CHARACTERIZATION OF COMPONENTS OF THE ECDYSTEROID BIOSYNTHETIC PATHWAY IN THE OVARIES OF THE YELLOW FEVER MOSQUITO, *Aedes aegypti*

by

DOUGLAS HAROLD SIEGLAFF

(Under the Direction of Mark R. Brown)

ABSTRACT

A blood meal induces the ovaries of *Aedes aegypti* mosquitoes to produce ecdysteroid hormones that regulate oogenesis. The ovaries of blood-fed females begin to produce ecdysteroids 6 h post blood meal (PBM), peak around 18 h PBM, and fall to pre-blood feeding levels by 48 h PBM. Various proteins involved in the intracellular transfer and biosynthesis of ecdysteroids in other insects have been identified and characterized. To begin a study of these processes in mosquito ovaries, complete cDNAs were cloned for putative orthologs of diazepam-binding inhibitor (DBI; *AedaeDBI*), StAR-related lipid transfer domain containing protein (Start1; *AedaeStart1*), aldo/keto reductase (A/KR; *AedaeA/KR*), adrenodoxin reductase (AR; *AedaeAR*), 22-hydroxylase (CYP302a1; *AedaeCYP302a1*), 2-hydroxylase (CYP315a1; *AedaeCYP315a1*) and 20-hydroxylase (CYP314a1; *AedaeCYP314a1*). As shown by RT-PCR analysis, transcripts for all seven genes were present in ovaries and other tissues both before and PBM. As determined by real-time PCR analysis, gene transcript abundance for *AedaeCYP302a1* and *AedaeCYP315a1* was significantly greater (9 and 12 fold, respectively) in ovaries 18 h PBM relative to that in ovaries from females not blood fed (NBF) or 2 h PBM. *AedaeDBI*, *AedaeStart1*, *AedaeA/KR* and *AedaeAR* also displayed higher transcript levels in ovaries at 18h

PBM; although, *AedaeDBI* transcripts were greatest at 48 h PBM. In contrast, gene transcript abundance for *AedaeCYP314a1* decreased PBM. Two separate control points of ecdysteroid biosynthesis, enzyme modification and sterol transfer, were addressed by studying expression and localization of AedaeCYP302a1 and AedaeStart1 protein in *A. aegypti* ovaries of NBF and blood-fed females. AedaeCYP302a1 was present in ovaries and other tissues both before and PBM. AedaeCYP302a1 increased in ovaries of blood-fed females and remained high 48 - 60 h PBM despite a lack of ovarian ecdysteroid production at these times. The levels of AedaeStart1 did not change in ovaries PBM. AedaeCYP302a1 localized predominantly to ovariole follicle cells, whereas AedaeStart1 was observed in both ovariole nurse and follicle cells. The information provided in this dissertation suggests that ovarian ecdysteroidogenesis in *A. aegypti* is partially regulated at the transcript and translational levels.

INDEX WORDS: Ecdysteroidogenesis, Diazepam-binding inhibitor, Start1, Aldo/keto reductase, Adrenodoxin reductase, Cytochrome P450, CYP302a1, CYP315a1, CYP314a1, Oogenesis, Insect, Culicidae

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DEDICATION

This work is dedicated to all that have helped, inspired, supported and comforted me during my graduate career at The University of Georgia.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

A jointed exoskeleton is the most characteristic feature shared by all species in the phylum Arthropoda, and it must be periodically cast-off and renewed to allow for growth. This morphogenetic event is primarily coordinated by polyhydroxylated sterols termed ecdysteroids (Jegla, 1990; Lachaise, 1990; Henrich et al., 1999). The monophyletic origin of the diverse organisms included in Arthropoda is currently under much debate, as earlier morphologically based relationships are either being corroborated or challenged with recent molecular data (Schmidt-Rhaesa et al., 1998; Ruppert et al., 2004). In the current review, the four arthropod taxa of Crustacea, Chelicerata, Myriapoda and Insecta comprise the discussion for it is within these taxa that the presence and biosynthesis of ecdysteroids has received the most attention (Gupta, 1990; Lachaise, 1990; Gilbert et al., 2002).

Arthropod gonads are derived from mesodermal epithelium, developing from embryonic coelomic cavities, and eventually become paired tubular structures that lie dorsally on each side of the gut opening to the exterior through a gonopore of which its segmental location depends on the taxon: Diplopoda (Myriapoda), anterior; Arachnida (Chelicerata) and Crustacea, middle; and Chilopoda (Myriapoda) and Insecta, posterior (Clarke, 1973; Ruppert et al., 2004). Arthropod gonads can also be single tubular structures located within the middle of the body cavity, along with other modifications (Conn, 2000), and such modifications or expansions to the general theme are described below within respective sections. The tubular structures branch into "follicles" in males and "ovarioles" in females and join to a common stem that develops toward

the vas deferens and oviduct, respectively (Conn, 2000). The spermatogonia or oogonia are located proximally at the blind end of the epithelial tubes (Conn, 2000). In the female this follicle epithelium has several functions during oogenesis including chorion deposition and ecdysteroid biosynthesis (Goltzene et al., 1978; Zhu et al., 1983; Conn, 2000). There is variation in gamete structure throughout the Arthropoda, but generally germ cells develop by receiving nutrients from associated nurse cells derived from the germ line or from the follicle epithelium itself which allows passage of nutrients from the hemolymph (Clarke, 1973).

Ecdysteroids have been isolated from the ovaries and eggs of species within all four subphyla (reviewed in Hoffman and Lagueux, 1985; Dorn, 1990; Subramoniam, 2000), but the presence of ecdysteroids in arthropod testes has only been confirmed in Insecta (Loeb et al., 2001). There are various functions assigned to ecdysteroids, either specifically synthesized or sequestered by arthropod gonads, such as the promotion of vitellogenesis, meitotic reinitiation, gametogenesis, and embryogenesis (Adiyodi and Adiyodi, 1970; Hagedorn, 1985; Lanot et al., 1988; Hagedorn, 1989; Lanot and Cledon, 1989; Lanot et al., 1989; Lomas and Rees, 1998; Subramoniam et al., 1999; Subramoniam, 2000; Rees, 2004). Note, in respects to the current review, ecdysteroids within gonads or eggs are considered separate from those within embryos, and this is based on the fact that the point at which *de novo* synthesis of ecdysteroids begins in embryos is difficult to define in many Arthropoda (Hoffmann and Lagueux, 1985; Dorn, 1990).

Ecdysteroid biosynthesis in arthropods has been reviewed frequently over the past two decades (e.g., Rees et al., 1980; Gilbert and Goodman, 1981; Rees, 1985; Rees and Isaac, 1985; Rees, 1989; Lachaise, 1990; Warren and Hetru, 1990; Grieneisen, 1994; Rees, 1995; Dauphin-Villemant et al., 1998; Gilbert et al., 2002). None addressed gonad ecdysteroid biosynthesis exclusively and instead relied on the observations made through the analysis of

ecdysteroidogenic tissues of both immature and adult arthropods. This review will address specifically ecdysteroid biosynthesis in arthropod gonads, and only when necessary will the observations obtained through analysis of other ecdysteroidogenic tissues (e.g., prothoracic glands of immature insects, Y-organs or crustaceans) be incorporated into the discussion.

Certain member groups of Arthropoda molt during their adult stage, retaining the same tissue responsible for ecdysteroid biosynthesis throughout its post-embryonic life (continual life molts: most Crustacea, Myriapoda, and Apterygota insects), and the maintenance of this tissue in the adult may alleviate the need for gonadal ecdysteroid biosynthesis during this stage. The absence of molts within the adult stage in other arthropods may necessitate gonadal ecdysteroid biosynthesis because the tissue responsible for ecdysteroid production during immature stages degenerates prior to or at the adult molt thus requiring another tissue to serve this function in the adult (pubertal molt: most Chelicerata and Pterygota insects) (Lachaise, 1990; Gilbert et al., 1996). Gonadal ecdysteroid biosynthesis, however, is proposed to occur in two members of the "continual life molt" group, the myriapod *Lithobius forficatus*, and the shore crab *Carcinus maenas* (see sections below). Further, a member of the "pubertal molt" group, the tick *Amblyomma hebraeum*, does not display ovarian ecdysteroid production (see section below). These observations suggest that the relationship between adult molts and gonadal ecdysteroid biosynthesis is not a steadfast definition throughout Arthropoda.

Various experimental approaches have been employed to determine whether a given tissue such as gonads synthesize ecdysteroids (Delbecque et al., 1990): removal of the tissue and consequential disappearance of whole body ecdysteroid titers, release of ecdysteroids within media in which the tissue is incubated, and presence of putative ecdysteroid precursor molecules within the tissue of interest. A more conclusive method to ascertain the biosynthetic capacity of a

given tissue is the use of radiolabeled ecdysteroid precursor molecules, preferably one used early in the biosynthetic pathway (Hoffmann et al., 1992). The most widely used method to establish the presence of ecdysteroids within a sample is through a radioimmunoassay (RIA), in which the sample ecdysteroids compete with radiolabled ecdysteroids for binding sites on anti-ecdysteroid serum by which ecdysteroid concentrations are calculated from a standard line (Reum and Koolman, 1989). Once the presence of ecdysteroids within a sample has been verified, the identification of isolated ecdysteroid(s) can be accomplished through techniques, such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), nuclear magnetic resonance spectroscopy (NMR), and mass spectral analysis (Rees and Isaac, 1985; Thompson and Lusby, 1989; Lafont and Beydon, 1990). All the above methods have been used to study ecdysteroid biosynthesis in arthropod gonads.

Conclusive evidence for gonadal ecdysteroid biosynthesis has essentially only been obtained in the class Insecta, whereas there is only limited evidence that these tissues are ecdysteroidogenic in other members of Arthropoda. The discussions below will first describe gonadal structure of the taxa (if different from that aforementioned or if relevant to the subsequent discussion); second, the evidence for gonadal ecdysteroid biosynthesis; and finally a brief description of the putative ecdysteroidogenic tissue(s) of the adult if the evidence for gonadal ecdysteroid biosynthesis is lacking.

Crustacea

Most Crustacea are gonochoric (separate male and female individuals) but some are hermaphroditic (male and female gonads present in single individual) (Ruppert et al., 2004). Fertilization is usually internal, but in some cases sperm transfer takes place externally (Ruppert

et al., 2004). Some crustaceans (brachyuran decapods - "crablike") have lobular gonads where single oocytes or numerous sperm are housed within each lobule (Conn, 2000).

The majority of members in this subphylum display "continual life molts," and it is presumed that they retain the tissue or gland producing the hormone that elicits this morphogenetic growth throughout their entire life (Gupta, 1990; Subramoniam, 2000). Other possible ecdysteroidogenic tissues have been suggested for this subphylum (e.g., ovaries); however, little work has been done to establish their biosynthetic capacity conclusively (Dorn, 1990; Lachaise, 1990; Subramoniam, 2000).

Ecdysteroids have been isolated from the ovaries of many members of this subphylum (Subramoniam, 2000), but, the assumed source of these ecdysteroids is the Y-organ, an epithelioid gland located in the cephalothorax of crustaceans that shares ultrastructural features common to steroid producing cells of insects and vertebrates (Spaziani, 1990). The activity of the Y-organ is under the negative control of a neuropeptide hormone, the molt-inhibiting hormone (MIH), which is produced within the X-organ and transported to the sinus gland for storage and eventual release, and both the X-organ and sinus gland are located within the eye stock of Crustacea (Spaziani et al., 1999). The sesquiterpenoid methyl farnesoate produced by the mandibular organ can stimulate ecdysteroid secretion by the Y-organ and positively effect reproductive growth, although such effects have not always been observed (Homola and Chang, 1997; Subramoniam, 2000).

Class Malacostraca: This class of Crustacea is the most studied in respects to ecdysteroid biosynthesis and the impact ecdysteroids have on reproductive development (esp., members of the order Decapoda; e.g., crabs, crayfish, lobsters, and shrimp) (Adiyodi and Adiyodi, 1970; Subramoniam et al., 1999; Subramoniam, 2000). Removal of the eye stock, thus

allowing ecdysteroid biosynthesis by the Y-organ, is a commonly used method to ascertain the influence ecdysteroids have on reproductive development in this group (Adiyodi and Adiyodi, 1970). Although a function for ecdysteroids in stimulating vitellogenesis is not shared by all Decapoda (Subramoniam, 2000), eye-stalk ablation in many species positively influences yolk deposition in the ovary (Adiyodi and Adiyodi, 1970).

Two studies do suggest that decapod ovaries may synthesize ecdysteroids. Ecdysteroid precursor molecules have been isolated from the shore crab *Carcinus maenas*, suggesting ecdysteroid biosynthesis may occur within this tissue (Hetru et al., 1978a; Hazel, 1986; Subramoniam, 2000). As opposed to the majority of decapods, the spider crab *Acanthony lunulatus* has a pubertal molt and Y-organ degeneration thereafter (Chaix and De Reggi, 1982). During adult reproductive development, the ovaries accumulate ecdysteroids leading the researchers to suggest that the ovaries themselves are the source of this hormone since the Y-organ is absent at this stage (Chaix and De Reggi, 1982).

Ecdysteroids have been isolated from ovaries of the amphipod *Orchestia gammarellus* and isopod *Armadillidium vulgare* (Blanchet et al., 1979; Suzuki et al., 1996). In *A. vulgare*, ovarian development and vitellogenin synthesis are inhibited following extirpation of the Y-organ (Suzuki, 1986; Suzuki, 1991), indicating that the Y-organ is the source of circulating ecdysteroids in the adult female.

Following secretion of ecdysteroids from primary ecdysteroidogenic tissues, so-called target tissue reactions, 20-hydroxylation or 3β-reduction, of ecdysteroid biosynthesis take place. These reactions have been observed in gonadal tissues of various decapod species. Ovaries and testes of the crabs, *Cancer antennarius* and *C. maenas*, and the crayfish, *Orconectes limosus*, are able to conduct "terminal hydroxylation" reactions of ecdysteroid biosynthesis when provided

radiolabeled precursors (e.g., 3β-reduction of 3-dehydroecdysone, or 20-hydroxylation of ecdysone) (Lachaise and Lafont, 1984; Bocking et al., 1995; Spaziani et al., 1997). Again, these reactions may be considered a target tissue activation (at least in insects; Smith, 1985), and thus are considered peripheral to ecdysteroid biosynthesis.

Chelicerata

All Arachnids (class of Chelicerata discussed here) are gonochoric, and fertilization is internal (Ruppert et al., 2004). Ecdysteroids have been isolated from ovaries and eggs of members of Chelicerta, but no such identification has been made for the testes of this subphylum. A definitive ecdysteroidogenic gland has not been identified in any order of Chelicerata, but many tissues have been proposed based on their cellular ultrastructure, changes in the ultrastructure with molting cycles, and the ability to secrete ecdysteroids *in vitro* (Diehl et al., 1986; Juberthie and Bonaric, 1990; Foelix, 1996; Rees, 2004).

Aranea: Ecdysteroid accumulation in the ovaries of two species of spiders, *Coelotes terrestris* and *Tegenaria domestica*, correlated with vitellogenic events (Trabalon et al., 1992), but the source of ecdysteroids was not described. That ecdysteroids promote vitellogenesis has been reported in a single spider species, *Tegenaria atrica*, but was hypothesized based on the finding that 20-hydroxyecdysone applied to unmated *T. atricia* promoted yolk accumulation within its ovaries (Pourie and Trabalon, 2003).

The ecdysteroidogenic gland in Aranea is located within the prosoma and is comprised of numerous masses of cells divided into lateral and posterior groups (Juberthie and Bonaric, 1990). The ecdysteroidogenic nature of this gland is based on the observation that the cellular ultrastructure changes with molting cycles (Juberthie and Bonaric, 1990). Whether the

ecdysteroidogenic gland serves as the source of ecdysteroids during reproductive development in adult spiders has not been addressed.

Acari: Sperm production in ticks is divided into spermatogenesis (development of haploid spermatids) and spermiogenesis (maturation of spermatids), both of which are positively influenced by ecdysteroids (Lomas and Rees, 1998). The ovaries of the ixodid ticks *Ornithodoros moubata*, *Rhipicephalus appendiculatus*, and *Amblyomma hebraeum* accumulate ecdysteroids during ovary maturation as assessed by RIA and fate of injected radiolabled ecdysteroids (Connat et al., 1984; Connat et al., 1985; Connat et al., 1987; Magee et al., 1996). The ovaries of *O. moubata* and *A. hebraeum* conduct the terminal 20-hydroxylation reactions when provided radiolabeled ecdysone (Connat et al., 1984; Connat et al., 1986; Connat et al., 1987), but as suggested above, this should be considered a reaction peripheral to ecdysteroid biosynthesis.

There is no direct evidence that the either testes or ovaries of ticks synthesize ecdysteroids, and more importantly, the source of circulating ecdysteroids within either the immature or adult stages is not well defined (Juberthie and Bonaric, 1990; Lomas and Rees, 1998). Ovaries of the ixodid tick *A. hebraeum* did not secrete ecdysteroids when incubutated *in vitro*, but instead ecdysteroid biosynthesis was observed by integumental tissue (Lomas et al., 1997). Lomas et al. (1997) further showed that ecdysteroid biosynthesis by the integument is modulated by a peptide factor from the synganglia. In addition to the integument, lateral organs, retrocerebral complex, and peritracheal glands have been suggested as putative ecdysteroidogenic tissues in ticks, as based on cellular ultrastructural evaluations (Binnington, 1986; Juberthie and Bonaric, 1990).

Three separate cell groups within mites have been the suggested ecdysteroidogenic tissue, and ultrastructural changes within one group located in the hemolymphatic sinus correlate with morphogenetic events in two species of mites (Juberthie and Bonaric, 1990). A function for ecdysteroids in mite reproduction has not been reported, and ecdysteroid titers have only been reported for the nymphal stages of the chicken mite, *Dermanyssus gallinae* (Chambers et al., 1996).

Opiliones: The ovaries of the harvestman ("daddy longlegs") *Phalangium ravennae* secrete both ecdysone and 20-hydroxyecdysone when cultured *in vitro*, but interestingly the oenocytes within the femora were found to be the main source of both of these ecdysteroids (Romer and Gnatzy, 1981).

A tissue described as a dorsoventral stripe that runs in two branches from the esophagus to the endosternite has be suggested as ecdysteroidogenic for its cellular ultrastructure changes with the molting cycle of four species of Opiliones (Juberthie and Bonaric, 1990). Even though this tissue persists in the adult, its function in ecdysteroid biosynthesis needs clarification (Juberthie and Bonaric, 1990).

Scorpiones: Ecdysteroids have been isolated from the eggs of a scorpion, *Leiurus quinquestriatus*, and the levels fluctuate over 10-fold from egg to embryo, as assessed by RIA (Jegla, 1990). Jegla (1990) pointed out, however, that the age of the eggs within this study was not defined, and thus one can not rule out that the ecdysteroids are from *de novo* synthesis by the embryo itself.

Three separate tissues have been suggested as the ecdysteroidogenic gland in scorpions: paired blind end-organs, glomus of the coxal gland, and "anterior endocrine cells" at the base of the pedipalps, and all three are based on gross morphology or similarity in its body location to

ecdysteroidogenic glands in other Chelicerata (Juberthie and Bonaric, 1990). No evidence has been acquired to substantiate any of these tissues as an ecdysteroidogenic gland.

Myriapoda

Myriapods are gonochoric, and fertilization occurs externally through deposition of spermatophores ("sperm packet") by the male with subsequent acquisition by the female (Brusca and Brusca, 1990). In most myriapods, the ovary and testes are single tubular structures (Chilopoda, Diplopoda, and Pauropoda), but in Symphyla the gonads are paired (Brusca and Brusca, 1990).

Given that the majority of species within this subphylum display "continual life molts," the presumed ecdysteroidogenic gland is likely retained throughout its life history. Chilopoda are the most studied members of this subphylum in respects to ecdysteroid biosynthesis, and it is only within this group that the possibility of ecdysteroid biosynthesis by gonads has been suggested.

Chilopoda: The ovaries of the centipede *Lithobius forficatus* secrete both ecdysone and 20-hydroxyecdysone when incubated *in vitro* (Leubert et al., 1982), and both testes and ovaries were shown to convert the commonly used ecdysteroid precursor 5β-ketodiol into ecdysone (Descamps et al., 1990). These results suggest that both gonad types are capable of synthesizing ecdysteroids in this species. These observations are quite intriguing for the putative ecdysteroidogenic gland would still be present throughout the adult molts (Dorn, 1990; Seifert, 1990).

Other evidence suggest that the so-called "lymphatic strands" surrounding the salivary glands function as the ecdysteroidogenic glands in at least *L. forficatus*: ultrastructural similarities with the prothoracic glands of insects, secretion of ecdysteroids when cultured *in*

vitro, cell ultrastructural changes during molting cycles, and cell populations within these tissues display immunohistochemical staining with anti-ecdysteroid antibodies (Seifert, 1990). Other ecdysteroidogenic glands from other chilopod species have been studied (Seifert, 1990).

Symphyla: Ecdysteroids have been identified from a single species in this class, *Hanseniella ivorensis* (Jegla, 1990), but no ecdysteroidogenic gland has yet been proposed (Seifert, 1990).

Diplopoda: The presence of ecdysteroids within millipedes has not yet been reported (Gupta, 1990; Seifert, 1990), but given that they do go through ecdysis (Ruppert et al., 2004), the presence of this steroid hormone may be assumed.

Despite that the presence of ecdysteroids has not been observed and reported for this group, the collar gland is proposed to be ecdysteroidogenic based on changes in cellular ultrastructural with molting cycles (Seifert, 1990). No further evidence as to its function has been provided.

Pauropoda: Nothing has been reported in relation to the function of ecdysteroids within this class of Myriapoda (Seifert, 1990).

Hexapoda

Insecta (Apterygota, Pterygota): Insects are gonochoric with fertilization usually occurring internally, but if external, a spermatophore is the means by which females access sperm for fertilization (Ruppert et al., 2004). Asexual reproduction does occur in some species of insects (e.g., polyembryony in parasitic wasps of Lepidoptera) as does parthenogenesis (development of egg without fertilization; e.g., aphids and honey bees) (Conn, 2000).

The paired insect testes typically are comprised of a series of testicular tubules (follicles) bound by a mesodermal sheath (Davey, 1985), and within these follicles spermatogenesis and

spermiogenesis takes place (Ruppert et al., 2004). The sperm mature longitudinally down the follicle with the mature sperm at the distal end of the follicle (Ruppert et al., 2004). Spermatogenesis is completed prior to the adult molt in most insect species, and the spermatozoa are stored either within testis or seminal vesicles organized together into spermiodesms (Retnakaran and Percy, 1985). Ecdysteroids have been observed to promote spermatogenesis in many insect species (Hagedorn, 1985), and may serve to promote growth of somatic tissue in the genital tract of male insects (Loeb, 1991a).

The paired insect ovaries typically consist of bundles of ovarioles surrounded by muscle and tracheoles (Ruppert et al., 2004). Each ovariole consists of a primary "vitellarium" in which the oocyte develops and a germarium at its distal end in which primordial germ cells produce subsequent oogonia (Ruppert et al., 2004). Insect ovaries can be divided into two categories, panoistic in which all oogonia become oocytes, and meroistic in which oogonia will either become an oocyte or develop into nurse cells that supply the oocyte with developmentally important macromolecules such as ribosomal RNA (King and Buning, 1985). Meroistic ovaries are subdivided further into telotrophic in which the nurse cells are located in the germarium, and polytrophic in which nurse cells and the oocyte are together within each ovariole (King and Buning, 1985). Insect ovarioles reach maturity either before or after morphogenesis into the adult, the timing of which may be related to the acquisition of adequate nutrient stores during immature stages, in which case ovaries develop before adult morphogenesis (Sehnal et al., 1996). Sehnal et al. (1996) use the examples of the silkworm *Bombyx mori* in which ovarioles reach maturity during the pupal stage, whereas mosquitoes such as *Aedes aegypti* require a blood meal in the adult to attain the necessary amount of nutrients for ovariole maturation.

The majority of research on gonadal ecdysteroid biosynthesis has been conducted with members of this arthropod class, and thus a more restrictive criterion will be used as "proof" of ecdysteroid biosynthesis within this tissue. The presence of ecdysteroids in ovaries and testes has been observed in insect species from many orders (Hagedorn, 1983; Hagedorn, 1985), but given the many insect tissues considered as ecdysteroidogenic (Redfern, 1989; Delbecque et al., 1990), the simple presence of ecdysteroids within the gonads is not definitive proof of ecdysteroid biosynthesis by the tissue.

Apterygota: Ovaries of the firebrat *Thermobia domestica* accumulate ecdysteroids during reproductive development, but the source is assumed to be the cephalic ventral gland, a tissue homologous to the prothoracic glands of immature stages of Pterygota insects (Rojo De La Paz et al., 1983; Bitsch and Bitsch, 1988). This tissue is maintained throughout the life of this species (Rojo De La Paz et al., 1983; Bitsch and Bitsch, 1988). The maintenance of the cephalic ventral gland, "continual life molts," and the presumed lack of gonadal ecdysteroid biosynthesis in *T. domestica* resemble the crustacean condition and may represent the primitive state of ecdysteroid biosynthesis in Insecta.

