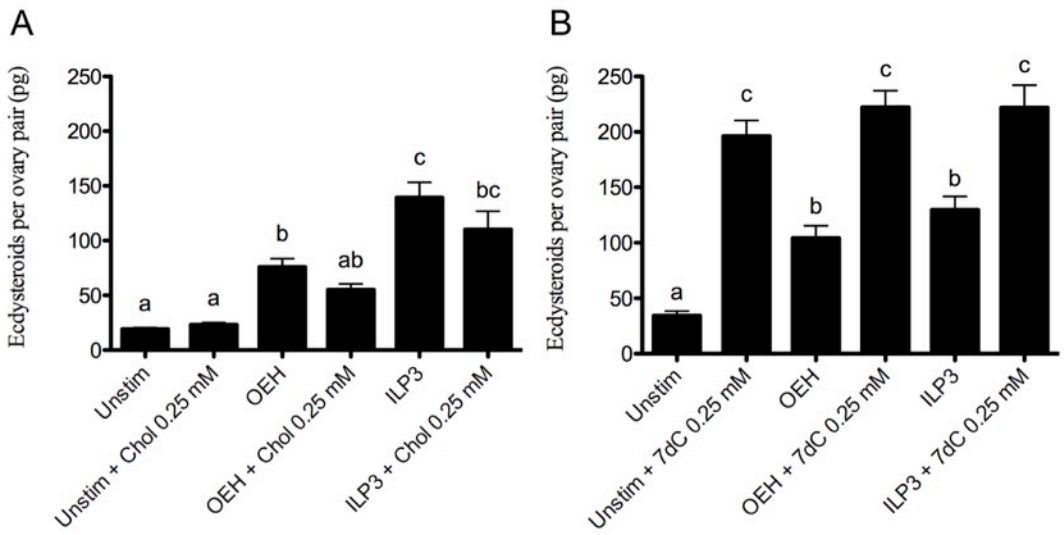
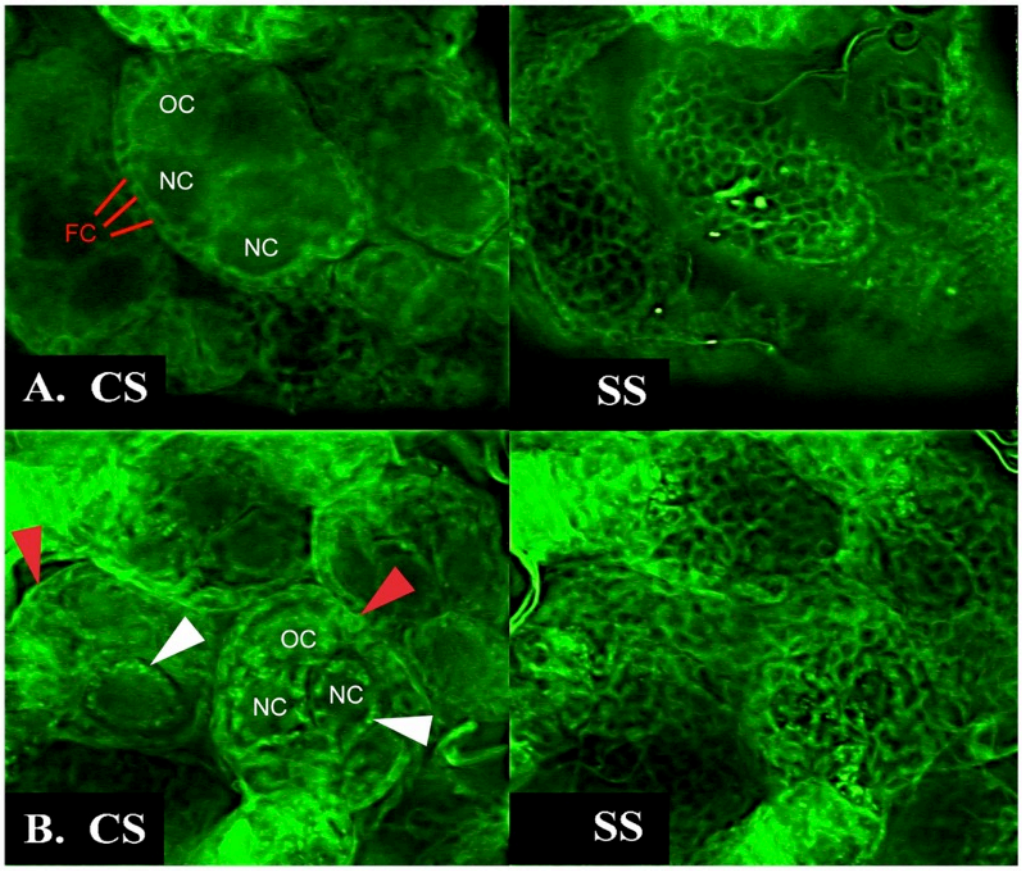