Pterygota: The evidence for gonadal ecdysteroid biosynthesis within this subclass of Insecta is much more convincing. A decrease in ecdysteroid titer following the removal of ovaries (ovariectomy) has been observed in the grasshopper *Locusta migratoria* (Lagueux et al., 1977), the lubber grasshopper *Romalea microptera* (Hatle et al., 2003), the house cricket *Acheta domesticus* (Renucci and Strambi, 1981), the cockroaches *Blattella germanica* and *Periplaneta americana* (Weaver et al., 1984; Romana et al., 1995), the kissing bug *Rhodnius* sp. (Ruegg et al., 1981), the silkworm *Bombyx mori* (Hanaoka and Ohnishi, 1974), and the wax moth *Galleria mellonella* (Bollenbacher et al., 1978). Ovaries incubated *in vitro* have secreted or accumulated

ecdysteroids in the cricket *Gryllus bimaculatus* (Hoffmann et al., 1992; Lorenz et al., 1995), *B. germanica* (Romana et al., 1995), the termite queens of *Macrotermes bellicosus* and *M. subhyalinus* (Delbecque et al., 1978), *L. migratoria* (Lagueux et al., 1977; Goltzene et al., 1978), the cockroach *Nauphoeta cinerea* (Zhu et al., 1983), *G. mellonella* (Bollenbacher et al., 1978), the mosquito *A. aegypti* (Hagedorn et al., 1975; Hagedorn et al., 1979; Hanaoka and Hagedorn, 1980; Sieglaff et al., 2005), the fly *Drosophila melanogaster* (Rubenstein et al., 1982; Schwartz et al., 1985; Bownes, 1989), the blowfy *Phormia regina* (Maniere et al., 2000; Maniere et al., 2004), the housefly *Musca domestica* (Adams et al., 1997; Adams and Li, 1998), and the beetle *Tenebrio molitor* (Taibi et al., 2003). The testes of tobacco budworm *H. virescens* (Loeb et al., 1982), European corn borer *Ostrinia nubilalis* (Gelman et al., 1988), cabbage armyworm *Mamestra brassica* (Shimizu et al., 1985), and cotton leafworm *Spodoptera littoralis* (Jarvis et al., 1994) have been shown to produce ecdysteroids either by secreting immunoreactive ecdysteroids into media in which they are incubated or converting putative ecdysteroid biosynthesis precursors into ecdysone or 20-hydroxyecdysone. Immunoreactive ecdysteroids have also been observed in the testes of the adult blowfly *Calliphora vicina* and the cricket *G. bimaculatus*, but the source of the ecdysteroids was not defined in either study (Koolman et al., 1979; Hoffmann, 1982).

The follicle cells of insect ovarioles are the assumed source of ecdysteroids, because preparations of this cell population from *N. cinerea* and *L. migratoria* secrete ecdysteroids when incubated *in vitro* (Goltzene et al., 1978; Zhu et al., 1983), and immunohistochemical staining of ecdysteroids was observed in the follicle cells of *L. migratoria* ovarioles (Glass et al., 1978). The inner testicular sheath has been the proposed site for ecdysteroid production in *H. virescens* testes based on the immunostaining of this region by ecdysteroid antibodies (Loeb, 1986).

The pathways of ecdysteroid biosynthesis occurring in insect gonads are based primarily on the isolation of putative ecdysteroid precursors from the ovaries of *L. migratoria*, *Schistocerca gregaria*, and *B. mori* (Ohnishi et al., 1977; Goltzene et al., 1978; Hetru et al., 1978; Hetru et al., 1978a; Ohnishi et al., 1981; Dinan and Rees, 1981a; Hetru et al., 1982). Some steps within these proposed pathways have been corroborated following metabolic conversion of radiolabeled ecdysteroid precursors to terminal ecdysteroids (e.g., ecdysone and 20-hydroxyecdysone) by insect ovaries and testes. These radiolabel studies lend significant support to the proposed ecdysteroid biosynthesis pathway occurring in arthropod gonads.

The pathway(s) of ecdysteroid biosynthesis in arthropod gonads

A generalized scheme for ecdysteroid biosynthesis occurring in arthropod gonads is presented in Figure 1.1 and the results of published radiolabel studies in Table 1.1. Within the text below, a bold number references the ecdysteroid precursor molecule and a bold letter the enzyme conducting the reaction, both depicted within Figure 1.1. The pathway is derived from the isolation of ecdysteroid precursors from the ovaries of *L. migratoria* (Hetru et al., 1978b), and work using radiolabeled precursors with the ovaries of *L. migratoria* (Hetru et al., 1982; Kappler et al., 1986; Haag et al., 1987; Fujimoto et al., 1989; Dolle et al., 1990; Rees, 1995), *S. gregaria* (Greenwood and Rees, 1982; Greenwood et al., 1984), *G. bimaculatus* (Hoffmann et al., 1992), and *D. melanogaster* (Warren et al., 1996a), and the testes of *S. littoralis* (Jarvis et al., 1994). The observation that ecdysteroid precursors have been isolated from crustacean ovaries and that myriapod gonads metabolized an ecdysteroid precursor to ecdysone prompted the more inclusive use of "arthropod gonads" within the above title (Hetru et al., 1978a; Hazel, 1986; Descamps et al., 1990; Subramoniam, 2000). The biosynthetic pathway would be incomplete if

studies conducted with prothoracic glands of immature insects and Y-organs of crustaceans were excluded, and thus were used to "fill-in" gaps in the hypothetical pathway.

Ecdysteroid biosynthesis is believed to mimic vertebrate steroidogenesis in that precursor molecules shuttle between the endoplasmic reticulum (ER) and the mitochondria during processing (Grieneisen, 1994; Rees, 1995; Gilbert et al., 2002). Such an assertion is based on early studies that found the enzymatic activity associated with ecdysteroid biosynthesis in subcellular preparations enriched in either microsomes (ER) or mitochondria (Kappler et al., 1989; Grieneisen et al., 1993; Rees, 1995). The localization of enzymes involved in ecdysteroid biosynthesis within these two organelles was later confirmed following the identification and characterization of four cytochrome P450 enzymes that conduct hydroxylation reactions of ecdysteroid biosynthesis (Petryk et al., 2003; Warren et al., 2004). Because of the resemblance to mammalian steroidogenesis, the literature for ecdysteroid biosynthesis in arthropods derives experimental methodologies, interpretation of results, and subsequent hypotheses from the more established and descriptive literature on mammalian steroidogenesis. None-the-less, ecdysteroid biosynthesis in arthropods is hypothesized to contain novel steps within its pathway not believed to occur in mammalian steroidogenesis , but because of the minute quantities, ephemeral nature, and relative instability of ecdysteroid precursors, the entire pathway has not been fully characterized (Rees, 1985; Grieneisen, 1994; Rees, 1995; Gilbert et al., 2002).

As in vertebrate steroidogenesis, cholesterol is the precursor of arthropod ecdysteroid biosynthesis (Lachaise, 1990; Grieneisen, 1994; Rees, 1995; Gilbert et al., 2002); but, unlike vertebrates, arthropods can not synthesize this molecule *de novo*, and must acquire it from their diet (Clayton, 1964; Kircher, 1982; Behmer and Nes, 2003). Insects can obtain sterols from plant or animal sources, which may require modification to cholesterol before it can be used for

ecdysteroid biosynthesis, a subject that has been addressed in numerous reviews (Clayton, 1964; Kircher, 1982; Rees, 1989; Svoboda and Feldlaufer, 1991; Grieneisen, 1994; Rees, 1995; Gilbert et al., 2002; Behmer and Nes, 2003). The extra- or intracellular source of cholesterol used in arthropod ecdysteroid biosynthesis is not known, but cholesterol used in mammalian steroidogenesis can be derived from three separate sources: (1) exogenous cholesterol transported to the cell via lipoproteins, (2) the plasma membrane, or (3) intracellular *de novo* synthesis (Grummer and Carroll, 1988; Liscum and Underwood, 1995; Thomson, 1998; Azhar et al., 2003). Given the lack of *de novo* cholesterol synthesis in arthropods, its source for ecdysteroid biosynthesis must either come from hemolymph lipoproteins or the plasma membrane. It has been shown in lepidopteran insects that cholesterol absorbed by the midgut is loaded into lipoproteins and transported to peripheral tissues for internalization (Chino and Gilbert, 1971; Jouni et al., 2002). Lipoprotein delivery to the ovaries of two members in this order has been reported (Kawooya and Law, 1988; Kawooya et al., 1988; Jouni et al., 2003).

The first step of ecdysteroid biosynthesis in the ecdysteroidogenic cell is the delivery of cholesterol (**1**) to the ER where the first enzymatic modification is conducted by the cytochrome P450 enzyme, 7,8-dehydrogenase (**A**), producing 7-dehydrocholesterol (**2**) (Grieneisen et al., 1993; Grieneisen, 1994). Because of the abundance of 7-dehydrocholesterol in ecdysteroidogenic glands and the lack of trophic hormonal stimulation of its synthesis, the delivery of cholesterol to the ER and its subsequent 7,8-dehydrogenation is not considered the rate limiting step of ecdysteroid biosynthesis in arthropods (Grieneisen et al., 1993; Grieneisen, 1994; Dauphin-Villemant et al., 1998). Various sterol transfer protein have been hypothesized to be responsible for the delivery of cholesterol to the ER or mitochondria, such as the diazepam-binding inhibitor, start1 and sterol-carrier protein 2 (Snyder and Van Antwerpen, 1998; Behmer and Nes, 2003;

Krebs and Lan, 2003; Roth et al., 2004), but none have been shown conclusively to mediate this process. Considerable evidence supports the role for the mammalian homologs of the three sterol transfer proteins in intracellular cholesterol transporter or promoters of steroidogenesis (Papadopoulos, 1993; Papadopoulos and Brown, 1995; Schroeder et al., 1996; Alpy et al., 2001; Schroeder et al., 2001; Zhang et al., 2002; Strauss et al., 2003; Uribe et al., 2003; Tuckey et al., 2004; Alpy et al., 2005). The three putative sterol transfer proteins identified in insects are expressed in insect ovaries, but their expression in insect testes has not been addressed (Snyder and Feyereisen, 1993; Kolmer et al., 1994; Krebs and Lan, 2003; Roth et al., 2004).

Following 7,8-dehydrogentaion at the ER, 7-dehydrocholesterol is transferred to the mitochondria, and this is considered the rate limiting step of insect ecdysteroid biosynthesis, on the basis of results attained through the analysis of ecdysteroid biosynthesis by *Manduca sexta* prothoracic glands (Grieneisen et al., 1993; Warren and Gilbert, 1996b). At the mitochondria, 7-dehydrocholesterol is transported into the inner mitochondrial membrane (IMM), where a series of reactions termed the "black box" take place, and various steps and precursor members within this "black box" have been proposed (Rees, 1985; Grieneisen, 1994; Rees, 1995; Gilbert et al., 2002). The modifications that occur within this "black box" involve A/B ring modification, introduction of the 6-keto group and 14-hydroxylation, but the order of such modifications has eluded characterization (Rees, 1989; Grieneisen, 1994). The time at which 14-hydroxylation of the ecdysteroid nucleus takes place has been difficult to determine (Grieneisen, 1994) and is confounded by the isolation of a "later" intermediate lacking the 14-C hydroxyl group from vitellogenic ovaries of *L. migratoria* (2,14,22,25-tetra-deoxy-ecdysone) (Hetru et al., 1978a; Hetru et al., 1982). A later study questioned the intermediacy of 2,14,22,25-tetra-deoxy-ecdysone given the lack of metabolism of this precursor to terminal ecdysteroids by this same

tissue (Haag et al., 1987). The timing of 14-hydroxylation and A/B ring modifications has been discussed in reviews (e.g., Rees, 1985; Rees, 1989; Grieneisen, 1994; Rees, 1995; Gilbert et al., 2002).

The ecdysteroid precursor following the "black box" modifications has alluded characterization, and the identity of the functional group on 3-C has been of much speculation (Grieneisen, 1994; Gilbert et al., 2002). The possibility of a 3-oxo-Δ^4 intermediate (**3**) following "black box" modifications has been addressed through both the metabolism of radiolabeled precursors by *S. gregaria* ovaries (Davies et al., 1981; Rees, 1985; Fujimoto et al., 1989), and the metabolism of such a 3-oxo-Δ^4 intermediate by *C. maenas* Y-organ preparations (Blais et al., 1996). When provided the 3-oxo-Δ^4 intermediate, *C. maenas* Y-organ preparations condutced a 5β-reduction, and the 5β-reductase (**B**) responsible was cytosolic requiring NADPH as a cofactor (Blais et al., 1996). No such 5β-reductase has yet been characterized from insects, but is a probable step following the "black box" (Gilbert et al., 2002).

The retention of the 3-oxo is a feature of ecdysteroids secreted by lepidopteran prothoracic glands (Kiriishi et al., 1990; Grieneisen, 1994) and the Y-organ of many crustaceans (see Dauphin-Villemant et al., 1997 and citations therein). The enzyme that conducts the 3β-reduction of secreted 3-oxo ecdysteroids has been characterized in numerous lepidopterans and later identified as 3-dehydroecdysone-3β-reductase (**G**) in *S. frugiperta* (Kiriishi et al., 1990; Chen et al., 1999). The fact that both 5β-diketol (**4a**) and its 3β-reduced product 5β-ketodiol (**4b**) have been isolated and metabolically converted by insect gonads (see Table 1), and the lack of 3β-reductase (**C**) activity in the hemolymph of non-lepidopteran insects (Kiriishi et al., 1990) strongly suggests that 3β-reduction may occur within gonads of various insect orders.

The subsequent hydroxylation reactions made to either 5β-diketol (**4a**) or 5β-ketodiol (**4b**) have been extensively studied (Kappler et al., 1989; Rees, 1995), and the hydroxylases responsible for these modifications represent the first proteins involved in the biosynthesis of ecdysteroids to be characterized fully (Warren et al., 2002; Petryk et al., 2003; Gilbert, 2004; Niwa et al., 2004; Warren et al., 2004). As early studies on enzymatic activity indicated (Meister et al., 1985; Kappler et al., 1986; Kappler et al., 1988), the identified cytochrome P450 enzymes conduct their hydroxylation reactions in a preferred sequence C25 → C22 → C2 (Warren et al., 2002; Niwa et al., 2004; Warren et al., 2004). With the isolation of various "terminal" ecdysteroids lacking one or more of these hydroxylated carbons from arthropod ovaries and eggs (Hoffman and Lagueux, 1985; Subramoniam, 2000; Sonobe and Yamada, 2004), the sequence of "terminal hydroxylation" reactions may not be steadfast. An example of such an "alternative" biosynthetic pathway is observed in *B. mori* in which ovarian ecdysteroids possess a hydroxyl group at C-20 ("final hydroxylation," see below) but lack hydroxyl groups at C-2 or C-22 (Ohnishi et al., 1977; Mizuno et al., 1981; Ohnishi et al., 1981). This observation has lead some investigators to purpose an alternative biosynthetic pathway that would result in a different sequence of "terminal hydroxylations" (Ohnishi et al., 1981; Sonobe and Yamada, 2004). As for the current review, the widely accepted privileged sequence (C-25 → C-22 → C-2) will be followed.

The cytochrome P450 enzymes conducting the "terminal hydroxylation" reactions of ecdysteroid biosynthesis have been localized to the ER (25-hydroxylase, CYP306a1) (**D**) or mitochondria (22-hydroxylase, CYP302a1) (**E**) (2-hydoxylase, CYP315a1) (**F**) (Petryk et al., 2003; Warren et al., 2004), further corroborating that ecdysteroid biosynthesis requires the transfer of ecdysteroid precursors between these organelles during processing (Kappler et al.,

1989; Rees, 1995; Gilbert et al., 2002). An increase in gene transcripts encoding the three cytochrome P450 enzymes conducting the "terminal hydroxylation" reactions correlates with increases in ecdysteroid titers during both embryonic and post embryonic development of *D. melanogaster* and *B. mori* (Chavez et al., 2000; Warren et al., 2002; Niwa et al., 2004; Warren et al., 2004; Parvy et al., 2005). Gene transcripts of 25-hydroxylase (CYP306a1) and 2-hydroxylase (CYP315a1) localize to both nurse and follicle cells of *D. melanogaster* ovarioles (Warren et al., 2002; Niwa et al., 2004; Warren et al., 2004), whereas gene transcripts for 22-hydroxylase (CYP302a1) were observed only within ovariole follicle cells of *D. melanogaster* (Chavez et al., 2000). Homologs of CYP302a1 and CYP315a1 were recently identified from the ovaries of *A. aegypti*, and gene transcripts encoding these cytochrome P450s were highest during peak ovarian ecdysteroid production following a blood meal, suggesting that ovary ecdysteroid production within *A. aegypti* may be modulated at the transcript level (Sieglaff et al., 2005). Indeed, it has been recently determined that the transcription factor, βFTZ-F1, regulates the expression of 25-hydroxylase and 22-hydroxylase in the ecdysteroidogenic tissue of larval *D. melanogaster*, ring glands (Parvy et al., 2005). The regulation of ecdysteroidogenic cytochrome P450 expression by βFTZ-F1 is concluded to be analogous to the regulation of steroidogenic cytochrome P450 expression by its vertebrate homolog, steroidogenic factor 1 (Parker and Schimmmer, 1997; Parker et al., 2002).

Enzymes responsible for providing reducing equivalents to cytochrome P450 enzymes in the ER and mitochondrial have been identified in insects, and they are required for ecdysteroid biosynthesis (Freeman et al., 1999; Horike et al., 2000). The enzyme responsible for providing reducing equivalents to mitochondrial cytochrome P450 enzymes, adrenodoxin reductase, is expressed in the nurse cells of *D. melanogaster* ovarioles (Freeman et al., 1999), whereas the

ovariole localization of the enzyme responsible for providing reducing equivalents to microsomal (ER) cytochrome P450 enzymes, cytochrome P450 enzymes NADPH-cytochrome P450 oxidoreductase, was not addressed in its original characterization (Horike et al., 2000). Whether either of these enzymes is expressed in testes has not been determined.

The "final" hydroxylation reaction at C-20 is the most studied reaction of ecdysteroid biosynthesis, and generally occurs in tissues peripheral to the original source of ecdysteroid biosynthesis (Smith, 1985). It is widely accepted that this reaction creates the most bioactive form of ecdysteroid, 20-hydroxyecdysone (**8**). This reaction is common to all arthropods, because 20-hydroxylated ecdysteroids are observed in species throughout Arthropoda (Gupta, 1990; Lachaise, 1990). 20-hydroxylase activity has been localized to both ER and mitochondrial preparations, depending on the species or tissue under study (Smith, 1985; Rees, 1995). The cytochrome P450 enzyme CYP314a1 of *D. melanogaster* was recently identified as a 20-hydroxylase (**H**), and its transcripts were observed primarily within tissues peripheral to the original source of ecdysteroids, the ring glands (e.g., Malpighian tubules, fat body) (Petryk et al., 2003). Gene transcripts of CYP314a1 were observed in both ovariole nurse and follicle cells, and its expression was shown to be required for oogenesis (Petryk et al., 2003). *D. melanogaster* CYP314a1 localizes to the mitochondria along with CYP302a1 and CYP315a1 (Petryk et al., 2003). A homolog of CYP314a1 was recently identified from the ovaries of *A. aegypti*, and in contrast to *A. aegypti* CYP302a1 and CYP315a1, gene transcript abundance of CYP314a1 did not increase in ovaries following a blood meal (Sieglaff et al., 2005). This observation agrees with the lack of significant 20-hydroxylase activity in *A. aegypti* ovaries following a blood meal (Smith and Mitchell, 1986), and the fact that ecdysone is the form of ecdysteroid secreted by *A. aegypti* ovaries (Hagedorn et al., 1975).

Metabolism of ecdysteroids by arthropod gonads

Ecdysteroids isolated from arthropod eggs are commonly conjugated to phosphate esters, long chain fatty acids (acylation), or glucosides, which represent either storage forms to be used later in embryonic development or inactivated waste products (Koolman, 1982; Thompson et al., 1990). Further modifications include hydroxylation at C-26, epimerization, and formation of ecdysteroid acids, which are generally viewed as inactivation processes (Lafont and Connat, 1989; Thompson et al., 1990). In insects, these conjugates can represent from 80-95% of ovary or egg ecdysteroids (Hsiao and Hsiao, 1979; Dinan and Rees, 1981a). In many insect species, such conjugated ecdysteroids represent a fraction of that synthesized by ovaries, and instead the majority of ovarian ecdysteroids are secreted into the hemolymph for the promotion of developmental events in other tissues (e.g., vitellogenin production by the fat body) (Hagedorn, 1985). These conjugated ecdysteroids have been characterized as high or low polars fractions, and such fractions have been isolated from the ovaries of crustaceans (Lachaise et al., 1981; Lachaise and Lafont, 1984; Wilder et al., 1990; Wilder et al., 1991; Suzuki et al., 1996; Subramoniam, 2000), Chelicerata (Diehl et al., 1986; Lomas and Rees, 1998; Rees, 2004), a myriapod (Leubert et al., 1982), insects (Lagueux et al., 1977; Hsiao and Hsiao, 1979; Dinan and Rees, 1981b; Rees and Isaac, 1985; Kappler et al., 1986; Slinger and Isaac, 1988; Thompson et al., 1990; Whiting et al., 1997; Tawfik et al., 1999; Sonobe and Yamada, 2004), and testes of insects (Hoffmann, 1982; Loeb et al., 1982). In *L. migratoria*, conjugation of ecdysteroids synthesized by the follicle cells takes place in the oocyte (i.e., ooplasm) (Kappler et al., 1986).

To identify ecdysteroids conjugates, isolated ecdysteroid fractions are treated with various enzyme mixtures that liberate the ecdysteroid for subsequent identification; e.g., enzyme cocktail of the snail *Helix pomatia*, phosphatases, and sulfatases (Hetru et al., 1985; Rees and

Isaac, 1985). Metabolism of arthropod ecdysteroids has been the subject of reviews (Koolman, 1982; Rees and Isaac, 1985; Lafont et al., 1986; Isaac and Slinger, 1989; Lafont and Connat, 1989; Lachaise, 1990; Thompson et al., 1990; Lomas and Rees, 1998), and this means of regulating ecdysteroid titers is suggested to occur throughout Arthropoda (Connat and Diehl, 1986; Lafont et al., 1986; Lafont and Connat, 1989).

Conjugated ecdysteroids within arthropod eggs are viewed as either maternal contributions for embryonic development or a means to sequester bioactive ecdysteroids (Lafont and Connat, 1989; Thompson et al., 1990; Lomas and Rees, 1998; Subramoniam, 2000). An association between embryonic development and commitment increase in free ecdysteroids (i.e., following hydrolysis) has been made for insects (Sall et al., 1983; Isaac and Rees, 1985; Tawfik et al., 1999; Sonobe and Yamada, 2004), but such a defined correlation between free ecdysteroids and embryonic development has not been observed for other arthropods such as crustaceans and acarines (Lomas and Rees, 1998; Subramoniam, 2000). Recently, an enzyme that releases stored ecdysteroid conjugates in *B. mori* eggs, ecdysteroid-phosphate phosphatase (EPPase), was identified and its activity correlated with both an increase in free ecdysteroid titers and embryonic development (Yamada and Sonobe, 2003). The observed increase in EPPase activity was associated with increases in abundance of its gene transcripts, suggesting regulation of its activity occurs at the transcriptional level.

Vertebrate-type steroids in the gonads of arthropods

Steroid hormones such as estradiol, testosterone, pregnenolone, and progesterone have been isolated from the ovaries of many arthropods (Ohnishi et al., 1985; Novak and Lambert, 1989; Bradbrook et al., 1990; Swevers et al., 1991a; Quinitio et al., 1994; Darvas et al., 1997). The significance of vertebrate-type steroids in Crustacea has been implicated in various studies

that observed a correlation between increasing steroid levels and reproductive developmental events such as vitellogenesis (Subramoniam, 2000). Such a function in insects, however, has been questioned (Ogiso and Ohnishi, 1986; Darvas et al., 1997; De Loof and Huybrechts, 1998). Enzymatic activity specific to the biosynthesis of vertebrate-type steroids has been observed in the gonads of insects and crustaceans (Young et al., 1992; Ghosh and Ray, 1993; De Loof and Huybrechts, 1998), but under closer review, enzymatic activity specific to the synthesis of vertebrate-type steroids was minimal or lacking in insect gonads (Swevers et al., 1991a; Swevers et al., 1991b). Swevers et al (1991a) suggest further that some of the observed enzymatic reactions may simply be a non-specific detoxification of xenobiotics. According to De Loof and Huybrechts (1998), the study of vertebrate-type steroids in insects is no longer an active area of research, and its significance needs much more clarification.

Regulation of ecdysteroid biosynthesis in arthropod gonads

The regulation of ecdysteroid biosynthesis in arthropod gonads has only been addressed in insects, and is no doubt related to the lack of evidence for ecdysteroid biosynthesis by gonads in non-insect arthropods. Regulation of ecdysteroid biosynthesis in prothoracic glands by the insect prothoracicotropic hormone (PTTH) and Y-organ by the crustacean molt-inhibiting hormone (MIH) has been addressed in recent reviews (Gilbert et al., 1996; Gilbert et al., 1997; Henrich et al., 1999; Spaziani et al., 1999; Gilbert et al., 2002; Gilbert, 2004), and again will only play a supportive role in the current discussion.

Gonadotropins that regulate ecdysteroid biosynthesis in insect gonads

Various gonadotropic hormones have been isolated from insects, but only a few have shown the ability to stimulate gonadal ecdysteroid biosynthesis directly or have been inferred by indirect evidence (De Loof et al., 2001). It has long been known that factors housed within cells

of insect brains and associated neurohemal organs regulate ovary maturation (Thomsen, 1952; Lea, 1967; Lea, 1972), and such a brain factor(s) from *A. aegypti*, termed egg development neurosecretory hormone (EDNH), was the first gonadotropin shown to stimulate ovary ecdysteroid production *in vitro* (Hagedorn et al., 1979). The *A. aegypti* EDNH was eventually identified, and is now called Ovary Ecdysteroidogenic Hormone I (OEH I) (Brown et al., 1998). OEH I was shown to be expressed within medial neurosecretory cells (MNC) of the *A. aegypti* brain (Brown et al., 1998), and the same cells were shown nearly thirty years earlier to be obligatory for ovary maturation in *A. aegypti* (Lea, 1967). Following a blood meal, the MNC of *A. aegypti* release OEH I which stimulates the ovaries to produce ecdysteroids (Hanaoka and Hagedorn, 1980; Greenplate et al., 1985).

Other gonadotropins have been identified in a few insect species, and either display direct evidence of stimulating ecdysteroid biosynthesis by gonads, indirectly through its ability to stimulate vitellogenesis (an event regulated by 20-hydroxyecdsone produced by the ovary), or the ability of unidentified chromatography fractions to stimulate gonadal ecdysteroid biosynthesis; example gonadotropins for these three cases are *L. dispar* testis ecdysiotropin (LTE), *L. migratoria* ovary maturing parsin (Lom OMP), and *Musca* ecdysteroidogenin (ESG), respectively (Girardie and Girardie, 1996; Adams et al., 1997; Wagner et al., 1997). Heterologous peptide hormones are also capable of stimulating ecdysteroid biosynthesis by insect gonads, such as bovine and porcine insulin acting on the ovaries of *A. aegypti* and *P. regina* (Brown et al., 1995; Graf et al., 1997; Riehle and Brown, 1999; Maniere et al., 2004), bombyxin II on the ovaries of *P. regina* (Maniere et al., 2004), and bovine angiotensin II acting on the testes of *L. dispar* (Loeb et al., 1998).

The trophic hormone that stimulates ecdysteroid biosynthesis in prothoracic glands of immature insects, prothoracicotropic hormone (PTTH), has been shown to regulate ecdysteroid biosynthesis through gene transcription, translation and phosphorylation events (Smith and Gilbert, 1989; Keightley et al., 1990; Rybczynski and Gilbert, 1994; Song and Gilbert, 1995; Rybczynski and Gilbert, 1995a; Gilbert et al., 1997; Song and Gilbert, 1997). In crustaceans, MIH regulation of Y-organ ecdysteroid biosynthesis also has been shown to occur at the levels of translation and post-translation (Lachaise and Somme, 1998; Sedlmeier and Siensche, 1998). Whether gonadotropin stimulated ecdysteroid biosynthesis in insect gonads is regulated at the gene transcription, translation or post-translational level has not yet been established. The means by which gonadotropins regulate gonadal ecdysteroid biosynthesis *in vivo* is also not known. Whether, gonadal ecdysteroid biosynthesis is regulated through pulsatile release of gonadotropins (Mizoguchi et al., 1990; Gilbert et al., 1996), through negative regulation by an ecdysteroidostatic hormone (Kelly et al., 1984; Hua et al., 1994; Adams and Li, 1998; Hua et al., 1999), or modulated by juvenile hormone (Birnbaum et al., 1984), needs further clarification. There are many studies that indirectly established the obligatory nature of gonadotropins in stimulating gonadal ecdysteroid biosynthesis and development of gonadal tissues (e.g., Hoffmann et al., 1980; Greenplate et al., 1985; Girardie and Girardie, 1996). Interestingly, the mosquito ovary itself may regulate its own biosynthesis of ecdysteroids by eliciting the release of OEH I (EDNH) from the MNC of the brain (Borovsky, 1982; Lea and Van Handel, 1982), but this ovary derived OEH I-releasing factor(s) has not yet been identified.

Signal transduction pathways regulating ecdysteroid biosynthesis in insect gonads

Studies of the signal transduction pathways regulating ovarian ecdysteroid biosynthesis have focused on the three species of Diptera *A. aegypti*, *P. regina*, and *D. melanogaster*.

Gonadotropic factors have been shown to elicit ovary ecdysteroid biosynthesis through cAMP-dependent and cAMP-independent signal transduction pathways (Shapiro, 1983; Maniere et al., 2000; Maniere et al., 2004). It has been determined that the cAMP-independent pathway involves insulin signaling, as demonstrated through both genetic studies and *in vitro* assays (Graf et al., 1997; Riehle and Brown, 1999; Tatar et al., 2001; Tu et al., 2002; Maniere et al., 2004). A single moth species (*L. dispar*) has been used to study ecdysteroid biosynthesis in testes and its regulation appears very complex, potentially involving multiple signaling pathways (Loeb et al., 1993; Loeb et al., 1994; Loeb et al., 1998; Loeb et al., 2001).

cAMP-dependent mechanism(s)

Two studies implicate cAMP-dependent mechanisms mediating ovarian ecdysteroid biosynthesis in insects: factors isolated from *A. aegypti* and *P. regina* heads promote cAMP accumulation within ovaries, and cAMP analogues can induce ovary ecdysteroid biosynthesis *in vitro* (Shapiro, 1983; Maniere et al., 2000). The gonadotropin factor stimulating ovary ecdysteroid biosynthesis through a cAMP-dependent mechanism resides outside of the pars intercerebralis (PI) of *P. regina* brains (Maniere et al., 2000). Interestingly, cells within the PI are implicated in insulin-like regulation of ovary ecdysteroid biosynthesis in *P. regina* (see below; Maniere et al., 2004). It has been established that cAMP-dependent ovarian ecdysteroid biosynthesis in *P. regina* is mediated through protein kinase A (PKA), as defined by the ability of Rp-cAMPs, a PKA antagonist, to inhibit ovarian ecdysteroid biosynthesis (Maniere et al., 2000). The signal transduction pathways through which cAMP or PKA flow are complex and not easily defined (Robinson-White and Stratakis, 2002), as is observed in steroidogenesis in the mammalian ovary (Wood and Strauss III, 2002). Thus, further research is required before a more

complete picture of the cAMP-dependent signal transduction mechanisms elicited by insect gonadotropins can be attained.

cAMP-independent mechanism(s)

Prior to the observation that ovarian ecdysteroid biosynthesis in *P. regina* can occur through a cAMP-independent mechanism (Maniere et al., 2000), heterologous insulins were shown to stimulate ecdysteroid biosynthesis by *A. aegypti* ovaries (Brown et al., 1995; Graf et al., 1997). Further studies firmly established the mechanism of insulin signaling in ovarian ecdysteroid biosynthesis in *A. aegypti* through use of pharmacological agents that disrupt this signaling pathway (Riehle and Brown, 1999). Such pharmacological disruptors would later establish the insulin signaling pathway as the cAMP-independent regulator of ecdysteroid biosynthesis in *P. regina* ovaries (Maniere et al., 2004). These pharmacological disrupters further suggest that insulin acts through a receptor tyrosine kinase (RTK) and phosphatidylinositol 3-kinase (PI3-K) for ovarian ecdysteroid biosynthesis in both *A. aegypti* and *P. regina* (Riehle and Brown, 1999; Maniere et al., 2004). Riehle and Brown (1999) excluded the involvement of mitogen-activated protein kinase (MAP), but no such restriction has yet been made in *P. regina* ovarian ecdysteroid biosynthesis. A later study showed that insulin treatment of *A. aegypti* ovaries elicites phosphorylation of a downstream component of PI3-K signaling, protein kinase B (PKB) (Riehle and Brown, 2003), an established downstream target in mammalian ovary steroidogenesis (Wood and Strauss III, 2002).

The first insulin-like peptide to be identified and characterized in insects was bombyxin, and now thirty-two separate *B. mori* bombyxin genes have been identified, comprising seven distinct families (Iwami, 2000). Bombyxin is expressed in the MNC of *B. mori* brains (Iwami, 2000), and recently, the MNC of *Anopheles gambiae* have been shown to contain peptides that

are recognized by anti-bombyxin II antibodies (Krieger et al., 2004). The MNC of *P. regina* are implicated in the insulin-like stimulation of ovary ecdysteroid biosynthesis, and its was observed that bombyxin II can stimulate ovarian ecdysteroid biosynthesis *in vitro* (Maniere et al., 2004). Bombyxin II-specific receptors have been observed in the ovaries of numerous lepidopteran insects (Fullbright et al., 1997a; Fullbright et al., 1997b). The bombyxin II receptor in *S. frugiperda* 9 ovarian cells (Sf9) disassociates into 90 and 116 kDa subunits when treated with the reducing agent dithiothreitol, resembling the molecular weights predicted for the reduced form of the *A. aegypti* mosquito insulin receptor (MIR) (Riehle and Brown, 2002). The expression of MIR is predominantly localized to the follicle cells of *A. aegypti* ovarioles (Riehle and Brown, 2002), the assumed source of ovariole ecdysteroids in insects (Goltzene et al., 1978; Zhu et al., 1983). The expression of MIR within follicle cells begins to diminish 24 -36 h PBM (Riehle and Brown, 2002), when ovarian ecdysteroid production starts to decline in blood-fed *A. aegypti* (Sieglaff et al., 2005). The pulsatile release of insulins such as bombyxin (Mizoguchi et al., 1990), and the temporal expression of their receptor (Riehle and Brown, 2002) may represent a means of regulating ecdysteroid production by insect ovaries. The observation that the ovaries of *D. melanogaster* mutants possessing lesions to specific components of the insulin-signaling cascade synthesize less ecdysteroids lends support for this hypothesis (Tatar et al., 2001; Tu et al., 2002).

Numerous physiological functions have been attributed to the action of insulin and insulin-like peptides in insects (Claeys et al., 2002), a feature well known in the mammalian literature (Ferrannini et al., 1999; Poretsky et al., 1999). Though it has been generally accepted that insulin stimulation of mammalian steroidogenesis occurs through its synergistic action with the primary gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH)

(Sekar et al., 2000; Wood and Strauss III, 2002), its capability to promote mammalian steroidogenesis alone has been observed (Poretsky and Kalin, 1987; Nestler, 1998; Seto-Young et al., 2003).

Inhibition of ovarian ecdysteroid biosynthesis

One of the more intriguing findings on the regulation on *P. regina* ovary ecdysteroid biosynthesis is the inhibitory effect of calcium (Maniere et al., 2002). Calcium plays an essential role in PTTH stimulation of ecdysteroid biosynthesis in lepidopteran prothoracic glands (Girgenrath and Smith, 1996; Dedos and Fugo, 1999; Gilbert et al., 2000; Dedos and Fugo, 2001; Rybczynski and Gilbert, 2003). Such a requirement for calcium has also been observed in brain factor stimulation of ecdysteroid biosynthesis in *D. melanogaster* ring glands (Henrich, 1995). Ecdysteroid biosynthesis in the crustacean Y-organ also requires the presence of this ion (Sedlmeier and Siensche, 1998; Spaziani et al., 1999). The effect of calcium on PTTH stimulated ecdysteroid biosynthesis is hypothesized to occur through calcium-calmodulin-dependent adenylate cyclase, for *in vitro* stimulation of prothoracic glands by PTTH requires calcium within the incubation media whereas stimulation by cAMP analogs can be accomplished in a calcium-free medium (i.e., bypassing calcium-calmodulin-dependent adenylate cyclase) (Gilbert et al., 1996). Maniere et al. (2002) further showed that calcium's negative effect on *P. regina* ovarian ecdysteroid biosynthesis is achieved through its promotion of cAMP degradation by a calcium-calmodulin phosphodiesterase I (PDE I).

Other negative regulators of *P. regina* ovarian ecdysteroid biosynthesis are cyclic guanosine monophosphate (cGMP), cyclic GMP-dependent protein kinase (PKG), and possibly nitric oxide (NO) (Maniere et al., 2003). The factor regulating the inhibitory cGMP pathway was not derived from *P. regina* brains (Maniere et al., 2003), suggesting the factor may have an

abdominal origin as observed for ecdysteroidostatin in the housefly (Adams and Li, 1998). Interestingly, MIH-inhibition of Y-organ ecdysteroid biosynthesis is also mediated through cGMP (Sedlmeier and Siensche, 1998; Spaziani et al., 1999), and recently NO synthase (NOS) has been identified in a crustacean (Kim et al., 2004). Unfortunately, Kim et al. (2004) did not address NOS activity as it pertains to Y-organ ecdysteroid biosynthesis. Of relevance to the chapter topic, transcripts of this crustacean NOS were observed in both testis and ovaries, along with other non-gonadal tissues (Kim et al., 2004), strongly questioning its exclusive function in ecdysteroid biosynthesis.

Regulation of ecdysteroid biosynthesis in testes

Various signal transduction pathways have been implicated in the regulation of ecdysteroid biosynthesis stimulated by testis ecdysiotropin (TE) (Loeb et al., 2001). *L. dispar* testis ecdysiotropin (LTE) elicits G-protein coupled receptor (GPCR) cascades in promoting testes ecdysteroid biosynthesis with G_i, inositol triphosphate (IP_3), diacylglycerol (DAG) to protein kinase C (PKC) representing the primary cascade (Loeb, 1991b; Loeb et al., 1993; Loeb et al., 1994). A cascade involving PKA has also been implicated in the promotion of testes ecdysteroid biosynthesis by LTE, but its involvement appears to function only during the larval stage and not in the pupae (Loeb et al., 1994). Loeb et al. (1994) attribute this difference to intrinsic ecdysteroid biosynthesis by pupal testes (i.e., the testes have already been stimulated by LTE *in vivo* prior to their dissection from the animal). cAMP at high doses is inhibitory to ecdysteroid biosynthesis in testes stimulated by LTE (Loeb et al., 1993), and this cAMP-mediated inhibitory mechanism was later attributed to the action of angiotensin II (Loeb et al., 1998). LTE regulation of ecdysteroid biosynthesis in testes is thus postulated to be coordinated

through G_i and G_s signal transduction cascades (Loeb et al., 2001), but requires more experimentation to derive a clearer picture of all the interweaving signal transduction pathways.

Ecdysteroid biosynthesis by *A. aegypti* ovaries

The ovary of *A. aegypti* was one of the first tissues in adult arthropods shown to produce ecdysteroids (Hagedorn et al., 1975), and this observation led to investigations into ecdysteroid biosynthesis in the gonads of many other arthropod species. Since the discovery, much work has been done to decipherer the developmental events promoted by the ovarian ecdysteroid hormones, but very little has been done to address their actual biosynthesis. The majority of research on ecdysteroid biosynthesis and its regulation has been conducted with the prothoracic glands of *M. sexta* and the ring glands of *D. melanogaster* (Grieneisen, 1994; Gilbert et al., 2002; Gilbert, 2004; Parvy et al., 2005). The ovaries of *A. aegypti* comprise an exceptional model for studying ecdysteroid biosynthesis. As opposed to other insect models, a blood meal taken by adult female *A. aegypti* initiates ovary ecdysteroid biosynthesis *in vivo* which excludes all the complications that arise from *in vitro* assays or studies that stage ecdysteroidogenic activity based on known whole body titers (Hagedorn et al., 1975; Greenplate et al., 1985; Sieglaff et al., 2005).

The regulation of ecdysteroid biosynthesis in *M. sexta* prothoracic glands occurs at three levels: gene transcription, translation and post-translational modifications such as phosphorylation (Smith and Gilbert, 1989; Keightley et al., 1990; Rybczynski and Gilbert, 1994; Song and Gilbert, 1995; Song and Gilbert, 1997; Gilbert et al., 2000; Smith et al., 2003). Although gonadotropic stimulation of ecdysteroid biosynthesis in insect gonads has received much attention, the regulation of this biosynthetic process at the transcription or translational levels has not yet been addressed. Female *A. aegypti* provide an exceptional model to begin to

address these two fundamental questions because of the ready initiation of this biosynthetic process following a blood meal. The following chapters of this dissertation attempt to answer whether gene transcription or protein translation is the means by which ovarian ecdysteroid biosynthesis is regulated in blood-fed *A. aegypti*.

Chapter 2: *Expression of genes encoding proteins involved in ecdysteroidogenesis in the female mosquito, Aedes aegypti*.

To determine first whether the transcription of specific genes is a regulated step in ovarian ecdysteroid biosynthesis in *A. aegypti*, homologs for gene transcripts that encode proteins involved in ecdysteroid biosynthesis in other insects were identified from *A. aegypti* ovaries. The expression of these gene transcripts within ovaries before, during and after ovarian ecdysteroid production by blood-fed females would indicate whether the transcription of specific genes is requisite for ecdysteroid production (i.e., a regulated step). Further, gene transcripts that encode proteins involved in ecdysteroid biosynthesis have been reported to be expressed exclusively within so-called ecdysteroidogenic tissues, prothoracic glands of immature insects and adult ovaries (Warren et al., 2002; Niwa et al., 2004), a means of defining an ecdysteroid producing tissue. The expression of gene transcripts of the *A. aegypti* homologs within various tissues and the amount of ecdysteroids produced by these tissues in comparison to *A. aegypti* ovaries may indicate whether exclusivity of gene transcript expression is a means by the ovary becomes the primary ecdysteroidogenic tissue in blood-fed *A. aegypti*.

Chapter 2 appendix: *Ecdysteroid production by Aedes aegypti ovaries following in vitro stimulation by bovine insulin and a cAMP analog: effects on gene transcripts encoding proteins putatively involved in ecdysteroidogenesis*

Bovine insulin and a cAMP analog have the ability to stimulate ovarian ecdysteroid production by *A. aegypti in vitro* (Shapiro, 1983; Riehle and Brown, 1999). Whether the promotion of gene transcription is the means by which insulin or cAMP stimulates ovarian ecdysteroid biosynthesis in *A. aegypti* was answered by determining whether there is a correlation between ovarian ecdysteroid production *in vitro* and an increase in transcript abundance for any of the genes identified and characterized in chapter 2.

Chapter 3: *Expression of the cytochrome P450 enzyme putatively conducting the 22-hydroxylaztion reaction in Aedes aegypti ecdysteroid biosynthesis within ovaries during a gonotropic cycle*

To address whether ovarian ecdysteroid biosynthesis is regulated by increasing the "machinery" or enzymes of ecdysteroid biosynthesis, a protein involved in biosynthesis of ecdysteroids encoded by one of the gene transcripts identified and characterized in Chapter 2 was studied at the protein level in ovaries of blood-fed females. Gross changes in protein levels can be addressed through immunoblot analysis, and a more detailed analysis of expression within the ovariole can be determined with confocal microscopy.

Chapter 4: *Expression and localization of Start1 immunoreactivity in the ovaries of the yellow fever mosquito, Aedes aegypti*

To further establish the relationship between protein translation and intracellular sterol trafficking, the proposed related step of ecdysteroid biosynthesis (Grieneisen, 1994; Warren and Gilbert, 1996b), a putative sterol transfer protein encoded by one of the gene transcripts identified and characterized in chapter 2 could be studied using the same methods suggested above.

The results received from all four dissertation chapters may shed let on the regulation of ovarian ecdysteroid biosynthesis in blood-fed *A. aegypti*.

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Figure 1.1. A generalized pathway for ecdysteroid biosynthesis occurring in arthropod gonads, where red colored reaction stars indicate a cytochrome P450 mediated enzymatic reaction and the blue colored stars a reductase mediated enzymatic reaction. (**1**) cholesterol is converted to (**2**) 7-dehydrocholesterol by the microsomal cytochrome P450, (**A**) 7,8-dehydrogenase (Grieneisen et al., 1993; Warren et al., 1996a); (**2**) 7-dehydrocholesterol is then transferred to the mitochondria and goes through a series of "black box" reactions being converted to the hypothetical 3-oxo-Δ^4 intermediate (**3**) 14α-hydroxy-cholesta-4,7-diene-3,6-dione; (**3**) 14α-hydroxy-cholesta-4,7-diene-3,6-dione is then converted to (**4a**) 5β-diketol by the cytosolic enzyme (**B**) 5β-reductase (Blais et al., 1996); two pathways may now occur from this point forward, (**4a**) 5β-diketol moves forward to the first of the "terminal hydroxylation" reactions, or is converted to (**4b**) 5β-ketodiol by a yet unidentified (**C**) 3β-reductase; both ecdysteroid precursors would then be converted by the microsomal cytochrome P450, (**D**) 25-hydroxylase (CYP306a1) (Niwa et al., 2004; Warren et al., 2004) yielding (**5a**) 2,22-dideoxy-3-dehydroecdysone for "**4a**" and (**5b**) 2,22-dideoxyecdysone for "**4b**" both ecdysteroid precursors are then transferred back to the mitochondria where they are converted by the mitochondrial P450, (**E**) 22-hydroxylase (CYP302a1) (Chavez et al., 2000; Warren et al., 2002; Petryk et al., 2003) to (**6a**) 2-deoxy-3-dehydroecdysone for "**5a**" and (**6b**) 2 -deoxyecdysone for "**5b**"; both ecdysteroid precursors would then be converted by the mitochondrial P450, (**F**) 2-hydroxylase (CYP315a1) (Warren et al., 2002; Petryk et al., 2003) to (**7a**) 3-dehydroecdysone for "**6a**" and (**7b**) ecdysone for "**6b**"; both ecdysteroids would then be released into the cytosol in which "**7a**" would be converted to "**7b**" by (**G**) 3-dehydroecdysone 3β-reductase (Chen et al., 1999); "**7b**" would be then be secreted by the

"ecdysial" tissue and converted to (**8**) 20-hydroxyecdysone by the mitochondrial P450, (**H**) 20-hydroxylase (CYP314a1) (Petryk et al., 2003) within the cells of the target tissue.