Figure 4.6. Sterol supplementation of NBF mosquito ovaries ex vivo using MBCD preloaded with either (A) cholesterol (2.5 mM MBCD:0.25 mM cholesterol), or (B) 7-dehydrocholesterol (1.25 MBCD:0.25 mM 7-dehydrocholesterol). Letters indicate a statistical difference between groups at $p < 0.05$. A) $F_{5, 89} = 24.42$, $P < 0.0001$; B) $F_{5, 76} = 29.81$, $P < 0.0001$. (A) n = 15, (B) n = 9-15.



C.

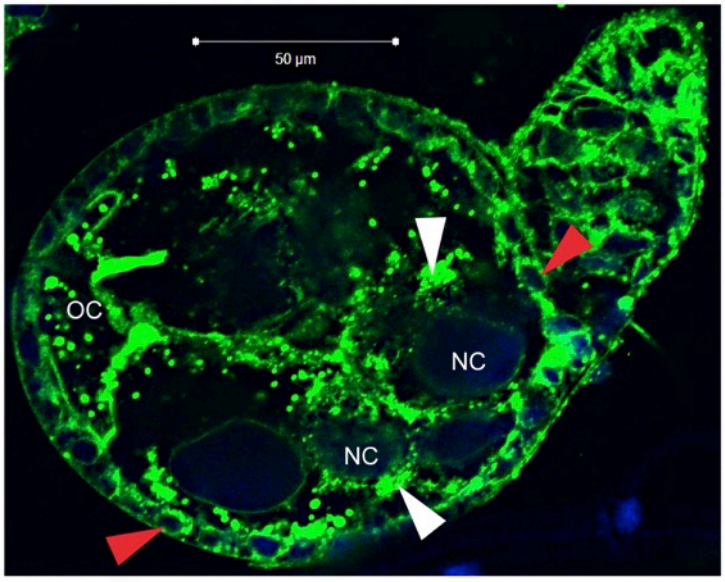


Figure 4.7. Distribution of labeled cholesterol (TF-cholesterol; green) loaded by MBCD in (A) unstimulated, and (B, C) ILP3 stimulated mosquito ovaries incubated ex vivo. Arrows indicate representative puncta in follicle cells (red) and nurse cells (white). Blue is Hoechst-stained nuclei. Cross section (CS), follicle cells (FC), nurse cells (NC), surface section (SS).