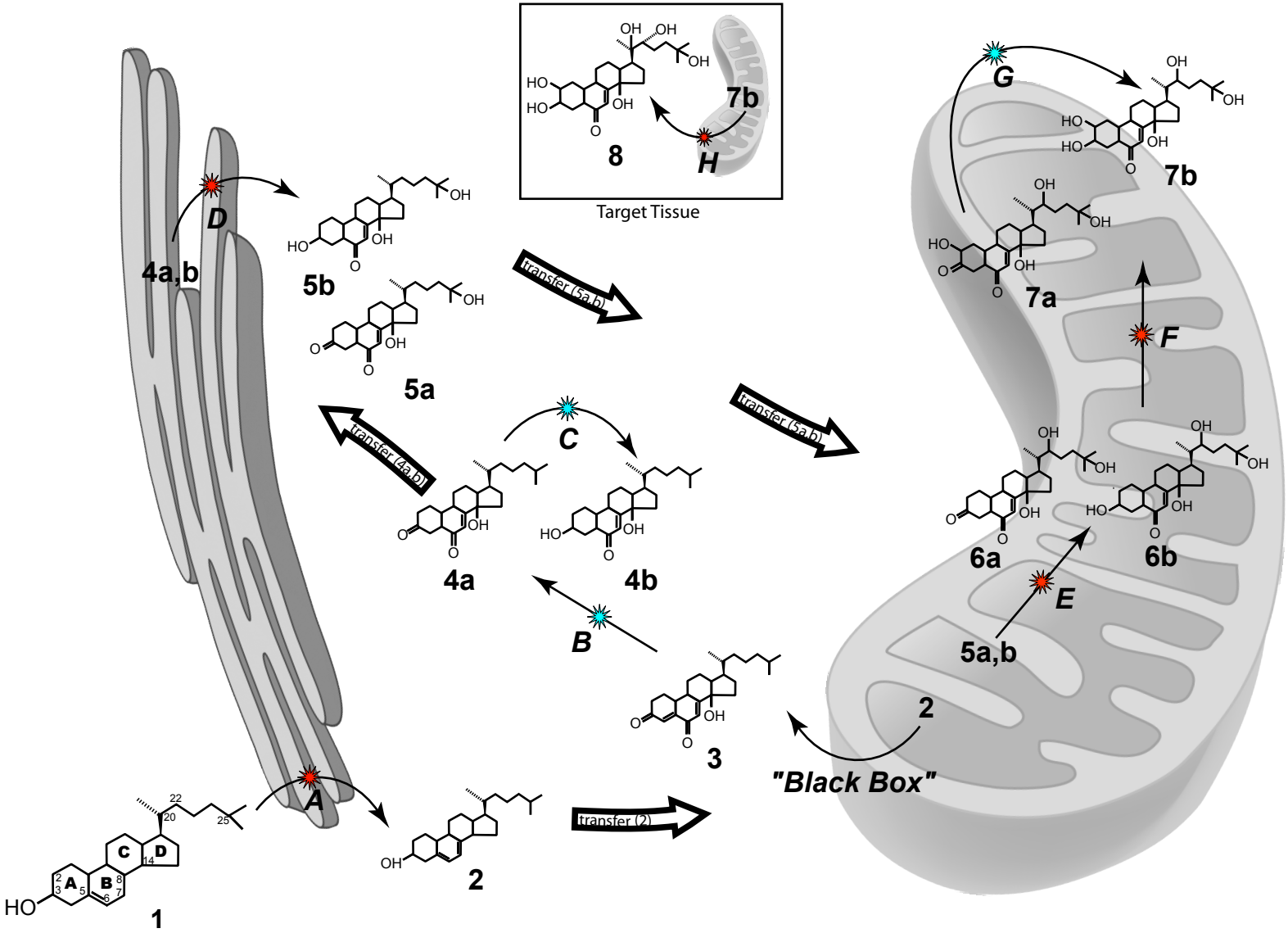
8
7b
H
Target Tissue
4a,b
D
5b
5a
transfer (5a,b)
transfer (4a,b)
C
4a
4b
B
3
A
2
1
transfer (2)
"Black Box"
G
7b
7a
F
6a
6b
E
5a,b
2

Table 1.1. Metabolism of radiolabeled ecdysteroid precursors into terminal ecdysteroids by arthropod gonads

Ecdysteroid Precursor	Terminal Ecdysteroid(s)	Tissue	Species	Reference
cholesterol	ecdysone 20-hydroxyecdysone	ovaries, iv	*L. migratoria*	Lagueux et al. 1977 Hetru et al. 1982
cholesterol	ecdysone ecdysone, 2-deoxyecdysone	ovaries, ic	*S. gregaria*	Greenwood and Rees 1982 Greenwood et al. 1984 Davies et al. 1981 Fujimoto et al. 1989
cholesterol	ecdysone	ovaries, iv	*G. bimaculatus*	Hoffman et al. 1992
cholesterol	ecdysone, 20-hydroxyecdysone	ovaries, ic	*A. aegypti*	Borovsky et al. 1982
cholesterol	2-deoxyecdysone	ovaries, ic	*D. melanogaster*	Warren et al. 1996a
7-dehydrocholesterol	ecdysone ecdysone, 2-deoxyecdysone	ovaries, iv ovaries, ic	*L. migratoria*	Dolle et al. 1990
7-dehydro-25-hydroxycholesterol	2-deoxyecdysone	ovaries, iv	*D. melanogaster*	Warren et al. 1996a
5β-diketol	ecdysone, 2-deoxyecdysone ecdysone, 20-hydroxyecdysone	ovaries, iv	*L. migratoria*	Hetru et al. 1982 Rees 1995
5β-ketodiol	ecdysone	ovaries, iv testes, iv	*L. forficatus*	Descamps et al. 1990
5β-ketodiol	ecdysone, 2-deoxyecdysone ecdysone, 20-hydroxyecdysone	ovaries, iv	*L. migratoria*	Hetru et al. 1982 Rees 1995
5β-ketodiol	ecdysone	ovaries, iv	*G. bimaculatus*	Hoffman et al. 1992
5β-ketodiol	ecdysone, 20-hydroxyecdysone	testes, iv	*S. littoralis*	Jarvis et al. 1994
2-deoxyecdysone	ecdysone	ovaries, iv	*L. migratoria*	Kappler et al. 1986
2-deoxyecdysone	ecdysone, 20-hydroxyecdysone	testes, iv	*S. littoralis*	Jarvis et al. 1994

Note, the identity of the terminal ecdysteroid is given and not possible conjugates. iv = *in vitro* conversion of ecdysteroid precursor; ic = ecdysteroid precursor was injected within the hemocoel of the animal and metabolized ecdysteroids were isolated from eggs as free or conjugated ecdysteroids

CHAPTER 2

EXPRESSION OF GENES ENCODING PROTEINS INVOLVED IN ECDYSTEROIDOGENESIS IN THE FEMALE MOSQUITO, *Aedes aegypti*[1]

Sieglaff, D. H., Adams-Duncan, K., and Brown, M. R. 2005. Insect Biochemistry and Molecular Biology 35(5): 471-490. Reprinted here with permission of the publisher

Abstract

A blood meal induces the ovaries of female *Aedes aegypti* mosquitoes to produce ecdysteroid hormones that regulate many processes required for egg maturation. Various proteins involved in the intracellular transport and biosynthesis of ecdysteroid precursors have been identified by analysis of *Drosophila melanogaster* mutants and by biochemical and molecular techniques in other insects. To begin examining these processes in mosquito ovaries, complete cDNAs were cloned for putative orthologs of diazepam-binding inhibitor (*DBI*), StAR-related lipid transfer domain containing protein (*Start1*), aldo/keto reductase (*A/KR*), adrenodoxin reductase (*AR*), and the cytochrome P450 enzymes, *CYP302a1* (22-hydroxylase), *CYP315a1* (2-hydroxylase) and *CYP314a1* (20-hydroxylase). As shown by RT-PCR, transcripts for all seven genes were present in ovaries and other tissues both before and following a blood meal. Expression of these genes likely supports the low level of ecdysteroids produced *in vitro* (7-10 pg /tissue/ 6 h) by tissues other than ovaries. Ovaries from females not blood fed and up to 6 h post blood meal (PBM) also produced low amounts of ecdysteroids *in vitro*, but by 18 h and 30 h PBM, ecdysteroid production was greatly increased (75-106 pg /ovary pair/ 6 h) and thereafter (48 and 72 h PBM) returned to low levels. As determined by real-time PCR analysis, gene transcript abundance for *AedaeCYP302* and *AedaeCYP315a1* was significantly greater (9 and 12 fold, respectively) in ovaries during peak ecdysteroid production relative to that in ovaries from females not blood fed or 2 h PBM. *AedaeStart1*, *AedaeA/KR* and *AedaeAR* also had high transcript levels in ovaries during peak ecdysteroid production, and *AedaeDBI* transcripts had the greatest increase at 48 h PBM. In contrast, gene transcription of *AedaeCYP314a1* decreased PBM. This study shows for the first time that transcription of a few key genes for proteins

involved in ecdysteroid biosynthesis is positively correlated with the rise in ecdysteroid production by ovaries of a female insect.

Keywords: oogenesis, radioimmunoassay, real-time PCR, diazepam-binding inhibitor, Start1 aldo/keto reductase, adrenodoxin reductase, cytochrome P450 enzyme

1. Introduction

Ecdysteroids are classically viewed as molting hormones produced by the prothoracic glands of immature and pupal stages of insects. The vast tissue changes that occur during the molting process are partially regulated by ecdysteroids, which promote RNA and protein synthesis, cell proliferation and differentiation, and cell death (Oberlander, 1985; Sehnal, 1989; Buszczak and Segraves, 2000). The prothoracic glands are considered the major site of ecdysteroid synthesis in immature insects, and most research on ecdysteroidogenesis has been conducted on the prothoracic glands of Lepidoptera, primarily the tobacco hornworm, *Manduca sexta*, and the silk moth, *Bombyx mori* (Henrich et al., 1999; Gilbert et al., 2002).

The detailed ecdysteroid biosynthetic pathway is not known, but it is believed to mimic vertebrate steroidogenesis in that precursor steroid molecules shuttle between the endoplasmic reticulum and the inner mitochondrial membrane during enzymatic processing (Rees, 1995; Gilbert et al., 2002). In contrast to the ability of vertebrate animals to synthesize the steroid precursor cholesterol *de novo*, insects must obtain it from their diet (Kircher, 1982). Cholesterol absorbed by the midgut is transported to peripheral tissues via the hemolymph by a transport protein, lipophorin (Soulages and Wells, 1994). Once inside the cell, various cholesterol "transporters" have been proposed to shuttle the ecdysteroid precursor to the organelles for modification. Diazepam-binding inhibitor (DBI) and the steroidogenic acute regulatory protein (StAR) have received the most attention (Snyder and Van Antwerpen, 1998; Henrich et al., 1999; Gilbert et al., 2002; Roth et al., 2004). Cytochrome P450 enzymes catalyzing the final steps of ecdysteroid biosynthesis have been identified by mutations in the “Halloween” gene family of *Drosophila melanogaster*: *phantom* (CYP306a1; 25-hydroxylase), *disembodied*

(CYP302a1; 22-hydroxylase); *shadow* (CYP315a1; 2- hydroxylase); and *shade* (CYP314a1; 20-hydroxylase) (Chavez et al., 2000; Gilbert et al., 2002; Warren et al., 2002; Petryk et al., 2003; Gilbert, 2004; Niwa et al., 2004; Warren et al., 2004). Another *D. melanogaster* mutant, *dare*, led to the identification of adrenodoxin reductase, which provides reducing equivalents to mitochondrial cytochrome P450s (Freeman et al., 1999). In other insect species, the identification of transport proteins and enzymes involved in ecdysteroidogenesis has been accomplished by other molecular techniques, such as protein isolation and sequencing, cDNA-library screening, and PCR in combination with cloning (Lafont, 2000; Gilbert et al., 2002).

Ecdysteroid production by ovaries of female insects has been demonstrated in species from many orders, including mosquitoes (Hagedorn et al., 1975; Hoffmann et al., 1980; Hagedorn, 1985; Hagedorn, 1989). The follicle cells of the ovarioles are the site of ecdysteroid biosynthesis in *Locusta migratoria* and the cockroach, *Nauphoeta cinerea* (Zhu et al., 1983; Lanot et al., 1989), but this has not yet been determined in higher orders of insects (e.g., Diptera). Functions for ecdysteroids in female insects include the induction of meiotic reinitiation and promotion of vitellogenesis (Hoffmann et al., 1980; Hagedorn, 1985; Lanot et al., 1989). In females of the yellow fever mosquito, *Aedes aegypti*, ecdysteroids also are known to induce secondary follicle separation, thus allowing for the sequential production of multiple egg batches (Beckemeyer and Lea, 1980).

A blood meal taken by female *A. aegypti* initiates ovary ecdysteroid production (Hagedorn et al., 1975; Greenplate et al., 1985; Borovsky et al., 1986). These ecdysteroids in turn stimulate the fat body to synthesize vitellogenin (Hagedorn and Fallon, 1973; Klowden, 1997; Martin et al., 2001), which is stored in the oocyte as vitellin, the major nutrient store for the developing mosquito larva (Raikhel, 1992). Following the blood meal, total body ecdysteroid

levels begin to increase 6 h post blood meal (PBM), peak around 18 h PBM, and fall to pre-blood feeding levels by 36 h PBM, when vitellogenin uptake has ended and chorion formation begins; finally eggs are oviposited by 60-72 h PBM (Hagedorn et al., 1975; Clements, 1992; Sappington and Raikel, 1999). Ecdysone is the major ecdysteroid secreted by *A. aegypti* ovaries (Hagedorn et al., 1975), and it is converted to the more active form, 20-hydroxyecdysone, by 20-hydroxylase in peripheral tissues such as the fat body (Hagedorn et al., 1975; Smith and Mitchell, 1986). This is the only enzyme involved in ecdysteroid biosynthesis that has been partially characterized in *A. aegypti*.

To further investigate ecdysteroidogenesis in female *A. aegypti*, transcripts for seven genes known to be involved in ecdysteroidogenesis in other insects were cloned and characterized from ovary cDNA. Next, RT-PCR analysis was performed to determine whether these genes were expressed in other body regions and tissues or exclusively in ovaries of females, and whether they were expressed before and after a blood meal. Similarly, these tissues and body regions were tested for ecdysteroid production *in vitro.* Finally, real-time PCR was used to quantify changes in transcript abundance for each of the genes in ovaries during a gonotropic cycle, and thus to correlate their expression with ovary ecdysteroid production following a blood meal.

2. Materials and Methods

2.1 *Insects*

Aedes aegypti (UGAL strain) were maintained at 27°C with a photoperiod of 16 h light: 8 h dark. Larvae (ca. 200/pan) were fed a mixture of ground rat chow/lactalbumin/brewers yeast (1:1:1) daily. Adults were fed at will on a 10% sucrose solution on the third day post eclosion and were provided distilled water all other days. To initiate a gonotropic cycle, females were fed

on an anesthetized rat until engorged, separated from unfed and partially fed individuals, and maintained on distilled water until needed.

2.2 *Cloning of cDNAs*

Ovaries were dissected from non-blood fed (NBF) and blood-fed females in a saline solution (128 mM NaCl, 4.7 mM KCl, and 1.9 mM $CaCl_2$), placed directly into RNAlater™ (Sigma) incubated at 4°C overnight, and then stored at -80°C until processed. Total RNA was extracted using the RNeasy mini kit (Qiagen), and cDNA was synthesized from total RNA using superscript II™ (Invitrogen) and a Not I$(dT)_{17}$ primer. Both insect and vertebrate protein sequences were used to design degenerate primers for diazepam-binding inhibitor (DBI) and adrenodoxin reductase (AR) (see Supplemental Table 2.1). Such primers also were designed from protein sequences for *D. melanogaster* Start1 (CG3522), *Spodoptera littoralis* 3-dehydroecdysone 3β-reductase, (AJ131966), and *D. melanogaster* CYP302a1 (22-hydroxylase, CG12028) (see Supplemental Table 2.1). Nested PCR was used to amplify products from ovary cDNA for these genes using Titanium™ Taq DNA polymerase (BD Biosciences). PCR products were gel purified using a GenElute™ Minus EtBr spin column (Sigma), cloned into pCR-TOPO vectors (TOPO TA cloning kit, Invitrogen), sequenced (ABI Prism 3100 Genetic Analyzer), and identified following a TBLASTN search (NCBI). Gene specific primers were then used to amplify the 3' and 5' ends by PCR (see Supplemental Table 2.2 and 2.3). The 5' ends of *DBI*, *Start1*, *AR*, and *A/KR* were obtained from a previtellogenic *A. aegypti* whole body cDNA library (kindly provided by A. S. Raikhel), and the 5' end of *CYP302a1* was obtained from PBM ovary cDNA using the 5'/3' RACE Kit, 2nd Generation (Roche). Contigs spanning the 5' and 3' untranslated regions (UTR) of all genes were formed by aligning all cloned PCR products using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Partial nucleotide sequences for putative orthologs to *D. melanogaster CYP315a1* (2-hydroxylase, CG14728) and *D. melanogaster CYP314a1* (20-hydroxylase, CG13478) were gleaned from the online *A. aegypti* cDNA library (http://tigrblast.tigr.org/) as AABED11TV and NABNQ88TF, respectively. Gene specific primers were used to amplify fragments of *CYP315a1* and *CYP314a1* by PCR to verify their existence in PBM *A. aegypti* ovaries. Gene specific primers then were used to amplify the 3' ends by PCR, and the 5' ends were obtained as described for *CYP302a1* (see Supplemental Table 2.2 and 2.3). Contigs for *CYP315a1* and *CYP314a1* were then formed as described above.

To verify the nucleotide sequences of the derived contigs, cDNAs spanning the open reading frames (ORF) for the genes were amplified from 18 h or 48 h PBM ovary cDNA by PCR using a proofreading DNA polymerase (FastStart High Fidelity PCR System or Expand High Fidelity PCR system, Roche) (see Supplemental Table 2.4 for gene specific primers, their respective annealing temperatures (Tm), amplicon size, and PCR programs). To minimize amplification of genomic DNA, total RNA was treated with Dnase-I (Qiagen), and mRNA was isolated with the GenElute™ mRNA miniprep kit (Sigma), from which cDNA was synthesized using the Advantage® RT-for-PCR kit and oligo(dT) as the primer (BD Biosciences Clontech). The cDNAs were cloned as above and sequenced by Retrogen Inc. (ABI 3730 sequencers; San Diego, CA.). Contigs of the PCR products described previously were compared to the ORF products obtained with the proofreading DNA polymerase.

2.3 Tissue distribution of transcripts for proteins involved in ecdysteroidogenesis

Head, thorax (without wings and legs), abdominal wall, gut (without blood) and ovaries were dissected from NBF and 18 h PBM females for the extraction of total RNA with TRIzol® (Invitrogen) that was then treated with Dnase-I (TURBO DNA-*free*™, Ambion). Whole males

were processed for total RNA in the same way. The synthesis of cDNA used total RNA (1 μg), oligo(dT) as the primer, and the Advantage® RT-for-PCR kit. PCR was conducted with Titanium™ Taq DNA polymerase using the same primer sets as specified in Supplemental Table 4, except *AedaeDBI* (forward primer 5'-GGTGAAGACCTTCACGAAAC-3' and the reverse primer 5'-GATACTTGGCCGACAGTTC-3'). The final volume for each PCR was 20 μl (equivalent to 50 ng total RNA/PCR), and the number of cycles chosen for each of the genes was based upon the intensity of the product band obtained from the 18 h PBM ovary (30 or 40 cycles). To verify the absence of genomic DNA contamination, 50 ng total RNA template was subjected to PCR with the different gene primer sets. Products for the *A. aegypti* ovary very-low-density lipoprotein receptor, AaLpRov (Cheon et al., 2001), and actin, Aaeact-1 (Ibrahim et al., 1996), were amplified by PCR to confirm the integrity of the tissue and cDNA preparations. Tissues from three separate cohorts were subjected to RT-PCR as described above.

2.4 *Ecdysteroid production by isolated tissues and body parts*

Modified procedures for *in vitro* tissue ecdysteroid production and the ecdysteroid radioimmunoassay (RIA) were followed (Sappington et al., 1997; Riehle and Brown, 1999). Ovary pairs with the last two abdominal segments and a small portion of the hindgut were dissected from females (3-5 days post eclosion) NBF and 2, 6, 18, 30, 48, and 72 h PBM in buffered medium. As controls, the above tissues without ovaries, heads, thoraces (without legs and wings), abdominal walls, and guts were prepared similarly from females NBF, 18 h PBM, and 30 h PBM. For each experiment, triplicates of four ovary pairs or four body parts/tissues were placed in 60 μl of medium in a polypropylene tube lid and incubated for 6 h at 27° C. Each experiment was replicated with females from three different cohorts. After incubation, 50 μl of medium were collected, stored at -80° C, and later subjected to RIA. The anti-ecdysteroid rabbit

serum (AS 4919, a gift from P. Poncheron, Université P. et M. Curie, Paris, France) recognizes ecdysone and 20-hydroxyecdysone equally (Porcheron et al., 1989), as verified with our RIA. For RIA, each tube contained 50 µl of a stock [23,24-^{3}H(N)]ecdysone solution (= [^{3}H]ecdysone; 12,000-13,000 counts/minute (cpm)/50 µl; PerkinElmer, Boston, MA), 50 µl of antiserum diluted to 1:35,000-45,000 (final dilution for bound to free [^{3}H]ecdysone cpm ratio (Bound / Free = 1), and 50 µl of sample or 20-hydroxyecdysone standard. Triplicate tubes were set up for each of the 20-hydroxyecdysone standards (1, 5, 10, 25, 50, 100, 250, 500, and 1000 pg). After overnight incubation at 4° C, bound and free radiolabeled ecdysone were separated by the ammonium sulfate method, and pellets in tubes were dispersed in water and scintillation fluid and counted in a scintillation counter (Beckman). For each RIA, a standard curve was plotted from the averaged Bound / Free (Y axis) and log values for the 20-hydroxyecdysone standards (X axis). The quantity of immunoreactive ecdysteroids in samples was calculated from a regression equation for the linear portion (10-250 pg) of the standard curve; samples were diluted when necessary to stay within this range. Sample values reported for each tissue treatment are presented as "ecdysteroid pg", because the secreted ecdysteroid species are unknown, and the values are means of triplicate treatments from three experiments (N = 9).

2.5 *Real-time PCR analysis of gene transcripts*

Twenty ovary pairs were dissected from 4-5 day post eclosion females, NBF and 2, 6, 18, 30, 48, and 72 h PBM, placed into RNAlater™ (Sigma), held at 4° C overnight, and then stored at -80° C until processed. As described above, total RNA was extracted and treated with Dnase-I, mRNA isolated and cDNA synthesized. Real-time PCR was conducted on a Rotor-Gene RG-3000 (Corbett Research) using the program: 95°C for 3 min, 95°C for 20 sec 65°C for 20 sec 72°C for 20 sec for 45 cycles, followed by melting curve analysis (see Supplemental Table 2.5

for primers, amplicon size, and corresponding protein sequence). For the analysis of gene transcript abundance from NBF and PBM ovaries, template cDNA-specific master mixes were prepared with IQ™ SYBR® Green Supermix (Biorad), nanopure water, and template cDNA (0.048 ovary pair equivalents/PCR) for the seven time points of a gonotropic cycle and a non-template control to which the primers were added (100 nmol final concentration/PCR). The total volume for each PCR was 10 μl, and each template cDNA reaction had four internal replicates to address pipetting error.

To estimate the number of transcripts at each time point, a standard curve was derived with PCR using plasmids with the gene ORF as template. In brief, PCR master mixes were prepared as above with serially diluted plasmid (1 ng, 100 pg, 10 pg, 1 pg, 100 fg or 10 fg/PCR) to which primers were added (100 nmol final concentration/PCR). The standard line was then used to calculate the transcript copy number as described by Paton et al., 2000. Real-time PCR data from three separate cohorts were analyzed with the RotoGene 4.6 software (Corbett Research) and was used to estimate transcript copy numbers at each of the time points. Real-time PCR samples that did not display a product as defined by melting curve analysis were excluded from analysis. Melting curve analysis confirmed the amplification of a single product in the real-time PCR reactions containing cDNA template, and no product was observed in the non-template controls.

2.6 Statistical analysis

Ecdysteroid production by isolated tissues and body parts and the real-time PCR results were analyzed by ANOVA, and their means separated by the Tukey-Kramer HSD test (SAS JMP 5.0.1a, SAS Institute Inc., Cary, N.C.).

3. Results

3.1 *Isolation of cDNAs encoding proteins involved in ovarian ecdysteroidogenesis*

Degenerate and gene specific primers for seven genes were used to amplify products from *A. aegypti* ovary cDNA by PCR. Once partial sequences were obtained, gene specific primers were used to amplify larger PCR products that allowed for the assembly of contigs spanning the 5' and 3' UTRs for all seven cDNA sequences. In addition, gene specific primers were used to amplify the entire ORFs of the seven genes by PCR to confirm the predicted contigs. Only two gene transcripts had two different contigs predicted (see Discussion). For each of the predicted *A. aegypti* ORFs, deduced protein sequences are provided with their homologs (PILEUP with BOXSHADE, Wisconsin GCG package; GenBank accession numbers in captions): AedaeDBI (Fig. 2.1), AedaeA/KR (Fig. 2.3), AedaeAR (Fig. 2.4), AedaeCYP302a1 (22-hydroxylase; Fig. 2.5), AedaeCYP315a1 (2-hydroxylase; Fig. 2.6), and AedaeCYP314a1 (20-hydroxylase; Fig. 2.7). The protein sequence for AedaeStart1a and its relevant homologs (Fig. 2.2) were aligned with CLUSTAL W 1.74 program (ch.EMBnet.org). In all instances, the best matches received in BLAST searches conducted with the *A. aegypti* protein sequences were to sequences in the *A. gambiae* genome database (http://www.ensembl.org/Anopheles_gambiae/).

The deduced *A. aegypti* proteins were analyzed for predicted pI and MW ($[M+H]^+$; http://au.expasy.org.tools/peptide-mass.html), protein motifs (http://motif.genome.ad.jp), transmembrane helices (TMHMM; http://www.cbs.dtu.dk/services/TMHMM/), and subcellular sorting signals (SignalP V2.0.b2, http://www.cbs.dtu.dk/services/SignalP-2.0/; MitoProt II 1.0a4; http://www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter) (Fig. 2.1-2.7). AedaeAR, AedaeCYP302a1, AedaeCYP315a1 and AedaeCYP314a1 are all predicted to have mitochondrial

N-terminal targeting sequences. AedaeDBI and AedaeA/KR do not possess either a signal peptide or a subcellular sorting signal; whereas, AedaeStart1a and AedaeStart1b (see Discussion) are predicted to have a signal peptide with cleavage occurring between A42-L43.

3.2 Tissue distribution of transcripts for proteins involved in ecdysteroidogenesis

As shown by RT-PCR, transcripts for all seven genes were present in ovaries, other tissues, and body regions of females (Fig. 2.8), and these results were consistent for all three cohorts. All but *AedaeCYP315a1* and *AedaeCYP314a1* also were evident in males. Importantly, the *AaLpRov* PCR product was seen only in ovaries (Fig. 2.8), which had not been reported in its original characterization (Cheon et al., 2001), and the *actin* product was in all tissues and body regions (Fig. 2.8), thus confirming the integrity and consistency of the tissue/body part cDNA preparations from all cohorts. With RT-PCR, variations in PCR product intensity obtained from tissue and body part cDNAs may reflect a qualitative difference in transcript abundance of a particular gene, but this variation also may be due to the dilution of transcripts from a subset of cells within a body part or whole body, as for males.

3.3 *Ecdysteroid production by isolated tissues and body parts*

Given that transcripts for all seven genes were present in different female tissues and body parts before and after a blood meal, the capability of these tissues/body parts from 18 h PBM females to produce ecdysteroids *in vitro* was determined. At this time whole body ecdysteroid levels are highest in female *A. aegypti* (Hagedorn et al., 1975). Notably, thoraces, abdomens, and guts from 18 h PBM females produced measurable levels of ecdysteroids, but not heads. Ovaries from the same females produced at least 12 times more ecdysteroids than these tissues/body parts (Fig. 2.9A). Ecdysteroid production by control preparations of the last two abdominal segments and hindgut (without ovaries) from 18 h PBM females (Fig. 2.9B) was

similar to that of other tissues (Fig. 2.9A) and ovaries from NBF females or females at 2, 6, 48, and 72 h PBM (Fig. 2.9B).

To assess the capacity of ovaries to produce ecdysteroids throughout the gonotropic cycle, *in vitro* assays were conducted with ovaries taken from females at different times PBM. Again, ovaries from 18 h PBM females produced the greatest amount of ecdysteroids (106.7 ± 6.9 pg/ovary pair/6 h) in comparison to those from NBF females or females at earlier or later times PBM (Fig. 2.9B). Ovaries from 30 h PBM females were still capable of ecdysteroid production but at a reduced amount (75.1 ± 4.7 pg/ovary pair/6 h).

3.4 *Real-time PCR analysis of gene transcripts for proteins involved in ovarian ecdysteroidogenesis*

Both rRNA and mRNA abundance rise and fall in *A. aegypti* ovaries during the gonotropic cycle (Clements, 1992; Banks et al., 1994; Sappington and Raikel, 1999), and the number of somatic follicle cells surrounding the nurse cell and oocyte approximately doubles following a blood meal (Laurence and Simpson, 1974). These facts required two adjustments to optimize quantification of gene transcripts by real-time PCR: (1) mRNA was isolated from total RNA to decrease the bias of increased rRNA, and (2) analysis of transcript abundance was based on ovary pair equivalents rather than a standard quantity of mRNA used in the reverse transcriptase reaction as is commonly done for non-developing cell lines or tissues.

All seven genes displayed changes in transcript abundance in ovaries following a blood meal (Fig. 2.10). Only *AedaeCYP302a1* and *AedaeCYP315a1* had statistically greater transcript numbers at the peak of ecdysteroid production, 18 h PBM, with 9 and 13 fold increases respectively (Fig. 2.10C), in comparison to those in NBF ovaries (Tukey-Kramer HSD $p \leq 0.05$). *AedaeStart1* (Fig. 2.10A) and *AedaeA/KR* (Fig. 2.10B) showed only a two-fold increase in

transcript abundance in 18 h PBM ovaries over that in NBF ones. *AedaeAR* transcript abundance was four-fold (Fig. 2.10B) and *AedaeDBI*, five-fold greater in 18 h PBM ovaries (Fig. 2.10A), than in NBF ovaries, but the differences were not statistically significant. There was a 262-fold increase in *AedaeDBI* transcripts in 48 h PBM ovaries, well after the peak of ecdysteroid production, relative to that in ovaries from NBF females. Furthermore, transcript abundance of all genes but *AedaeDBI* and *AedaeCYP314a1* was statistically greater in ovaries at 18 h PBM than at 2 h PBM (Tukey-Kramer HSD $p \leq 0.05$), which may be the more important comparison since the females all had blood meals.

4. Discussion

Ecdysteroids are key regulators of the massive gene expression required for yolk protein synthesis (Raikhel et al., 2002) and, ultimately, of the reproductive success by female *A. aegypti*. As demonstrated by Hagedorn et al. (1975) and in this paper, ovaries are the primary source of ecdysteroids in blood-fed female *A. aegypti*. Ovarian ecdysteroid production *in vitro* reaches a peak at 18 h PBM and continues at least until 30 h PBM, which is longer than reported by Hagedorn et al. (1975), but in agreement with others (Greenplate et al., 1985; Borovsky et al., 1986). The initiation of this process likely requires the activation of gene expression and synthesis of one or more proteins required for ecdysteroid biosynthesis, as indicated by the lag of six or more hours after blood ingestion by female *A. aegypti* before ovaries can sustain ecdysteroid secretion *in vitro*.

To elucidate the activation of this process, orthologues of seven genes encoding proteins putatively involved in ecdysteroidogenesis in other insects were identified from ovary cDNA. Subsequently, their transcript expression profiles were determined by real-time PCR before and during a gonotropic cycle. The highest transcript levels for *AedaeStart1*, *AedaeA/KR*, *AedaeAR*,

AedaeCYP302a1 (22-hydroxylase) and *AedaeCYP315a1* (2-hydroxylase) coincided with the peak in ovarian ecdysteroid production at 18 and 30 h PBM, whereas that of *AedaeCYP314a1* (20-hydroxylase) showed little change during the gonotropic cycle. *AedaeDBI* transcript abundance displayed a small increase during ovarian ecdysteroid production, and then at 48 h PBM, had the greatest increase observed for all gene transcripts. These trends indicate that the capacity of ovaries to produce ecdysteroids depends on increased transcript abundance of some but not all of the identified genes. Translation of these gene transcripts presumably results in increased amounts of protein or enzyme that in turn supports a greater capacity for ecdysteroid production. In this study, the synthesis and use of cDNA template for real time PCR as a defined and constant "ovary pair equivalent" is validated since the transcript expression profiles differed among the seven genes during a gonotropic cycle. If instead a constant amount of total RNA had been used for cDNA synthesis, these profiles in gene transcript abundance may have been minimized or even not revealed, given the great rise and fall in ovarian total RNA during this cycle.

In addition, transcripts for all genes were present in other female tissues and body parts before and after the females had a blood meal. These same tissues/body parts from blood-fed females subsequently were shown to produce low levels of ecdysteroids *in vitro*, thus suggesting that proteins from these genes also play a role in ecdysteroid production by non-ovarian tissues. Specific roles for the proteins encoded by ortholog genes identified herein for female *A. aegypti* have been characterized in steroidogenic tissues from other insects and animals and are reviewed below.

4.1 Intracellular trafficking of cholesterol and ecdysteroid precursors

For female *A. aegypti*, cholesterol comes from either stores attained during larval development or from a blood meal (Svoboda et al., 1982). Human plasma lipoproteins contain both cholesterol esters and free cholesterol (Gibbons et al., 1982; Oh, 1982), both of which have been shown to be metabolically useable by *A. aegypti* larvae (Golberg and De Meillon, 1948). Ovaries in female *A. aegypti* could acquire cholesterol needed for ecdysteroid biosynthesis either by a lipophorin receptor-mediated process or through "aqueous diffusion" (Kawooya and Law, 1988; Kawooya et al., 1988; Capurro Mde et al., 1994; Ford and Van Heusden, 1994; Van Heusden et al., 1997; van Heusden et al., 1998; Sun et al., 2000; Cheon et al., 2001; Jouni et al., 2003).

In ecdysteroidogenic cells, the first enzymatic modification of cholesterol is hypothesized to be its conversion to 7-dehydrocholesterol by a microsomal cytochrome P450 enzyme, cholesterol 7,8-dehydrogenase (Grieneisen et al., 1993; Rees, 1995; Warren et al., 1995; Gilbert et al., 2002). The next step is believed to be the rate limiting step of ecdysteroidogenesis, and it involves the transport of 7-dehydrocholesterol to the inner mitochondrial matrix for further processing (Warren and Gilbert, 1996b; Henrich et al., 1999; Gilbert et al., 2002).

In vertebrates, various proteins have been implicated in cholesterol transport to the inner mitochondrial matrix during acute steroidogenesis (Papadopoulos, 1993; Stocco and Clark, 1996; Thomson, 1998; Christenson and Strauss, 2001). These include DBI and proteins with the START domain, StAR and MLN64, both of which have been studied in insects (Snyder and Feyereisen, 1993; Snyder and Antwerpen, 1997; Snyder and Van Antwerpen, 1998; Henrich et al., 1999; Gilbert et al., 2002; Roth et al., 2004).

4.1.1. *AedaeStart1*

Recently, a gene encoding a protein with a START domain, *Start1*, was characterized in *D. melanogaster* at the transcript and protein level (Roth et al., 2004), but prior work had revealed StAR-immunoreactive proteins on western blots of *M. sexta* prothoracic gland extracts (Henrich et al., 1999). Now, apparent orthologs of *Start1* have been identified in *A. aegypti* as *AedaeStart1a*/*b* and in *A. gambiae* as *AnogaStart1*. The deduced amino acid sequences of the mosquito and *D. melanogaster* proteins are highly similar (Fig. 2.2). Like Start1, AedaeStart1a possesses the "insert coding sequence" (Roth et al., 2004), with the START domain split into E202-G316 and V457-L545 (Pfam E values of 10^{-8} and 10^{-9}, respectively; Marchler-Bauer et al., 2003). Interestingly, a PILEUP conducted with AedaeStart1a and its orthologs did not produce the “insert coding region” split of the START domain as depicted in the multiple sequence alignment of Roth et al. (2004). Consequently, we conducted a multiple sequence alignment using the program specified by Roth et al. (2004), which resulted in the split of the START domain (Fig. 2.2).

The START domain of the probable Start1 ortholog in humans, MLN64, promotes steroidogenesis (Watari et al., 1997; Zhang et al., 2002; Tuckey et al., 2004) and is known to be involved in intracellular cholesterol trafficking (Alpy et al., 2001; Zhang et al., 2002). The greatest identity between AedaeStart1a and human MLN64 is within the "Mental" domain in the N-terminal half, which is hypothesized to contain four transmembrane domains (Alpy et al., 2001; Alpy et al., 2002; Zhang et al., 2002; Roth et al., 2004). Given the high sequence identity and similarity between human MLN64 and the insect Start1s (Fig. 2.2), it is likely that the insect Start1s are involved in the endosomal trafficking of cholesterol to acceptor membranes, such as

the mitochondria, in the same way as MLN64 (Bose et al., 2000a; Alpy et al., 2001; Zhang et al., 2002; Tuckey et al., 2004).

During the cloning of *AedaeStart1*, two possible contigs were derived, one represented by AedaeStart1a (GenBank AY947545) and the other AedaeStart1b (GenBank AY947546). AedaeStart1b is predicted to contain 60 substituted amino acids and an additional 21 amino acids at the C-terminus, all of which reside within the region of R435-S574+. AedaeStart1b also possesses the "insert coding region," with the START domain split into E212-G316 and V457-E515 (Pfam E values of 10^{-8} and 10^{-5}, respectively). It is not known whether the different *AedaeStart1s* represent two different genes, splice variants, or different alleles. In prior and later sections, the gene transcript is referred to as *AedeaStart1* since the primers used in both RT-PCR and real-time PCR would amplify products common to *AedaeStart1a* and *AedaeStart1b*.

During the gonotropic cycle of female *A. aegypti*, *AedaeStart1* transcript abundance did not change substantially in ovaries, although it reached a peak in ovaries at 18 h PBM (Fig. 2.10A), as does ovarian ecdysteroid production *in vitro*. In *D. melanogaster*, *Start1* mRNA was only observed by *in situ* hybridization in the nurse cells of stage 10 egg chambers (Roth et al., 2004), which is the final vitellogenic stage of oogenesis (Spradling, 1993). The expression of *Start1* is not specific to ovaries in female *A. aegypti*, as described above, and a similarly broad tissue expression was observed for *D. melanogaster* Start1 (Roth et al., 2004), and for the human orthologue MLN64 (Watari et al., 1997). Based on these results, other functions for Start1 in cholesterol metabolism beyond promoting ecdysteroidogenesis are suggested.

For these two dipteran females, expression of this gene and others encoding proteins involved in ecdysteroid production will depend on the timing of this process during oogenesis. Egg chambers in *D. melanogaster* develop asynchronously and those in *A. aegypti*,

synchronously. Buszczak et al. (1999) suggested that the individual ovarioles of *D. melanogaster* progress through oogenesis by autocrine control of ecdysone production via the timing of the expression of proteins involved in ecdysteroidogenesis. In *A. aegypti*, the primary follicles of the paired ovaries develop in a single batch, thus the timing and duration of ecdysteroidogenesis would depend on the global regulation of gene transcription and translation.

4.1.2. *AedaeDBI*

Although there is considerable evidence for the involvement of DBI in mammalian steroidogenesis (Krueger and Papadopoulos, 1990; Boujrad et al., 1993; Papadopoulos, 1993; Papadopoulos and Brown, 1995), its function is controversial (Knudsen et al., 1993; Gossett et al., 1996). Related DBIs have been characterized in *M. sexta*, *D. melanogaster*, and *B. mori* (Snyder and Feyereisen, 1993; Kolmer et al., 1994; Snyder and Antwerpen, 1997; Snyder and Van Antwerpen, 1998; Matsumoto et al., 2001), and its transcripts and protein product were observed in various tissues, including ovary. During vitellogenesis in female *D. melanogaster*, DBI transcripts and protein were localized in nurse cells, oocytes, and follicle cells (Kolmer et al., 1994). In *M. sexta* larvae, high DBI transcript and protein levels were observed in prothoracic glands during ecdysteroid biosynthesis, and incubation of prothoracic glands with anti-DBI antibodies significantly decreased ecdysteroid production *in vitro* (Snyder and Van Antwerpen, 1998).

Another suggested function for DBI is to promote lipid metabolism in vertebrates (Knudsen et al., 1993; Rasmussen et al., 1993; Rasmussen et al., 1994; Gossett et al., 1996; Swinnen et al., 1998) and insects (Kolmer et al., 1994; Snyder and Antwerpen, 1997). Given this possible function, the expression of *AedaeDBI* in non-ovarian tissues and body parts of male and female *A. aegypti*, as described above, was not surprising. DBI expression was observed in

tissues of *D. melanogaster* that metabolize fatty acids or require high amounts of energy input to function (Kolmer et al., 1994) and in representative tissues from *M. sexta* (Snyder and Feyereisen, 1993). Moreover, DBI has been observed in every mammalian tissue examined, and consequently has been assigned numerous functions such as promoting steroidogenesis or secretion of steroids and long-chain fatty acid metabolism (Knudsen et al., 1993; Gossett et al., 1996; Knudsen et al., 2000).

In our study, *AedaeDBI* transcript abundance increased during ovarian ecdysteroid production, but the greatest peak occurred 48 h PBM (Fig. 2.10A), well after the deposition of protein and lipid within the developing oocyte (Ziegler and Ibrahim, 2001; Briegel et al., 2003). The majority of lipid synthesis occurs within the insect's fat body (Beenakkers et al., 1985), and lipids are shuttled by lipophorin to the developing oocytes (Beenakkers et al., 1985; Soulages and Wells, 1994). As well, *A. aegypti* ovaries can synthesize lipids after a blood meal (Ziegler, 1997), and an increase in fatty ester deposition begins 48 h PBM in *A. aegypti* ovaries (Troy et al., 1975). AedaeDBI may thus be involved in lipid metabolism during later stages of oocyte maturation.

4.2. Modification of the steroid nucleus by the ecdysteroid biosynthetic enzymes

The next proposed step of ecdysteroid biosynthesis is the oxidation of 7-dehydrocholsterol and has been termed the "black box" because it has yet to be characterized (Rees, 1995; Gilbert et al., 2002). A subsequent modification is thought to be the reduction of diketol to ketodiol by 3-dehydoecdysone 3β-reductase (3DE3BR) (Rees, 1995; Gilbert et al., 2002). This enzymatic step occurs in the hemolymph of lepidopteran larvae, where 3DE3BR reduces the 3-dehydroecdysone secreted by prothoracic glands to ecdysone (Kiriishi et al., 1990; Nomura et al., 1996; Chen et al., 1999). In a comparative study, the ovaries of blood-fed *A.*

aegypti did not secrete 3-dehydroecdysone, and hemolymph in such females had no 3DE3BR activity (Kiriishi et al., 1990). These results suggest that this step of ecdysteroid modification may occur within the ovaries.

4.2.1. AedaeA/KR

The identification of an aldo/keto reductase in *A. aegypti* ovaries was based on PCR amplification with degenerate primers to conserved amino acid regions shared by the 3DE3BR of *S. littoralis* (Chen et al., 1999) and related genes/proteins found in the genome databases of *D. melanogaster* and *A. gambiae*. In *S. littoralis*, 3DE3BR gene expression was associated with an increase in the corresponding enzyme activity in the last larval instar (Chen et al., 1996; Chen et al., 1999). A 3DE3BR homolog was characterized from the cabbage looper, *Trichoplusia ni* (Lundstrom et al., 2002), and the recombinant *T. ni* 3DE3BR homolog was able to reduce the ecdysteroid, 3-dehydro-makisterone A, to makisterone. Despite this demonstrated activity, the authors believe that its function in ecdysteroidogenesis to be "conjectural", presumably because the protein is localized near the cuticular surface of the epidermis and upregulated following bacterial challenge (Lundstrom et al., 2002).

Transcripts for *AedaeA/KR* were obtained from ovary cDNA, and the encoded protein is identified as an aldo/keto reductase and not 3DE3BR for now, because six additional genes for aldo/keto reductases were gleaned later from the *A. gambiae* genome database. Other such aldo/keto reductases likely exist in *A. aegypti* ovaries, and any one of these proteins or AedaeA/KR may function as a 3DE3BR. AedaeA/KR has 41.3% identity and 53.7% similarity to *S. littoralis* 3DE3BR (Fig. 2.3), which is comparable to the 3DE3BR homolog of *T. ni* and *S. littoralis* 3DE3BR (44.1% identity and 52.2% similarity).

In the current study, there is a positive correlation between *AedaeA/KR* transcript abundance in ovaries and the rise and fall of ovary ecdysteroid production PBM *in vitro* (Fig. 2.10B). The level of *AedaeA/KR* transcripts in NBF ovaries was about half that in 18 h PBM ovaries, but the levels in 2, 48, and 72 h PBM ovaries were significantly lower. Most notable was the *AedaeA/KR* transcript increase in 6 h PBM ovaries that preceded the capacity for ecdysteroid secretion *in vitro*. In total, these results suggest early expression of this gene may be required for activation of ecdysteroidogenesis in ovaries and its increased expression results in an A/KR that plays a key role. Because *AedaeA/KR* transcripts were detected in males and other tissues and part from females, the protein may have other functions, including bacterial defense as in Lepidoptera (Lundstrom et al., 2002).

4.2.2. AedaeAR

Adrenodoxin reductase (AR) is a mitochondrial protein that transports electrons from NADPH to adrenodoxin, which in turn donates them to the cytochrome P450 enzymes responsible for steroid modification. An increase in AR expression is a common feature of mammalian ovary and placenta steroidogenesis (Hanukoglu and Hanukoglu, 1986; Hanukoglu et al., 1990; Tuckey and Sadleir, 1999; Tuckey and Headlam, 2002). For insects, an AR gene, *dare*, was identified first in *D. melanogaster*, where its expression is required for entrance into the vitellogenic stage of oogenesis. It is first evident in nurse cells of ovaries at stage 6-7 and increases through stage 10 (Buszczak et al., 1999; Freeman et al., 1999). In the current study, a related AR was shown to be expressed in *A. aegypti* ovaries, and the protein is predicted to localize to the mitochondria (Fig. 2.4), as shown for *D. melanogaster* AR (Freeman et al., 1999). *AedaeAR* transcript abundance was highest in 18 h PBM ovaries (Fig. 2.10B), the peak of *in vitro* ecdysteroid production. Gene transcripts for AedaeAR were observed in males and every

tissue/body part of females (Fig. 2.8), which is consistent with the expression of *dare* in *D. melanogaster* (Freeman et al., 1999). These results suggest that AedaeAR may provide reducing equivalents to mitochondrial cytochrome P450 enzymes in all mosquito tissues and in this way be required for ovarian ecdysteroidogenesis.

4.2.3. Cytochrome P450 enzymes

The final hydroxylation steps of ecdysteroid biosynthesis are catalyzed by cytochrome P450 enzymes that were first characterized in *D. melanogaster* as the "Halloween" genes (Gilbert et al., 2002; Warren et al., 2002; Petryk et al., 2003; Niwa et al., 2004; Warren et al., 2004). Conceptually, these steps are catalyzed in the order of 25-hydroxylase (*phantom*/CYP306a1)→ 22-hydroxylase (*disembodied*/CYP302a1)→ 2-hydroxylase (*shadow*/CYP315a1), resulting in the synthesis of ecdysone (Kappler et al., 1988; Warren et al., 2002; Niwa et al., 2004; Warren et al., 2004). Ecdysone is then converted to 20-hydroxyecdysone by 20-hydroxylase (*shade*/CYP314a1) (Petryk et al., 2003). Mutations in these genes inhibited oogenesis and disrupted embryonic development (specifically cuticle deposition), and the wild-type gene products were localized in the larval prothoracic glands, adult ovary, developing egg and embryo (Buszczak et al., 1999; Freeman et al., 1999; Buszczak and Segraves, 2000; Chavez et al., 2000; Warren et al., 2002; Petryk et al., 2003; Niwa et al., 2004; Warren et al., 2004).

Studies have suggested that tissues other than ovaries produce ecdysteroids in *D. melanogaster* females (Handler, 1982; Bownes, 1989) and that they do not (Rubenstein et al., 1982). For a time, there even was a controversy as to whether or not ovaries in *D. melanogaster* produced ecdysteroids (Handler, 1982; Rubenstein et al., 1982; Schwartz et al., 1985; Bownes, 1989; Warren et al., 1996a). Regardless, expression of the "Halloween" genes has yet to be

profiled in ovaries during oogenesis in *D. melanogaster*, and there remains the possibility that other tissues in females express these genes only at specific times and, as a result, produce ecdysteroids that have specific autocrine functions.

Cytochrome P450 enzymes are named by applying the symbol "CYP" followed by a family number in which the same number is used for sequences with >40% identity, which is then followed by a letter for the subfamily where the same letter is shared with sequences with >55% identity (Werck-Reichhart and Feyereisen, 2000). These criteria were used to identify the putative *A. aegypti* orthologs of the above cytochrome P450 enzymes along with comparison with those annotated for *D. melanogaster* and *A. gambiae* at "The P450 Site at INRA" (http://p450.antibes.inra.fr/) (Figures 2.5-7). With online programs, the subcellular localization of these *A. aegypti* enzymes was predicted from their N-terminal protein sequences and has yet to be definitively established. Moreover, it should be noted that a single amino acid substitution can change the enzyme's substrate specificity (Feyereisen, 1999), thus the biosynthetic activity of the putative *A. aegypti* ortholog proteins should be confirmed in future studies.

4.2.4. AedaeCYP302a1 (22-hydroxylase)

The gene that encodes 22-hydroxylase was the first cytochrome P450 specifically involved in ecdysteroidogenesis to be identified and characterized in an insect (Chavez et al., 2000; Warren et al., 2002), and now, orthologs from *A. gambiae* (Ranson et al., 2002) and *A. aegypti* have been identified. Transcript abundance of *AedaeCYP302a1* was greatest in ovaries at 6, 18, and 30 h PBM (Fig. 2.10C), coinciding with the onset and peak in ovarian ecdysteroid production, and indicates that this enzyme likely has an important role, as described for *D. melanogaster* CYP302a1 (Chavez et al., 2000; Warren et al., 2002). The increased transcript abundance in 6 h PBM ovaries, which cannot sustain ecdysteroid secretion *in vitro*, suggests

early expression of this gene may be a requisite step for the activation of ecdysteroidogenesis, as with *AedaeA/KR*. Transcripts of *AedaeCYP302a1* in males and other tissues/body parts from females, along with the demonstration of *in vitro* ecdysteroid production by some of these tissues/body parts, suggests that this enzyme plays a similar role in other tissues.

In *D. melanogaster*, *disembodied* is expressed in the larval ring/prothoracic gland complex and follicle cells of ovaries beginning at stage 8, when vitellogenesis begins, and by stage 11, its expression levels diminish (Chavez et al., 2000). Transfected cells expressing *D. melanogaster* CYP302a1 catalyzed the 22-hydroxylation of an ecdysteroid precursor (Warren et al., 2002), thus confirming its role in ecdysteroid biosynthesis. Apparently, this enzyme is not essential for the completion of oogenesis, because *disembodied* mutants can complete oogenesis but are negatively affected by this lesion later in embryogenesis (Chavez et al., 2000). This phenomenon is explained by the observation that *D. melanogaster* CYP302a1 is expressed within the somatic cells of the ovary (i.e., follicle cells) and not the germline cells (Chavez et al., 2000).

In our study, two clones of *AedaeCYP302a1* were obtained from PCR products amplified from *A. aegypti* ovary cDNA using the same primer pair (Supplemental Table 2.4) but displayed different ECOR-I digestion patterns. The ORF of one clone encodes a protein with 515 amino acid residues (Figure 2.5), and the other, a 479 amino acid residue protein (GenBank accession number AY947550). The 515 amino acid protein has an additional 36 amino acids at the C-terminus; otherwise, the sequences of the two proteins are essentially identical with only eight amino acid substitutions. These two clones may represent different alleles or splice variants.

4.2.5. AedaeCYP315a1 (2-hydroxylase)

The identification of another cytochrome P450 followed shortly after the discovery of *disembodied*, and it was shown to conduct the 2-hydroxylation reaction of ecdysteroidogenesis (Warren et al., 2002). Like CYP302a1, orthologs from *A. gambiae* (Ranson et al., 2002) and *A. aegypti* have now been identified. In female *A. aegypti*, an increase in *AedaeCYP315a1* transcript abundance was evident only in 18 h PBM ovaries (Fig. 2.10C), which coincides with the peak of ovarian ecdysteroid production. Transcripts for this gene also were present in other female tissues that produced ecdysteroids, but not in males.

The expression levels of CYP315a1/*shadow* coincide with the rise and fall in ecdysteroid production by prothoracic glands in *D. melanogaster* larvae, and in females, transcripts were present in the follicle and nurse cells of ovaries (Warren et al., 2002). *D. melanogaster* CYP315a1 has a mitochondrial targeting signal at the N-terminus (Chavez et al., 2000), as does AedaeCYP315a1, and a later study showed that the *D. melanogaster* protein was localized to this organelle (Warren et al., 2002).