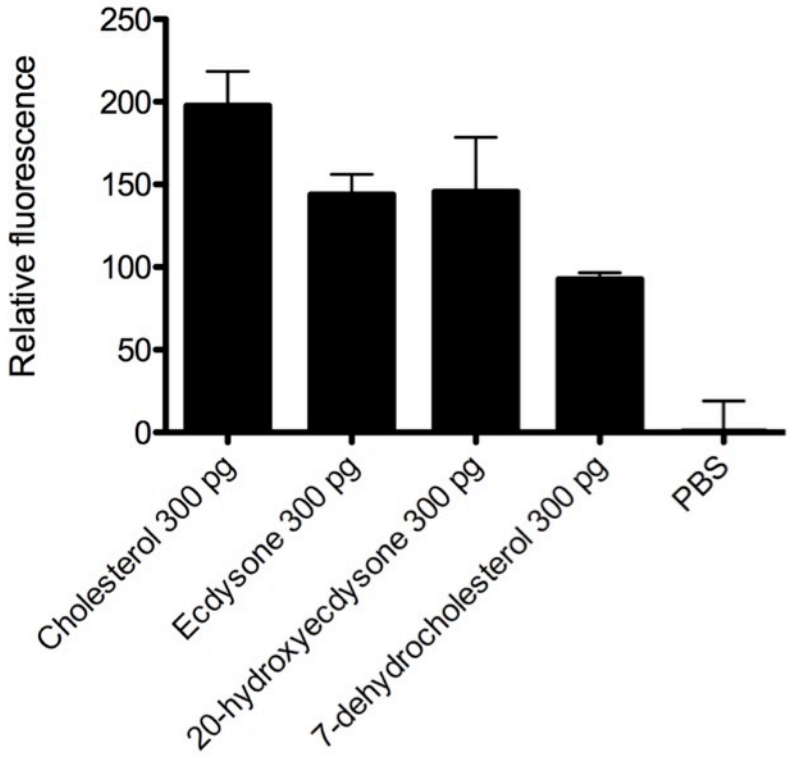


Figure 4.S1. Filipin III fluorescence in the presence of several relevant sterols. All values background corrected using mean filipin in PBS value. Filipin added to a final 1:100 dilution of kit stock concentration to wells containing 300 pg of sterol or PBS. n = 9.

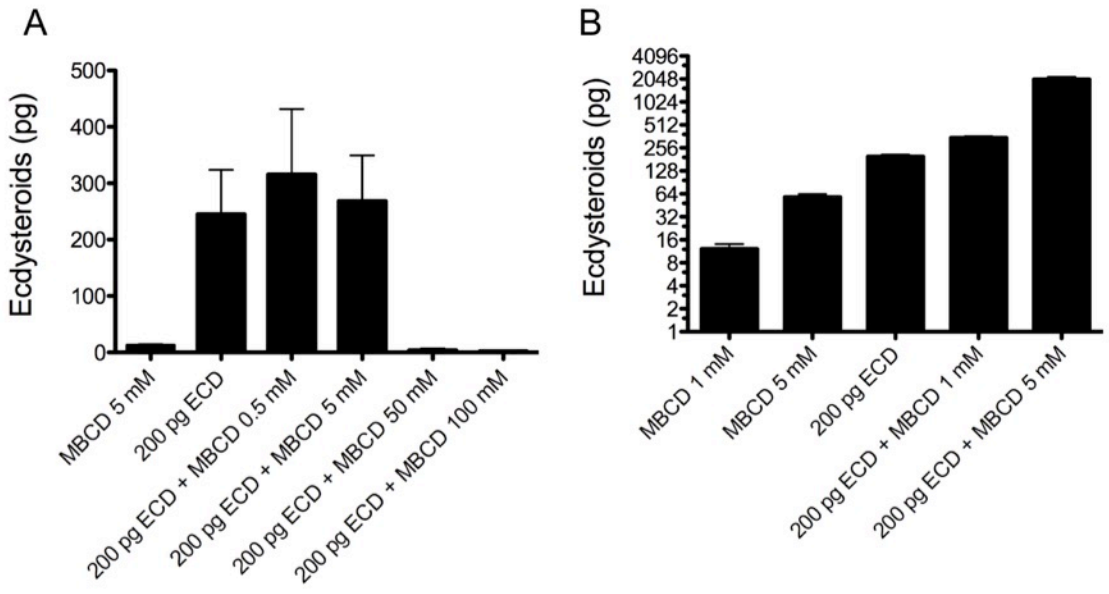


Figure 4.S2. Impact of MBCD on (A) radioimmunoassay, and (B) enzyme-linked immunoassay (displayed in log 2 scale). Ecdysone (ECD), methyl-beta-cyclodextrin (MBCD). (A) n = 6, (B) n = 12-17.