4.2.6 AedaeCYP314a1 (20-hydroxylase)

The cytochrome P450 responsible for the final hydroxylation step of ecdysteroidogenesis was identified as CYP314a1/*shade* of *D. melanogaster* (Petryk et al., 2003), and orthologs have been identified from *A. gambiae* (Ranson et al., 2002) and *A. aegypti*. As reported for other insects, the modification of ecdysone to 20-hydroxyecdsone occurs largely in the target tissues. *D. melanogaster* CYP314a1 was shown to catalyze this reaction in a transfected cell system, and it was localized in the mitochondria (Petryk et al., 2003), as is predicted for AedeaCYP314a1.

In contrast to the other two genes for cytochrome P450 enzymes, transcript abundance of *AedaeCYP314a1* did not increase in ovaries as the gonotropic cycle progressed in *A. aegypti* females (Fig. 2.10C), and it displayed the most specific tissue expression, i.e. abdominal wall,

gut and ovary. In female *D. melanogaster*, CYP314a1/*shade* is expressed within the nurse and follicle cells of the ovary and in other tissues, and analysis of transgenic adult females suggests that its expression specifically within the ovary is required for oogenesis (Petryk et al., 2003). In *D. melanogaster* larvae, CYP314a1/*shade* is expressed in the midgut, epidermis and fat body, sites with high 20-hydroxylase activity, and not expressed in tissues lacking 20-hydroxylase activity (e.g., ring glands, brains) (Petryk et al., 2003).

An earlier study of *A. aegypti* females reported that 20-hydroxylase activity in gut/Malpighian tubule/ovary homogenates decreased during the first 24 h PBM and remained low to 64 h PBM, but body wall/fat body homogenates showed a rise and fall in such activity from 12 to 32 h PBM (Smith and Mitchell, 1986). Also, it was shown that isolated ovaries at 16 h PBM possessed 20-hydroxylase activity and secreted 20-hydroxyecdysone *in vitro*. These results generally support the expression of *AedaeCYP314a1* in ovaries, gut, and abdominal wall of females, as described above, and it should be noted that no transcripts were present in males (Fig. 2.8). Interestingly, 20-hydroxylase activity was present in both the mitochondrial and microsomal fractions of processed female abdomens (Smith and Mitchell, 1986). In part, this agrees with the prediction that AedaeCYP314a1 would be localized in the mitochondria, but the deduced N-terminus contains many hydrophobic residues, which could serve as an anchor for a microsomal cytochrome P450 (Feyereisen, 1999).

4.3 Ecdysteroidogenesis and gene expression in other female tissues and males

Tissues in the thorax and abdomen of female *A. aegypti* can produce detectable quantities of ecdysteroids *in vitro* following a blood meal (Fig. 2.9A). This phenomenon may explain why low levels of vitellogenin were found in isolated and ovariectomized abdomens of *A. aegypti* females with a blood meal (Van Handel and Lea, 1984), since 20-hydroxyecdysone is a primary

activator of vitellogenin synthesis in this species (Raikhel et al., 2002). In fourth instar larvae and pupae of *A. aegypti*, these same body regions were found to be the *de novo* source, both *in vitro* and *in vivo*, of circulating ecdysteroids (Jenkins et al., 1992), whereas the prothoracic glands of larvae failed to produce ecdysteroids *in vitro*. In a variety of insect species and developmental stages, tissues other than prothoracic glands and gonads are known to produce ecdysteroids (Delbecque et al., 1990; Sakurai et al., 1991).

The detection of transcripts for all seven genes in the thorax, abdomen, and guts of females (Fig. 2.8) in part supports our finding that these tissues are capable of limited ecdysteroid production after a blood meal. Of particular relevance, the larval gut, integument and carcass of *L. migratoria* and the blowfly, *Calliphora vicina*, were shown to synthesize ecdysteroids from the precursor 5β-ketodiol, suggesting the presence of enzymes that conduct the terminal hydroxylation reactions on C-25, C-22 and C-2 (Meister et al., 1985; Meister et al., 1987) and supporting our finding that *AedaeCYP302a1* and *AedaeCYP315a1* are expressed in same tissues of female *A. aegypti*. Transcripts for many of the genes were also present in males (Fig. 2.8). In a few Lepidoptera, the testes are the presumed source of ecdysteroids in males (Loeb et al., 2001), but this has not been reported for any dipterans. As a caveat, it must be noted that the presence of gene transcripts in a particular tissue does not signify their inevitable translation, as exemplified by presence of transcripts and the absence of 3DE3BR and CYP306a1 (25-hydroxylase) in tissues of *S. littoralis* (Chen et al., 1999) and *D. melanogaster* (Warren et al., 2004), respectively.

Throughout this paper, the term “ecdysteroid production” by tissues has been used to describe the secreted or released ecdysteroids measured with the RIA. The term, “ecdysteroidogenesis”, implicitly means *de novo* biosynthesis of ecdysteroids from sterols, and

this has not been demonstrated. Tissues may be releasing ecdysteroids that were sequestered from that previously synthesized (Redfern, 1989) or were acquired from hemolymph after secretion by a different tissue (Isaac and Slinger, 1989), the ovaries in this instance. There is ample evidence for ecdysteroidogenesis by ovaries of other insect species (Zhu et al., 1983; Lanot et al., 1989; Warren et al., 1996a), and presumably, this occurs in the ovaries of *A. aegypti*. Definitive evidence for ecdysteroidogenesis in ovaries and other tissues in *A. aegypti* will be attained when the conversion of a radiolabeled sterol precursor into ecdysone or 20-hydroxyecdsone is demonstrated (Hetru et al., 1982; Dolle et al., 1990; Warren et al., 1996a).

4.4 Regulation of ovarian ecdysteroid production

Although ecdysteroid biosynthesis has been investigated for decades, it is only recently that a number of genes encoding proteins shown or thought to be involved in this process have been identified. During the same time, the stimulation of this process by neuropeptides has received considerable attention because the signal transduction pathways activated by such peptides can result in direct modifications of one or more proteins that facilitate or inhibit steroid transport and biosynthesis or affect the transcription or translation of one or more genes for such proteins. Numerous studies have addressed the specific proteins and signal transduction events that follow the acute stimulation of ecdysteroidogenesis in prothoracic glands by the neuropeptide, prothoracicotropic hormone (Smith and Gilbert, 1989; Rybczynski and Gilbert, 1994; Song and Gilbert, 1995; Rybczynski and Gilbert, 1995a; Song and Gilbert, 1996; Song and Gilbert, 1997; Smith et al., 2003). Both gene transcription and translation are required for ecdysteroid synthesis by the prothoracic glands in *M. sexta* (Smith et al., 1986; Smith and Gilbert, 1989; Keightley et al., 1990). In mammals, tissue-specific gene expression of cytochrome P450 enzymes is believed to define the steroidogenic capacity of adrenal and

gonadal tissue (Waterman and Simpson, 1989; Hanukoglu, 1992; Omura and Morohashi, 1995). Likewise, it has been suggested that the cytochrome P450 enzymes responsible for the three terminal steps of ecdysteroid biosynthesis in *D. melanogaster* and one in *B. mori* are expressed in a tissue and developmental specific manner (Chavez et al., 2000; Warren et al., 2002; Petryk et al., 2003; Gilbert, 2004; Niwa et al., 2004; Warren et al., 2004).

The ovary of *A. aegypti* is an exceptional model for defining the regulation of ecdysteroid production, since it can be stimulated *in vivo* by providing females with a blood meal or *in vitro* by using neuropeptides and pharmacological agents (Brown et al., 1998; Riehle and Brown, 1999). The neuropeptide, ovary ecdysteroidogenic hormone, is known to have this effect both *in vivo* and *in vitro* (Brown et al, 1998), and insulin-like peptides acting through an insulin signalling pathway also may be involved (Riehle and Brown, 1999; Riehle et al., 2002; Krieger et al., 2004). Future research will determine whether these neuropeptides and other agents affect gene expression in ovaries, by focusing on the genes described herein and characterizing their proteins. In time, this approach may even lead to the identification of new components in the biosynthetic and regulatory pathways of this fundamentally important process in insects.

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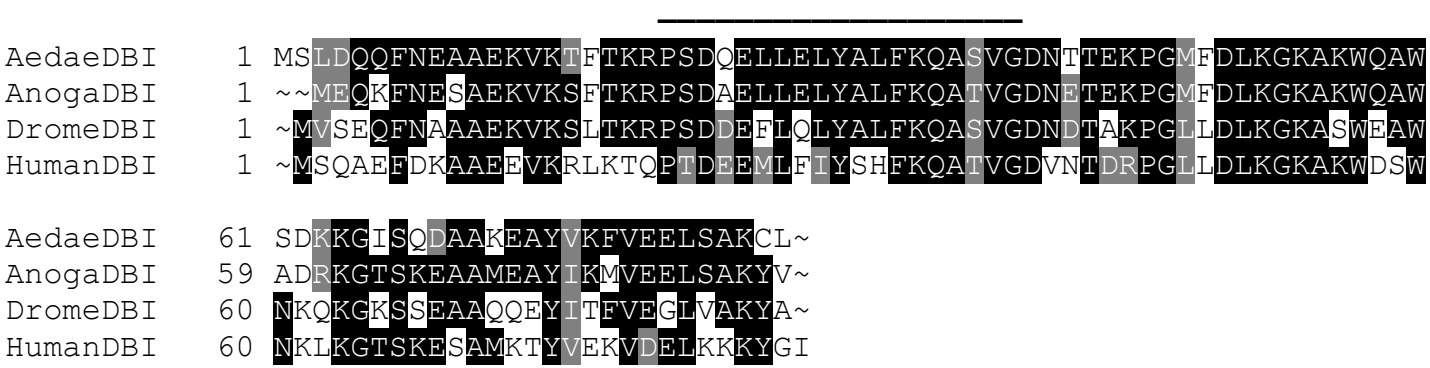
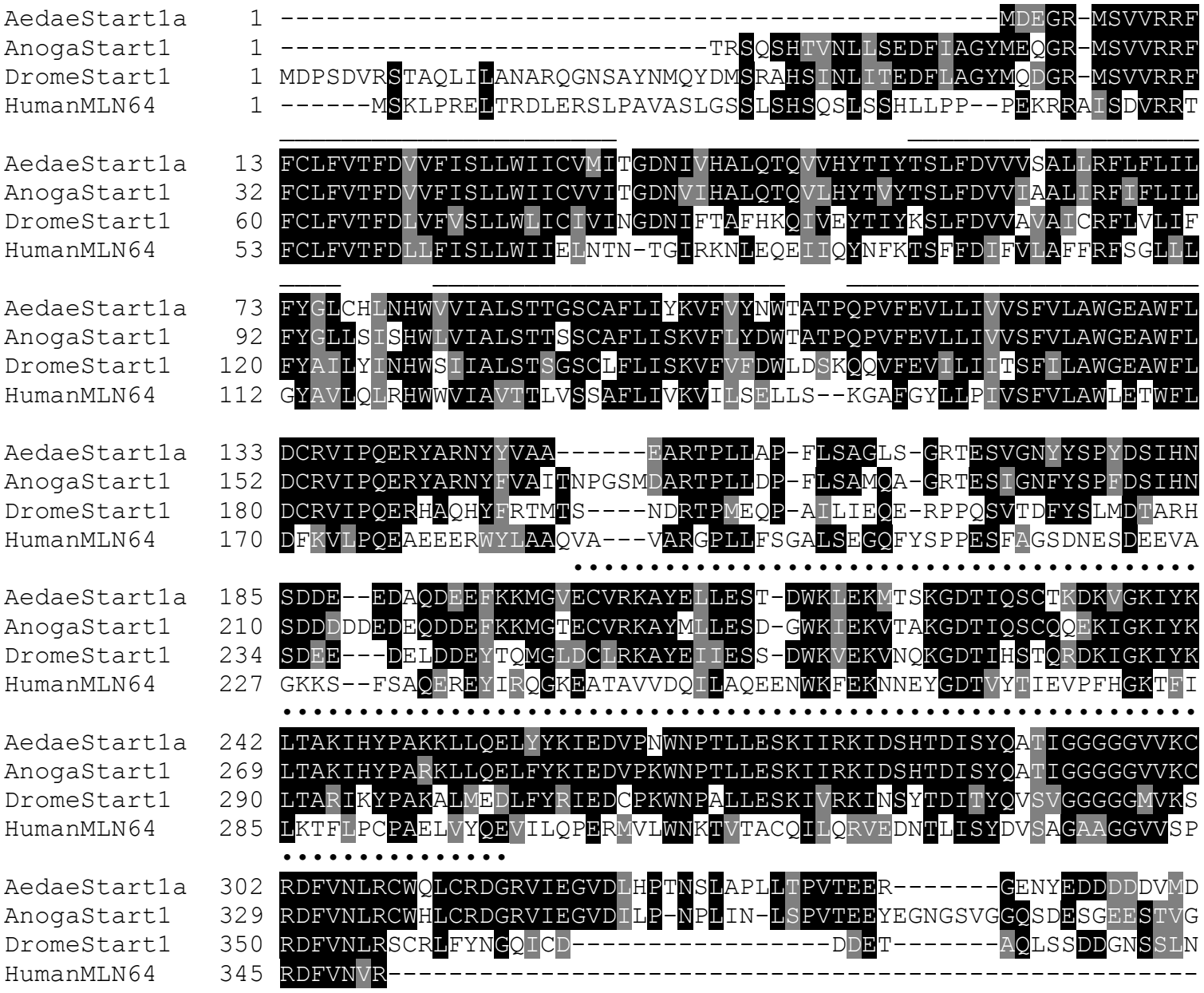


Figure 2.1. Protein sequence of the *A. aegypti* diazepam-binding inhibitor, AedaeDBI, translated from a 502 bp cDNA (GenBank AY947544) with a single ORF of 87 amino acids and predicted to have a pI of 6.26 and a MW of 9836.2 Da. Sequence alignment of AedaeDBI with putative orthologues of *A. gambiae*, AnogaDBI (XM_308405; 80.7% identity, 86.7% similarity), *D. melanogaster*, DromeDBI (X75596; 66.3% identity, 47.7% similarity), and human DBI (AF139542; 50% identity, 61.3% similarity). The solid overbar specifies the Acyl-CoA-binding protein signature [PTLV]-[GSTA]-X-[DENQ]-X-[LMFK]-X_2-[LIVMFY]-Y-[GSA]-X-[FY]-K-Q-[GSA]-[ST]-X-G.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AnogaStart1 1 ----------TRSQSHTVNLLSEDFIAGYMEQGR-MSVVRRF
DromeStart1 1 MDPSDVRSTAQLILANARQGNSAYNMQYDMSRAHSINLITEDFLAGYMQDGR-MSVVRRF
HumanMLN64 1 ------MSKLPRELTRDLERSLPAVASLGSSLSHSQSLSSHLLPP--PEKRRAISDVRRT
AedaeStart1a 13 FCLFVTFDVVFISLLWIICVMITGDNIVHALQTQVVHYTIYTSLFDVVVSALLRFLFLIL
AnogaStart1 32 FCLFVTFDVVFISLLWIICVVITGDNVIHALQTQVLHYTVYTSLFDVVIAALIRFIFLIL
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HumanMLN64 53 FCLFVTFDLLFISLLWIIELNTN-TGIRKNLEQEIIQYNFKTSFFDIFVLAFFRFSGLLL
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DromeStart1 120 FYAILYINHWSIIALSTSGSCLFLISKVFVFDWLDSKQQVFEVILIITSFILAWGEAWFL
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AnogaStart1 152 DCRVIPQERYARNYFVAITNPGSMDARTPLLDP-FLSAMQA-GRTESIGNFYSPFDSIHN
DromeStart1 180 DCRVIPQERHAQHYFRTMTS----NDRTPMEQP-AILIEQE-RPPQSVTDFYSLMDTARH
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DromeStart1 290 LTARIKYPAKALMEDLFYRIEDCPKWNPALLESKIVRKINSYTDITYQVSVGGGGGMVKS
HumanMLN64 285 LKTFLPCPAELVYQEVILQPERMVLWNKTVTACQILQRVEDNTLISYDVSAGAAGGVVSP
AedaeStart1a 302 RDFVNLRCWQLCRDGRVIEGVDLHPTNSLAPLLTPVTEER-------GENYEDDDDVMD
AnogaStart1 329 RDFVNLRCWHLCRDGRVIEGVDILP-NPLIN-LSPVTEEYEGNGSVGGQSDESGEESTVG
DromeStart1 350 RDFVNLRSCRLFYNGQICD----------------DDET-------AQLSSDDGNSSLN
HumanMLN64 345 RDFVNVR----------

Figure 2.2. Protein sequence of the *A. aegypti* StAR-related lipid transfer domain containing protein, AedaeStart1, translated from a 2099 bp cDNA (GenBank AY947545) with a single ORF of 574 amino acids and predicted to have a pI of 5.24 and a MW of 64680.4 Da. Sequence alignment of AedaeStart1a with the two dipteran orthologues of *A. gambiae*, AnogaStart1 (EAA03945; 74.9% identity and 81.6% similarity) and *D. melanogaster*, DromeStart1 (AAF47232; 51.1% identity, 60.7% similarity), and human MLN64 (Q14849; 42.8% identity, 52.3% similarity). Note, sequences were aligned using the program CLUSTAL W 1.74 (http://www/ch.embnet.org/software/ClustalW.html). The solid overbars signify four putative transmembrane regions and solid circles, the predicted START domain.

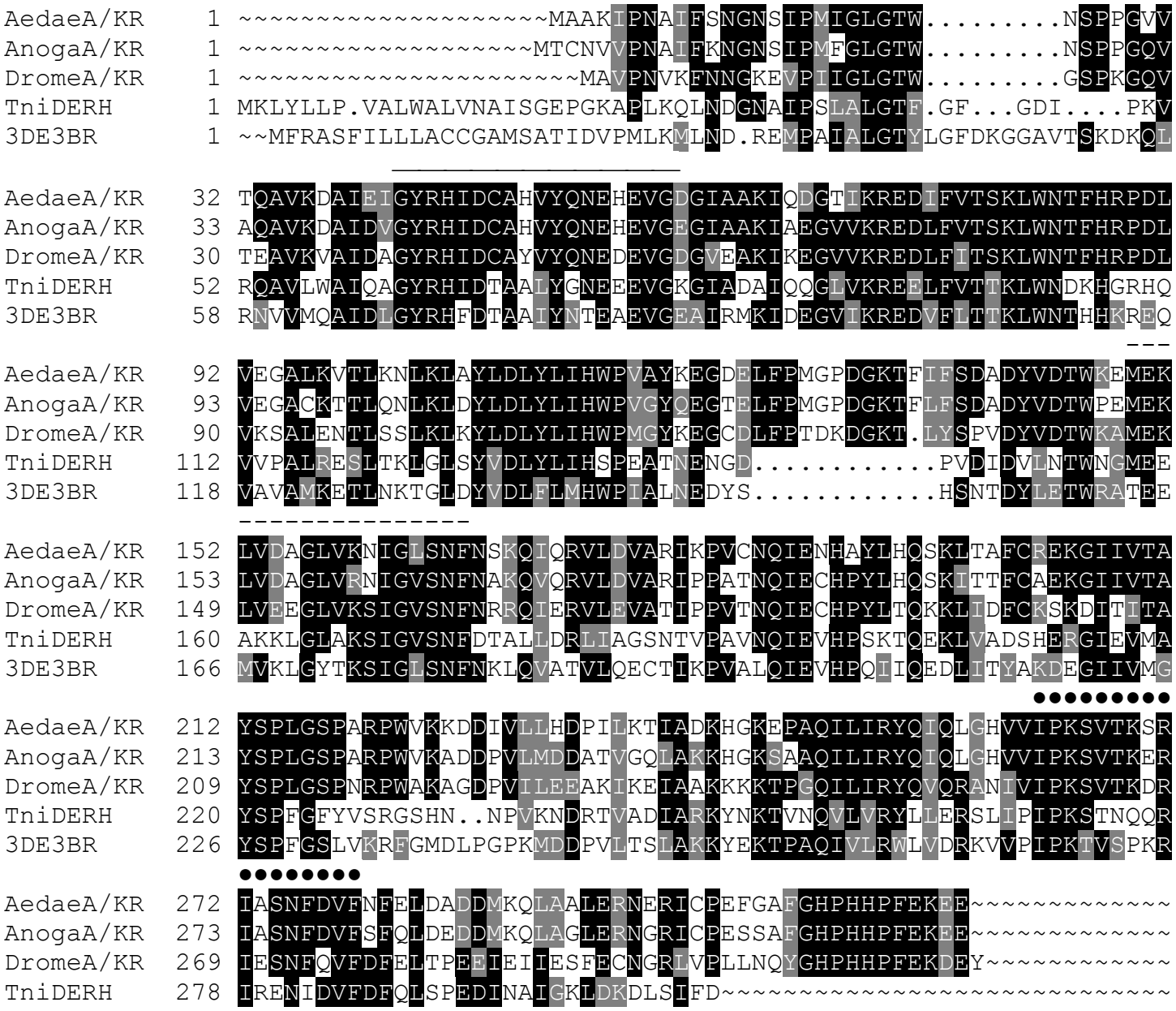
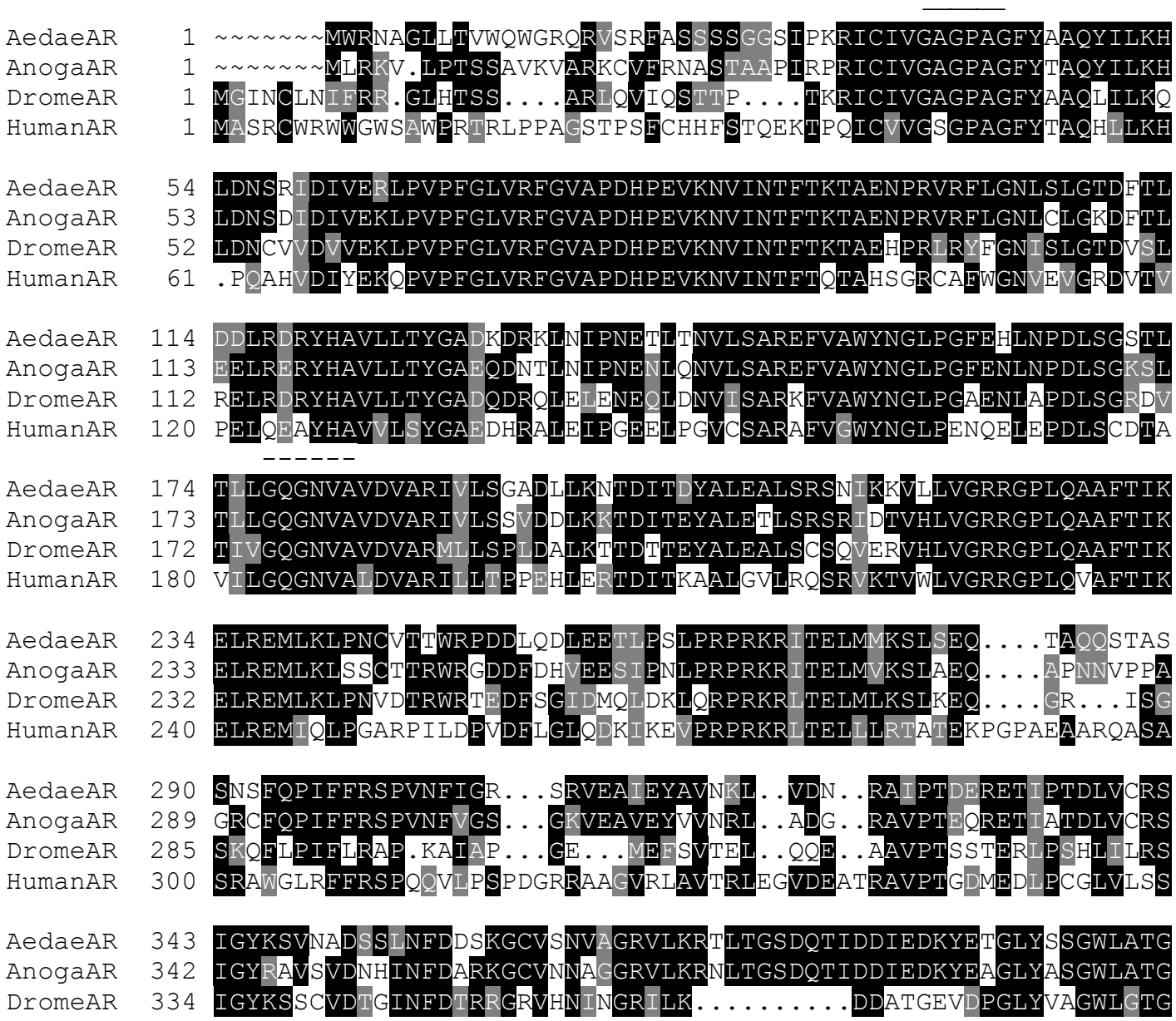

AedaeA/KR
AnogaA/KR
DromeA/KR
TniDERH
3DE3BR

Figure 2.3. Protein sequence of the *A. aegypti* aldo/keto reductase, AedaeA/KR, translated from a 1277 bp cDNA (GenBank AY947547) with a single ORF of 318 amino acids and predicted to have a pI of 6.4 and a MW of 35789.1 Da. Sequence alignment of AedaeA/KR with the dipteran homologues of *A. gambiae*, AnogaA/KR (EAA03870; 82.5% identity, 86.9% similarity) and *D. melanogaster*, DromeA/KR (CG6084-PA; 63.9% identity, 73.5% similarity), and for comparison *Trichoplusia ni* 3-dehydroecdysone 3β-reductase homolog, TniDERH (AF409102; 42.5% identity; 50.4% similarity) and *Spodoptera frugiperta* 3-dehydroecdysone 3β-reductase, 3DE3BR (AJ131966; 41.3% identity, 53.7% similarity). The solid overbar specifies the aldo/keto reductase family signature 1: G-[FY]-R-[HSAL]-[LIVMF]-D-[STAGC]-[AS]-X_5-E-X_2-[LIVM]-G, the dotted overbar the family signature 2: [LIVMFY]-X_9-[KREQ]-X-[LIVM]-G-[LIVM]-[SC]-N-[FY], and the solid circle overbar the putative active site signature: [LIVM]-[PAIV]-[KR]-[ST]-X_4-R-X_2-[GSTAEQK]-[NSL]-X_2-[LIVMFA].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AnogaAR 1 ~~~~~~~MLRKV.LPTSSAVKVARKCVFRNASTAAPIRPRICIVGAGPAGFYTAQYILKH
DromeAR 1 MGINCLNIFRR.GLHTSS....ARLQVIQSTTP....TKRICIVGAGPAGFYAAQLILKQ
HumanAR 1 MASRCWRWWGWSAWPRTRLPPAGSTPSFCHHFSTQEKTPQICVVGSGPAGFYTAQHLLKH
AedaeAR 54 LDNSRIDIVERLPVPFGLVRFGVAPDHPEVKNVINTFTKTAENPRVRFLGNLSLGTDFTL
AnogaAR 53 LDNSDIDIVEKLPVPFGLVRFGVAPDHPEVKNVINTFTKTAENPRVRFLGNLCLGKDFTL
DromeAR 52 LDNCVVDVVEKLPVPFGLVRFGVAPDHPEVKNVINTFTKTAEHPRLRYFGNISLGTDVSL
HumanAR 61 .PQAHVDIYEKQPVPFGLVRFGVAPDHPEVKNVINTFTQTAHSGRCAFWGNVEVGRDVTV
AedaeAR 114 DDLRDRYHAVLLTYGADKDRKLNIPNETLTNVLSAREFVAWYNGLPGFEHLNPDLSGSTL
AnogaAR 113 EELRERYHAVLLTYGAEQDNTLNIPNENLQNVLSAREFVAWYNGLPGFENLNPDLSGKSL
DromeAR 112 RELRDRYHAVLLTYGADQDRQLELENEQLDNVISARKFVAWYNGLPGAENLAPDLSGRDV
HumanAR 120 PELQEAYHAVVLSYGAEDHRALEIPGEELPGVCSARAFVGWYNGLPENQELEPDLSCDTA
AedaeAR 174 TLLGQGNVAVDVARIVLSGADLLKNTDITDYALEALSRSNIKKVLLVGRRGPLQAAFTIK
AnogaAR 173 TLLGQGNVAVDVARIVLSSVDDLKKTDITEYALETLSRSRIDTVHLVGRRGPLQAAFTIK
DromeAR 172 TIVGQGNVAVDVARMLLSPLDALKTTDTTEYALEALSCSQVERVHLVGRRGPLQAAFTIK
HumanAR 180 VILGQGNVALDVARILLTPPEHLERTDITKAALGVLRQSRVKTVWLVGRRGPLQVAFTIK
AedaeAR 234 ELREMLKLPNCVTTWRPDDLQDLEETLPSLPRPRKRITELMMKSLSEQ....TAQQSTAS
AnogaAR 233 ELREMLKLSSCTTRWRGDDFDHVEESIPNLPRPRKRITELMVKSLAEQ....APNNVPPA
DromeAR 232 ELREMLKLPNVDTRWRTEDFSGIDMQLDKLQRPRKRLTELMLKSLKEQ....GR...ISG
HumanAR 240 ELREMIQLPGARPILDPVDFLGLQDKIKEVPRPRKRLTELLLRTATEKPGPAEAARQASA
AedaeAR 290 SNSFQPIFFRSPVNFIGR...SRVEAIEYAVNKL..VDN..RAIPTDERETIPTDLVCRS
AnogaAR 289 GRCFQPIFFRSPVNFVGS...GKVEAVEYVVNRL..ADG..RAVPTEQRETIATDLVCRS
DromeAR 285 SKQFLPIFLRAP.KAIAP...GE...MEFSVTEL..QQE..AAVPTSSTERLPSHLILRS
HumanAR 300 SRAWGLRFFRSPQQVLPSPDGRRAAGVRLAVTRLEGVDEATRAVPTGDMEDLPCGLVLSS
AedaeAR 343 IGYKSVNADSSLNFDDSKGCVSNVAGRVLKRTLTGSDQTIDDIEDKYETGLYSSGWLATG
AnogaAR 342 IGYRAVSVDNHINFDARKGCVNNAGGRVLKRNLTGSDQTIDDIEDKYEAGLYASGWLATG
DromeAR 334 IGYKSSCVDTGINFDTRRGRVHNINGRILK.....DDATGEVDPGLYVAGWLGTG

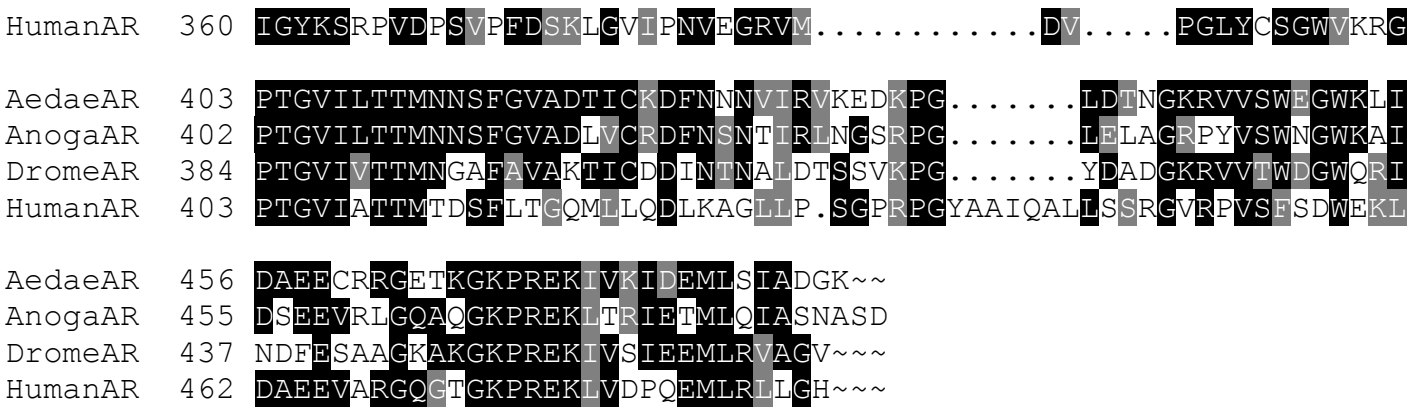
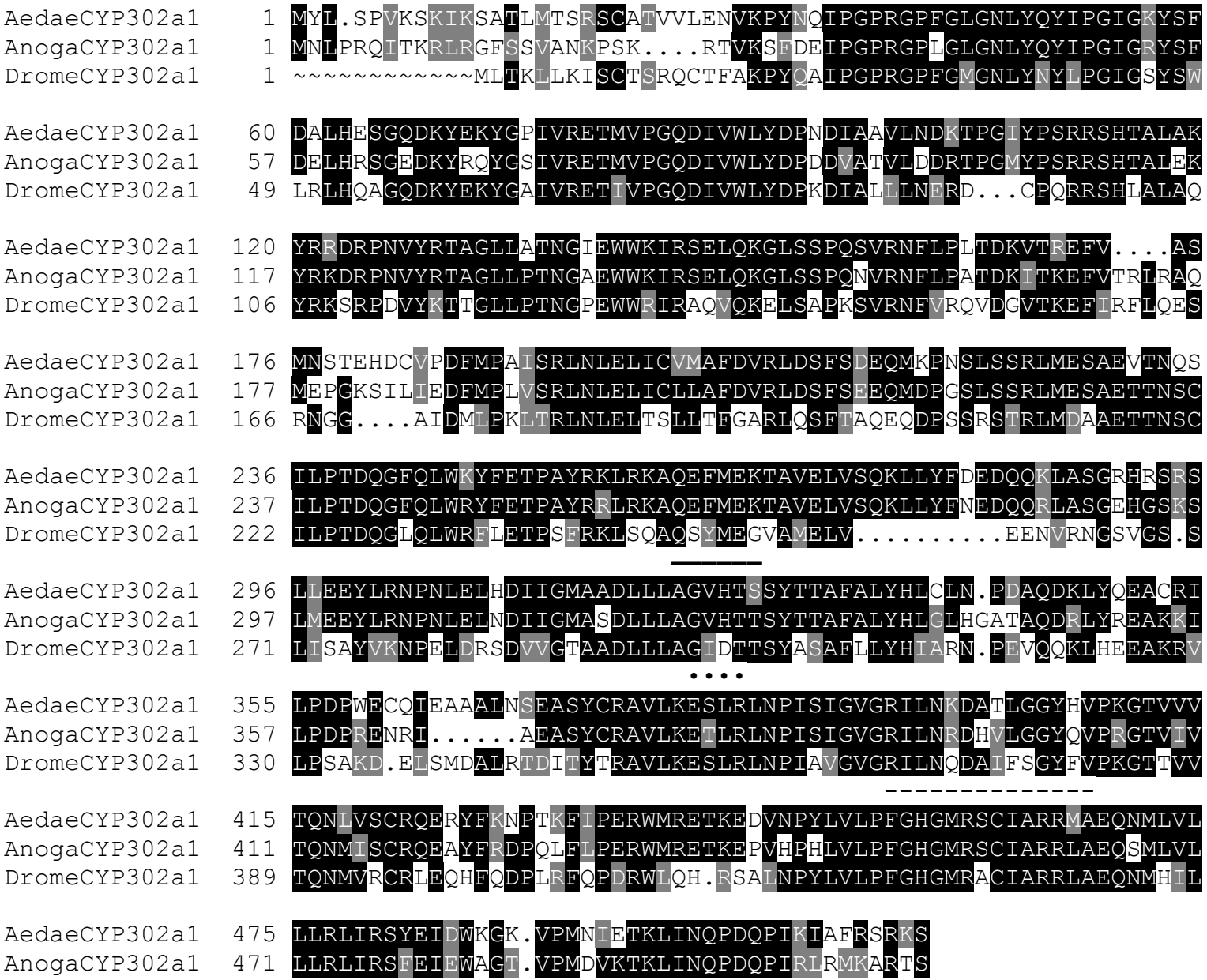


Figure 2.4. Protein sequence of the *A. aegypti* adrenodoxin reductase, AedaeAR, translated from a 1754 bp cDNA (GenBank AY947548) with a single ORF of 486 amino acids and predicted to have a pI of 8.6 and a MW of 53982.5 Da. Sequence alignment of AedaeAR with the insect orthologues of *A. gambiae*, AnogaAR (EAA11924; 74.3% identity, 79.7% similarity) and *D. melanogaster*, DromeAR (AAD50819; 63.7% identity, 70.6% similarity), and human AR (AAA51669; 52.6% identity, 60.9% similarity). The solid overbar specifies the FAD-binding site with the signature G-X-G-X_2-G, and the dotted overbar specfies the NADPH-binding site with the signature G-X-G-X_2-A, both of which are characteristic of this flavoenzyme.


AedaeCYP302a1 1 MYL.SPVKSKIKSATLMTSRSCATVVLENVKPYNQIPGPRGPFGLGNLYQYIPGIGKYSF
AnogaCYP302a1 1 MNLPRQITKRLRGFSSVANKPSK....RTVKSFDEIPGPRGPLGLGNLYQYIPGIGRYSF
DromeCYP302a1 1 ~~~~~~~~~~~~MLTKLLKISCTSRQCTFAKPYQAIPGPRGPFGMGNLYNYLPGIGSYSW
AedaeCYP302a1 60 DALHESGQDKYEKYGPIVRETMVPGQDIVWLYDPNDIAAVLNDKTPGIYPSRRSHTALAK
AnogaCYP302a1 57 DELHRSGEDKYRQYGSIVRETMVPGQDIVWLYDPDDVATVLDDRTPGMYPSRRSHTALEK
DromeCYP302a1 49 LRLHQAGQDKYEKYGAIVRETIVPGQDIVWLYDPKDIALLLNERD...CPQRRSHLALAQ
AedaeCYP302a1 120 YRRDRPNVYRTAGLLATNGIEWWKIRSELQKGLSSPQSVRNFLPLTDKVTREFV....AS
AnogaCYP302a1 117 YRKDRPNVYRTAGLLPTNGAEWWKIRSELQKGLSSPQNVRNFLPATDKITKEFVTRLRAQ
DromeCYP302a1 106 YRKSRPDVYKTTGLLPTNGPEWWRIRAQVQKELSAPKSVRNFVRQVDGVTKEFIRFLQES
AedaeCYP302a1 176 MNSTEHDCVPDFMPAISRLNLELICVMAFDVRLDSFSDEQMKPNSLSSRLMESAEVTNQS
AnogaCYP302a1 177 MEPGKSILIEDFMPLVSRLNLELICLLAFDVRLDSFSEEQMDPGSLSSRLMESAETTNSC
DromeCYP302a1 166 RNGG....AIDMLPKLTRLNLELTSLLTFGARLQSFTAQEQDPSSRSTRLMDAAETTNSC
AedaeCYP302a1 236 ILPTDQGFQLWKYFETPAYRKLRKAQEFMEKTAVELVSQKLLYFDEDQQKLASGRHRSRS
AnogaCYP302a1 237 ILPTDQGFQLWRYFETPAYRRLRKAQEFMEKTAVELVSQKLLYFNEDQQRLASGEHGSKS
DromeCYP302a1 222 ILPTDQGLQLWRFLETPSFRKLSQAQSYMEGVAMELV..........EENVRNGSVGS.S
AedaeCYP302a1 296 LLEEYLRNPNLELHDIIGMAADLLLAGVHTSSYTTAFALYHLCLN.PDAQDKLYQEACRI
AnogaCYP302a1 297 LMEEYLRNPNLELNDIIGMASDLLLAGVHTTSYTTAFALYHLGLHGATAQDRLYREAKKI
DromeCYP302a1 271 LISAYVKNPELDRSDVVGTAADLLLAGIDTTSYASAFLLYHIARN.PEVQQKLHEEAKRV
AedaeCYP302a1 355 LPDPWECQIEAAALNSEASYCRAVLKESLRLNPISIGVGRILNKDATLGGYHVPKGTVVV
AnogaCYP302a1 357 LPDPRENRI......AEASYCRAVLKETLRLNPISIGVGRILNRDHVLGGYQVPRGTVIV
DromeCYP302a1 330 LPSAKD.ELSMDALRTDITYTRAVLKESLRLNPIAVGVGRILNQDAIFSGYFVPKGTTVV
AedaeCYP302a1 415 TQNLVSCRQERYFKNPTKFIPERWMRETKEDVNPYLVLPFGHGMRSCIARRMAEQNMLVL
AnogaCYP302a1 411 TQNMISCRQEAYFRDPQLFLPERWMRETKEPVHPHLVLPFGHGMRSCIARRLAEQSMLVL
DromeCYP302a1 389 TQNMVRCRLEQHFQDPLRFQPDRWLQH.RSALNPYLVLPFGHGMRACIARRLAEQNMHIL
AedaeCYP302a1 475 LLRLIRSYEIDWKGK.VPMNIETKLINQPDQPIKIAFRSRKS
AnogaCYP302a1 471 LLRLIRSFEIEWAGT.VPMDVKTKLINQPDQPIRLRMKARTS

DromeCYP302a1 448 LLRLLREYELIWSGSDDEMGVKTLLINKPDAPVLIDLRLRRE

Figure 2.5. Protein sequence of the *A. aegypti* 22-hydroxylase, AedaeCYP302a1, translated from a 1726 bp cDNA (GenBank AY947549) with a single ORF of 515 amino acids and predicted to have a pI of 8.9and a MW of 58816.7 Da. Sequence alignment of AedaeCYP302a1 with the dipteran orthologues of *A. gambiae*, AnogaCYP0302a1 (XM_316034; 78.6% identity, 85.9% similarity; note, used annotation of R. Feyereisen available at (http://p450.antibes.inra.fr/) and *D. melanogaster*, DromeCYP302a1 (NM_080071; 56.5% identity, 67.6% similarity). The solid overbar specifies the "I-Helix" signature [AG]-G-X-[DE]-T-[TS], the dotted overbar the cytochrome P450 cysteine heme-iron ligand signature [FW]-[SGNH]-X-[GD]-X-[RKHPT]-X-C-[LIVMFAP]-[GAD], and the closed-circle overbar the possible "K-Helix" according to the signature E-X-X-R where X is commonly a hydrophobic aliphatic residue (Werck-Reichhart and Feyereisen 2000).


AedaeCYP315a1 1 ~~~~~~MKLMSSVA..SGKARPFQDLP
AnogaCYP315a1 1 ~~~~~~~~~~~~~~~~~~~~~~~~MLQRLRGTGSRFRC.TGGQQQRAGVAGDSRGAKPFAEMP
DromeCYP315a1 1 MTEKRERPGPLRWLRHLLDQLLVRILSLSLFRSRCDPPPLQRFPATELPPAVAAKYVPIP
AedaeCYP315a1 20 GPRRFPFLGTINDIIHLGNPKTLHLTISKHHIKYGPLFKIQI.GNVNAVFIKDPDMMRSV
AnogaCYP315a1 39 GPRRIPLLGMLNDVMQLGKPAELHLRISKYHEQYGDLVRLQI.GTQNAVFVRDPSLMRKT
DromeCYP315a1 61 RVKGLPVVGTLVDLIAAGGATHLHKYIDARHKQYGPIFRERLGGTQDAVFVSSANLMRGV
AedaeCYP315a1 79 FAYEGKFPKHPLPEAWTYFNEKRKCKRGLFFMDDEEWLHYRKLLNQPLLR.NTSWMIGPI
AnogaCYP315a1 98 FQLEGAYPRHPLPESWTYFNKKHDYQRGLFFMDGKEWLQSRQIFNKPMLK.DFHWMEEPI
DromeCYP315a1 121 FQHEGQYPQHPLPDAWTLYNQQHACQRGLFFMEGAEWLHNRRILNRLLLNGNLNWMDVHI
AedaeCYP315a1 138 KRVSDNTIKSLPHNAKHS.......DCKEKRFELHNVESVLYKWSIEVLLSVMLGSSYNE
AnogaCYP315a1 157 RGTCEATV...GHMQQTC.......D.SAAAFE..GIEAFLYQWSVEVVLSVMLGSAFTE
DromeCYP315a1 181 ESCTRRMVDQWKRRTAEAAAIPLAESGEIRSYELPLLEQQLYRWSIEVLCCIMFGTSVLT
AedaeCYP315a1 191 I.NAIKLNELVEQFSRTVYQIFMYSSKLMAVPPEIADRLQLDAWKQFERIVPESLAIANK
AnogaCYP315a1 204 CQQSAEFRRLVQQFSAVVYDIFRCSSELMNIPPAIADRLNVQPWQQFEKVVPETIRLATA
DromeCYP315a1 241 CP...KIQSSLDYFTQIVHKVFEHSSRLMTFPPRLAQILRLPIWRDFEANVDEVLREGAA
AedaeCYP315a1 250 IID..ISIDDIERG...DGLLSKLEDCIPSRDSIKRIFSDFIIAAGDTTAFATLWCLYLL
AnogaCYP315a1 264 IIE..FGIANAQTR...DGLLDLMMQKL.DKPLMMRIFIDFIIAAGDTTAFATVWALYLL
DromeCYP315a1 298 IIDHCIRVQEDQRRPHDEALYHRLQAADVPGDMIKRIFVDLVIAAGDTTAFSSQWALFAL
AedaeCYP315a1 305 AKNQAVQTMVR.DETKHDFLESPLIRATVKESLRLFPIAPFIGRFLATDAIIGDYCIPKN
AnogaCYP315a1 318 ASNANLQQSVRQDVLDSNTLECGAVKGVVRETLRLYPIAPFIGRFVEHESVFGAYALPKD
DromeCYP315a1 358 SKEPRLQQRLAKERATND...SRLMHGLIKESLRLYPVAPFIGRYLPQDAQLGGHFIEKD
AedaeCYP315a1 364 TLALLSLYSAGRDEVNFYLPNEFLPQRWLRRDDKNQSIIPFNANASLPFAIGSRSCIGRR
AnogaCYP315a1 378 TLVLLSLYSAGRDERFFAEPEAFNPYRW.QRTNAAESSTGRTPSASLPFAIGARSCIGQK
DromeCYP315a1 415 TMVLLSLYTAGRDPSHFEQPERVLPERWC....IGETEQVHKSHGSLPFAIGQRSCIGRR
AedaeCYP315a1 424 VALIQMQYLLSKILNEYRLTVLNDEEVDA...ELKLVTVPNKKVKLAFHKLQ~~~~~~~~

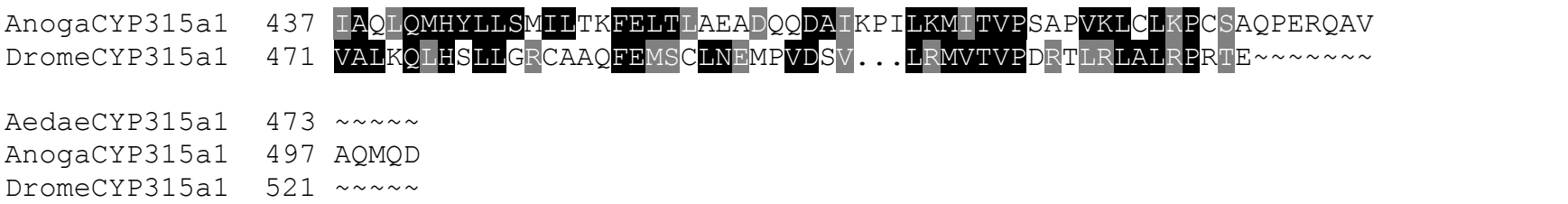


Figure 2.6. Protein sequence of the *A. aegypti* 2-hydroxylase, AedaeCYP315a1, translated from a 1576 bp cDNA (GenBank AY947551) with a single ORF of 472 amino acids and predicted to have a pI of 9.17 and a MW of 54320.27 Da. Sequence alignment of AedaeCYP315a1 with the dipteran homologues of *A. gambiae*, AnogaCYP315a1 (XM_310837**;** 51% identity, 62% similarity; note, used annotation of R. Feyereisen avaliable at http://p450.antibes.inra.fr/) and *D. melanogaster*, DromeCYP315a1 (AY079170; 42.4% identity, 56% similarity). The solid overbar specifies the "I-Helix" signature, the dotted overbar the cytochrome P450 cysteine heme-iron ligand signature, and the closed-circle overbar the possible "K-Helix."