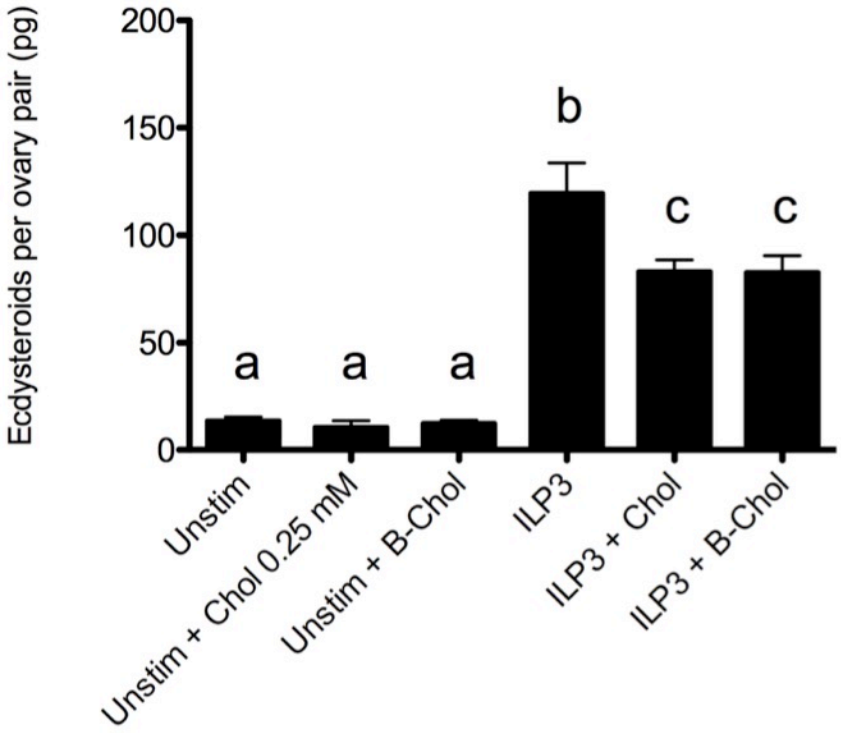


Figure 4.S3. Effects of BODIPY-cholesterol on mosquito ovary ecdysteroidogenesis ex vivo. Letters indicate a statistical difference between groups at $p < 0.05$. $F_{5, 58} = 54.98$, $P < 0.0001$. n = 6-12

CHAPTER 5

CONCLUSION

The yellow fever mosquito, *Aedes aegypti*, is an important vector of diverse pathogens for human and animal diseases. Furthermore, it is representative of mosquito vectors as a whole. Understanding the regulation of its reproduction provides new avenues of control for these vectors, specifically by making known possible targets for insecticide development. The work here expands on what was known before, elucidating important aspects of ecdysteroid quantification and production that were not known in insect hormone science, in three important areas.

The first chapter presented here provides a reliable method for the independent quantification of ECD and 20ECD apart from the need for HPLC purification. It also establishes a previously unreported problem with ecdysteroid analysis, namely, solvent salt integrations that lead to structural changes, and an inaccurate quantification of insect steroid hormones. Important question that remain to be pursued include: Is this only an artifact of methanol extraction of insects? Or are other parameters that have been taken for granted all these years effecting steroid hormone quantification? Physiologically, what does this mean for circulating ecdysteroids, which are exposed to the relatively high magnesium concentration in the insect's hemolymph?

The second chapter shows that signal transduction in the activation of mosquito ovarian ecdysteroidogenesis is a complex integration of nutrient and developmental signals not unlike what is seen in the prothoracic glands. Downstream or crosstalk of OEH and ILP signaling cascades enable extracellular calcium to modulate ecdysteroid synthesis via a store operated calcium entry mechanism. Additionally, this work suggests that there is a yet unidentified

receptor ligand pair that can activate ecdysteroidogenesis through cAMP-calcium signaling. Questions remaining to be explored are: Is there another important receptor and neuropeptide that may regulates ecdysteroid production in mosquito ovaries as implied by implied by this work? Is the regulation of hemolymph calcium indirectly related to ecdysteroid titer changes? Are there implications for salt marsh mosquitoes, whose hemolymph calcium levels are substantially higher than their freshwater counterparts?

The third chapter examines cholesterol distribution and mobilization that is important for steroid hormone synthesis. However, no work prior to this has demonstrated its mobilization in insect ovaries as a direct consequence of the activation of ecdysteroidogenesis. This leaves several questions ripe for study, namely, what is the role of the nurse cells to ecdysteroidogenesis? Do they supply the follicle cells with the needed cholesterol for ecdysteroid synthesis? Or do they themselves participate in steroid synthesis? Since cytoskeletal restructuring appears necessary to accomplish ecdysone production, it is an open question as to what signal mediates this restructuring.

As a whole, the work here paves the way forward for better understanding the underpinnings of mosquito reproduction. As a number of previous authors have indicated, these insights may have import beyond insect steroidogenesis, as there is a level of commonality between insect and vertebrate steroid hormone synthesis. However, there are also many facets of the work here that provide a solid foundation for further exploration.