AedaeCYP314a1 1 MSVTIILFYVFITAFMLLSYNPKPRKILESIKSFLLHLV.H.....LSKN
AnogaCYP314a1 1 MSVTIVLFYTFVTLFMFLSYNPKPKKIVESIRSFLLHLLQHNGDAGAGSGGLQ.AATACN
DromeCYP314a1 1 ~~~~~~~~~~~~~~~~~~~~~~~~~MAVILLLALALVLVCYCALHRHKLADIYLRPLLKN
AedaeCYP314a1 45 VVSTT.IHVPPDAMQMNQA..EPVYTVWDIPGPKRLPLVGTRWMY..YVGRYKLNKMQDA
AnogaCYP314a1 60 AAQTSPVSPPAPSPPPNDAGVPTVRSIWDIPGPRRLPLIGTKWRY..FFGRHRYAKVHET
DromeCYP314a1 36 TLLEDFYH..AELIQP.EAPKRRRRGIWDIPGPKRIPFLGTKWIFLLFFRRYKMTKLHEV
AedaeCYP314a1 100 FVDLHKRYGNIVLEF..DHVPIVNLFDRVDMEKVLKYPSKYPYRPPTEIVEYYRRSRPDR
AnogaCYP314a1 118 FMEMHRRYGPIMLDV..DTVPIVNLFDRADMEKVLRYPSRYPFRPPTEIVEVYRSSRPDR
DromeCYP314a1 93 YADLNRQYGDIVLEVMPSNVPIVHLYNRDDLEKVLKYPSKYPFRPPTEIIVMYRQSRPDR
AedaeCYP314a1 158 FASTGIVNTQGEQWHELRVKLTSGIMSRKLLQAFIPTLNEIADEFVTLIRQKRDSND.CV
AnogaCYP314a1 176 FGVTNLINAQGAKWHELRAKLTTGITSRRVLQAFIPSVNEICDDFVDLVRRQRADDG.TI
DromeCYP314a1 153 YASVGIVNEQGPMWQRLRSSLTSSITSPRVLQNFLPALNAVCDDFTELLRARRDPDTLVV
AedaeCYP314a1 217 KDFQDIANTVGLEIICCFVLGRRMGYMSGDKQKNEKFVKLAEAVKSTFMYISQSYYGVKL
AnogaCYP314a1 235 RNFQDIANSVGLEIICCLVLGRRMGYLTTNRQ.NAKFMRLAEAVKESFVYIGESYFGLKL
DromeCYP314a1 213 PNFEELANLMGLEAVCTLMLGRRMGFLAIDTKQPQKISQLAAAVKQLFISQRDSYYGLGL
AedaeCYP314a1 277 WKYLPTKLYRDYVRCEEIIYDTIAEIVNEALAE..EQEKCAADD...MRGIFLNILQSEG
AnogaCYP314a1 294 WKYVPTRLYSNFVRCEEIIYETIAEIVYEALEE..EQLNCPDND...VKHIFISILQTEG
DromeCYP314a1 273 WKYFPTKTYRDFARAEDLIYDVISEIIDHELEELKKSAACEDDEAAGLRSIFLNILELKD
AedaeCYP314a1 332 LDKKDKIAGIIDLIHAAIETFSNTLSFLLNNMTSHPERQARIASEFTS..DT.ITNNDLV
AnogaCYP314a1 349 LDTKEKISGIIDLITSAIETLSNTLSFLLHNLSQTVEYQREIAHEFAHCVQH.ITNEDLV
DromeCYP314a1 333 LDIRDKKSAIIDFIAAGIETLANTLLFVLSSVTGDPGAMPRILSEFCEYRDTNILQDALT
AedaeCYP314a1 389 NAAFTRACIKESYRISPTTPCLARILEEDFDLSGYQLKAGTVVLCHTRVACQNEQNFQQA
AnogaCYP314a1 408 SARFTKACIQESYRISPTTPCLARILEEDFQLSGYHLQAGTLVLCHTRVACLSEDNFQQA
DromeCYP314a1 393 NATYTKACIQESYRLRPTAFCLARILEEDMELSGYSLNAGTVVLCQNMIACHKDSNFQGA
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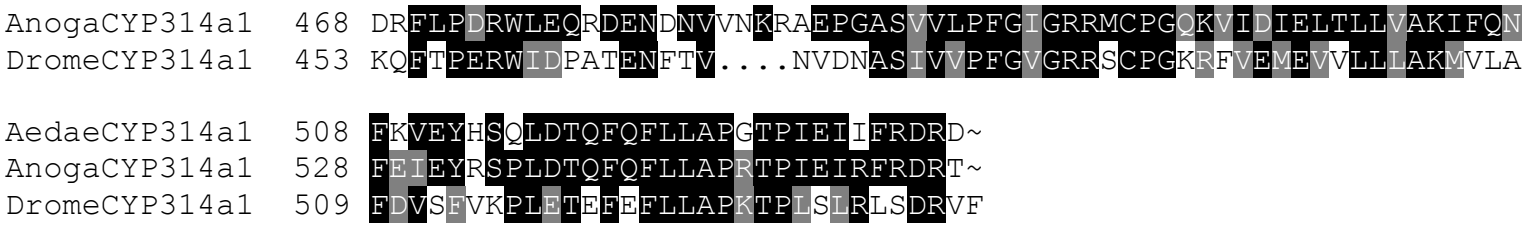


Figure 2.7. Protein sequence of the *A. aegypti* 20-hydroxylase, AedaeCYP314a1, translated from a 2229 bp cDNA (GenBank AY947552) with a single ORF of 538 amino acids and predicted to have a pI of 6.59and a MW of 62208.83Da. Sequence alignment of AedaeCYP314a1 with the dipteran orthologues of *A. gambiae*, AnogaCYP314a1 (EAA08077; 66.4% identity, 74.8% similarity; note, used annotation of R. Feyereisen avaliable at http://p450.antibes.inra.fr/) and *D. melanogaster*, DromeCYP314a1 (AF484414; 51.8% identity, 63.8% similarity). The solid overbar specifies the "I-Helix" signature, the dotted overbar the cytochrome P450 cysteine heme-iron ligand signature, and the closed-circle overbar the possible "K-Helix."

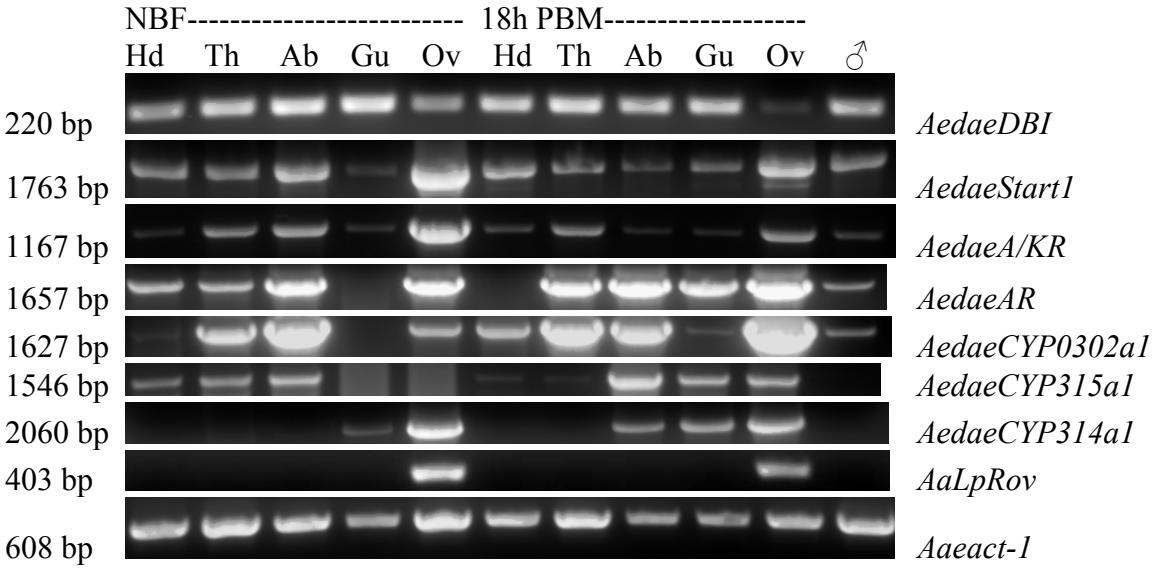
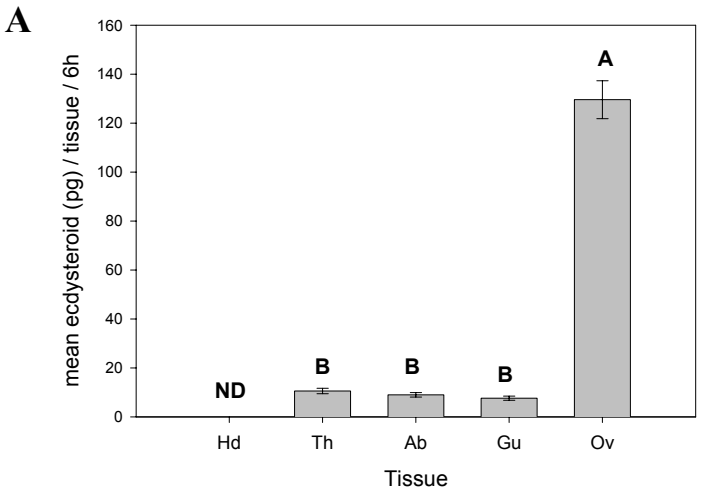


Figure 2.8. RT-PCR analysis of the tissue distribution of the seven gene transcripts characterized in the current study along with tissue specific and loading controls, mosquito ovarian lipophorin receptor (AaLpRov) and *A. aegypti* actin (Aaeact-1), respectively. Hd = head, Th = thorax, Ab = abdomen, Gu = gut, Ov ovary pair, ♂ = male *A. aegypti*.



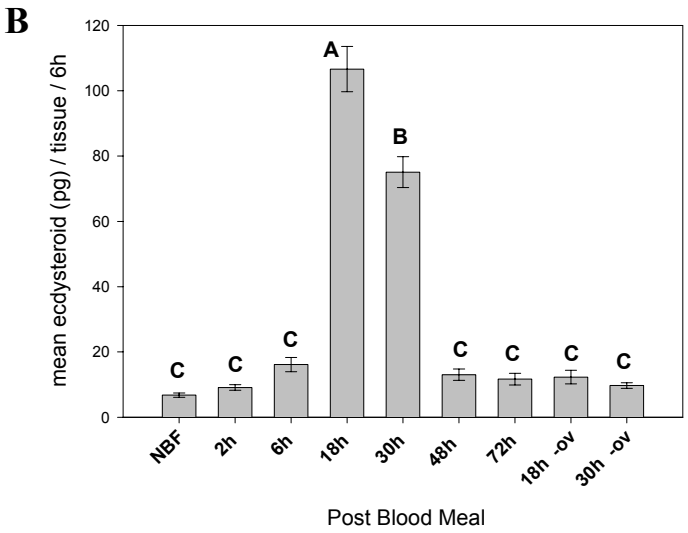
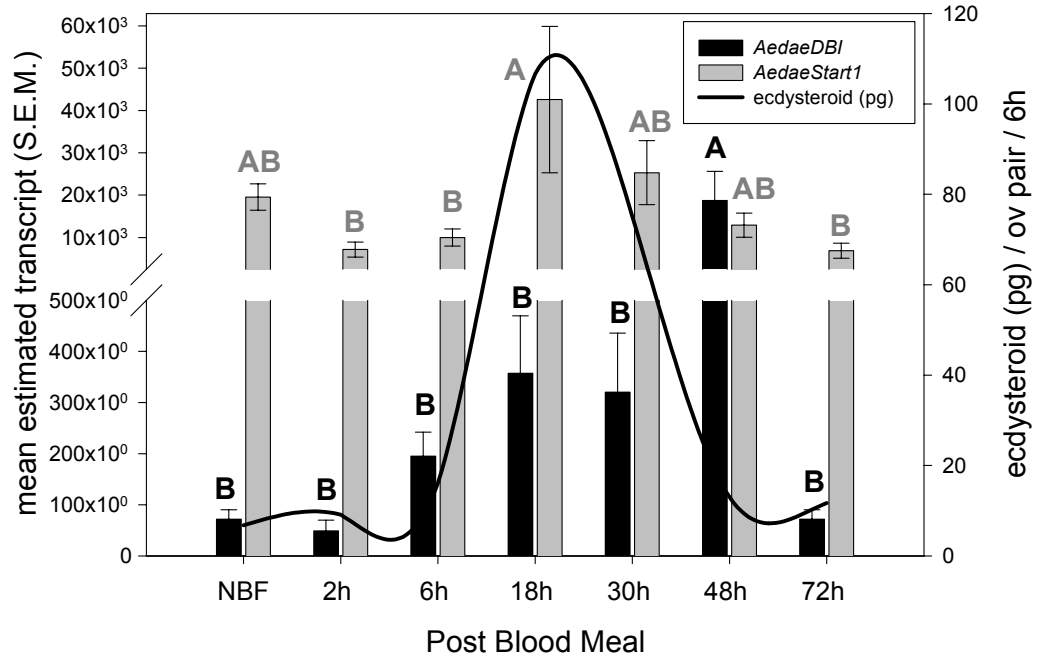


Figure 2.9. (**A)** Ecdysteroid production by various tissue and body parts of *A. aegypti* females 18h PBM as assessed by RIA. Hd = head, Th = thorax, Ab = abdomen, Gu = gut, and Ov = ovary pair. Amounts are presented as ecdysteroid (pg) produced by a single tissue equivalent in 6h (error bars = S.E.M.), or ND = not detectible. (**B)** Ecdysteroid production by ovary pairs at specific points during the gonotropic cycle as assessed by RIA. As a control, ecdysteroids produced by dissected tissues without the ovary pair (-ov) were assayed. Amounts are presented as ecdysteroid (pg) produced per ovary pair and "-ov" tissue in 6h (error bars = S.E.M.). Bars with the same letter are not significantly different (Tukey-Kramer HSD $p \leq 0.05$).

A


AedaeDBl
AedaeStart1
ecdysteroid (pg)
mean estimated transcript (S.E.M.)
ecdysteroid (pg) / ov pair / 6h
60x10³
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30x10³
20x10³
10x10³
500x10⁰
400x10⁰
300x10⁰
200x10⁰
100x10⁰
0
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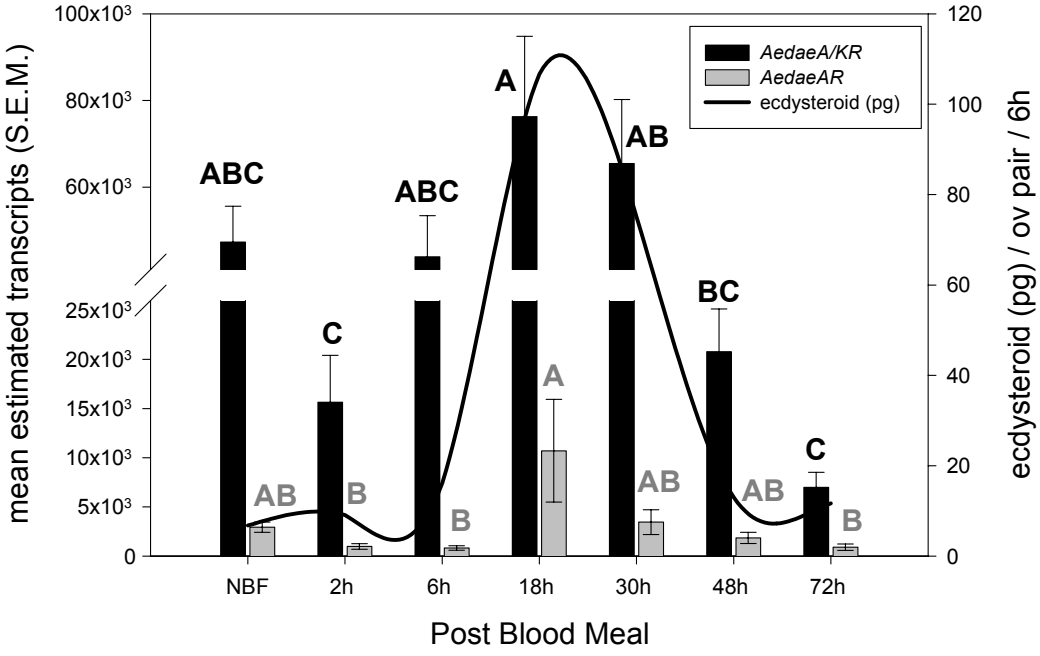
40
20
0
AB
B
B
A
AB
B
B
B
B
B
B
B
B
NBF
2h
6h
18h
30h
48h
72h
Post Blood Meal

B


AedaeA/KR
AedaeAR
ecdysteroid (pg)
mean estimated transcripts (S.E.M.)
ecdysteroid (pg) / ov pair / 6h
100x10³
80x10³
60x10³
25x10³
20x10³
15x10³
10x10³
5x10³
0
120
100
80
60
40
20
0
ABC
AB
C
B
ABC
B
A
A
AB
AB
BC
AB
C
B
NBF
2h
6h
18h
30h
48h
72h
Post Blood Meal

C

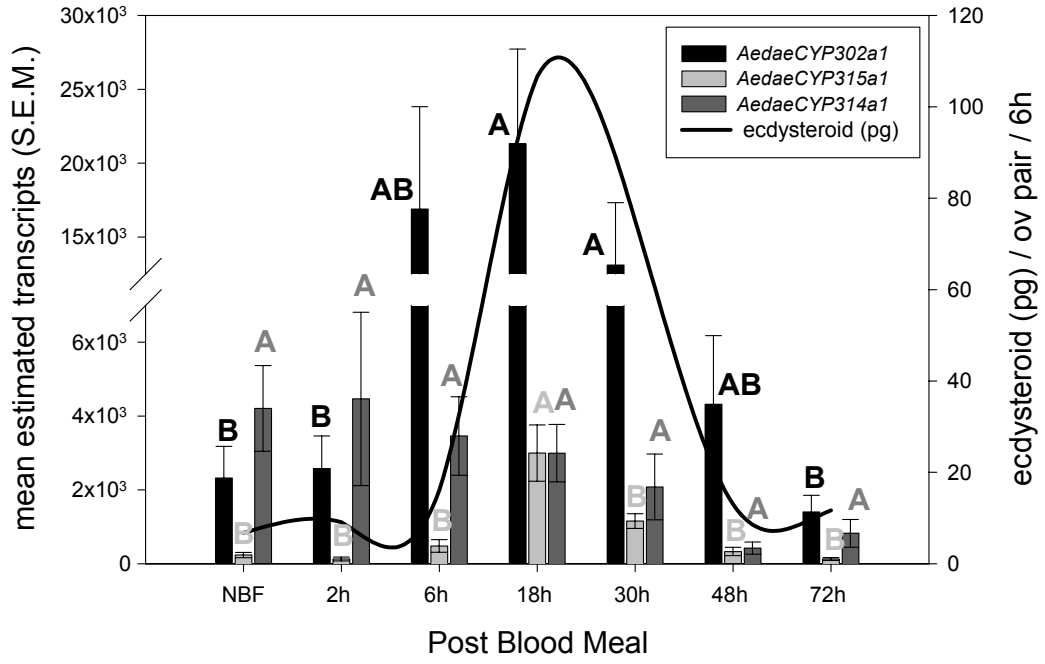


Figure 2.10. Real-time PCR analysis of gene transcripts in ovaries in non-blood fed (NBF) and those provided a blood meal (PBM). The graphs display mean estimated transcript copies ± S.E.M per 0.048 ovary pair equivalents (A) *AedaeDBI* and *AedaeStart1*, (B) *AedaeA/KR* and *AedaeAR*, and (C) *AedaeCYP302a1*, *AedaeCYP315a1* and *AedaeCYP314a1*. Bars with the same letter are not significantly different (Tukey-Kramer HSD $p \leq 0.05$). A smoothed line representing PBM ovary ecdysteroid production is superimposed over each graph for descriptive purposes (note: ecdysteroid measurements from current study).

Supplemental Table 2.1. Degenerate PCR primers used in the initial screen of *A. aegypti* ovary cDNA

Gene / "nested" PCR round	forward primer / **corresponding amino acids**	reverse primer / **corresponding amino acids**	*A. aegypti* amplicon
AedaeDBI / round 1	5'-TAYGCNYTNTTYAARCARGC-3' **Y A L F K Q A**	5'-(AGC)$(dT)_{18}$-3' **N/A**	Y30 – 3' UTR
AedaeDBI / round 2	5'-GAYYTNAARGGNAARGCNAAR-3' **D L K G K A K**	5'-(AGC)$(dT)_{18}$-3' **N/A**	D50 – 3' UTR
AedaeStart1 / round 1	5'-GARGAYTTYATHGCNGGNTAYATG-3' **E D F I A G Y M**	5'-ARNGTNGGRTTCCAYTTNGG-3' **[PKWNPTL]***	N/F – L271
AedaeStart1 / round 2	5'-TTYTGYYTNTTYGTNACNTTYGA -3' **F C L F V T F D**	5'-ACYTTYTCDATYTTCCA-3' **[WKIEKV]***	F13 – M221
AedaeA/KR / round 1	5'-GGNTAYMGNCAYATHGAYTGYGC-3' **G Y R H I D C A**	5'-ARNGGNSWRTANGCNGTNAC-3' **[VTAYSPL]***	G42 – L215
AedaeA/KR / round 2	same as round 1	5'-GGCCARTGDATNARRTANARRTC-3' **[DLYLIHWP]***	G42 – P116
AedaeAR / round 1	5'-GAYCAYCCNGARGTNAARAA-3' **D H P E V K N**	5'-TCYTTDATNGTRAANGCNGC-3' **[AAFTIKE]***	D79 – E234
AedaeAR / round 2	5'-AAYACNTTYACNAARACNGC-3' **N T F T K T A**	5'-TTDATNGTRAANGCNGCYTG-3' **[QAAFTIK]***	N88 – K233
AedaeCYP302a1 / round 1	5'-ATHYTNCCNACNGAYCARGG-3' **I L P T D Q G**	5'-CATNCCRTGNCCRAANGG-3' **[PFGHGM]***	I236 – M458

* amino acid sequence of the reverse compliment of the degenerate primers; **x** = amino acid differs from that deduced for the *A. aegypti* protein sequence; N/F = Not Found; N/A = Not Applicable

Supplemental Table 2.2. Gene-specific PCR primers used in 3' RACE of *A. aegypti* ovary cDNA

Gene / "nested" PCR round	**Gene-specific forward primer**	**reverse primer**	***A. aegypti* amplicon**
AedaeStart1 / round 1	5'-ACCCCGCAGCCCGTTTTC-3'	Not I anchor	T108 – 3'UTR
AedaeStart1 / round 2	5'-GAATGTGTTCGCAAGGCATACGAG-3'	Anchor(dT)R	E202 – 3'UTR
AedaeA/KR / round 1	5'-TGTACCAAAACGAGCATGAAG-3'	Not I anchor	Y52 – 3'UTR
AedaeA/KR / round 2	5'-CATGAAGTTGGCGATGGAATAG-3'	Anchor(dT)R	H56 – 3'UTR
AedaeAR / round 1	5'-ACACTAGACGACCTCAGGGATA-3'	Not I anchor	T112 – 3'UTR
AedaeAR / round 2	same as round 1	Anchor(dT)R	same
AedaeCYP302a1 / round 1	5'-GCTGTGGAAGTACTTCGAGACTCCAGC-3'	Not I anchor	L245 – 3'UTR
AedaeCYP302a1 / round 2	5'-AAAGACTGCCGTTGAGTTG-3'	Anchor(dT)R	K266 – 3'UTR
AedaeCYP315a1 / round 1	5'-CGCTTATTCGTGCAACAGTGAAGG-3'	Not I anchor	L327 – 3'UTR
AedaeCYP315a1 / round 2	5'-AGGAATCTCTCCGTCTGTTCCCTA-3'	Anchor(dT)R	E334 – 3'UTR
AedaeCYP314a1 / round 1	5'-GGTTATCAGCTCAAAGCTGGGACGGT-3'	Not I anchor	G422 – 3'UTR
AedaeCYP314a1 / round 2	5'-TAACACGTTTCTACCGGAGCGATG-3'	Anchor(dT)R1	N449 – 3'UTR

Not I anchor: 5'-AACTGGAAGAATTCGCGGCCGCAGGAA-3'

Anchor(dT)R: 5'- GGGGAGGCTCGAG$(dT)_{16}$-3'

Supplemental Table 2.3. Gene-specific PCR primers used in 5' RACE of *A. aegypti* ovary cDNA

Gene / "nested" PCR round	**forward primer**	**Gene-specific reverse primer**	***A. aegypti* amplicon**
AedaeDBI / round 1	LamdaZap1	5'-ATCCACCACACATATCCCATC-3'	5'UTR – 3'UTR
AedaeDBI / round 2	LamdaZap2	5'-CCATCTCGCGCAGTTCTATC-3'	5'UTR – 3'UTR
AedaeStart1 / round 1	LamdaZap1	5'-GCCTCCGCCGCGACATAAT-3'	5'UTR – E151
AedaeStart1 / round 2	LamdaZap2	5'-CAGACACAACCACGTCGAACAG-3'	5'UTR – S62
AedaeA/KR / round 1	LamdaZap1	5'-TACAGGTCCAAGTAGGCGAG-3	5'UTR – L110
AedaeA/KR / round 2	LamdaZap2	5'-CGATGGAACGTATTCCACAG-3'	5'UTR – H87
AedaeAR / round 1	LamdaZap1	5'-GTTACCCTGCCCTAGTAATGTCAG-3'	5'UTR – N180
AedaeAR / round 2	LamdaZap2	5'-CTGCCCTAGTAATGTCAGGGTACT-3'	5'UTR – Q178
AedaeCYP302a1 / round 1	Oligo(dT) anchor primer	5'-TACAGCCACACAATATCCTGACCG-3'	5'UTR – L90
AedaeCYP302a1 / round 2	Forward nested anchor primer	5'-GGGCATCGAAGCTGTATTTCCCTA-3'	5'UTR – A61
AedaeCYP315a1 / round 1a	Oligo(dT) anchor primer	5'-CGAGCATCACACTGAGCAGAACTT-3'	5'UTR – L184
AedaeCYP315a1 / round 2a	Forward nested anchor primer	5'-GTCGCTCACTCGTTTAATTGGTCC-3'	5'UTR – D142
AedaeCYP315a1 / round 1b	Oligo(dT) anchor primer	5'-CCCTCGTATGCAAACACACTTCTC-3'	5'UTR – E82
AedaeCYP315a1 / round 2b	Forward nested anchor primer	5'-GTCTAGGACCCGGAAGATCTTGAA-3'	5'UTR – R22
AedaeCYP314a1 / round 1	Oligo(dT) anchor primer	5'-CCTTGTGTGTTCACAATTCCGGTG-3'	5'UTR – Q167
AedaeCYP314a1 / round 2	Forward nested anchor primer	5'-ATAGCGCCCTACGTAGTACATCCA-3'	5'UTR – Y91

LamdaZap1: 5'-GCTATGACCATGATTACGCC-3'

LamdaZap2: 5'-TACGCCAAGCTCGAAATTAACC-3'

Oligo(dT) anchor primer: 5'-GACCACGCGTATCGATGTCGAC$(T)_{16}$(A/C/G)

Forward nested anchor primer: 5'-GACCACGCGTATCGATGTCGAC-3'

X = nucleotide different from that determined for the final contig (T = G in final contrig, C = T in final contig)

Supplemental Table 2.4. Primers used for the amplification of the ORF using a proof-reading DNA polymerase.

Transcript	Forward primer	Reverse primer	Amplicon	Tm
AedaeDBI	5'-AGGAATTCGGCACGAGTCTCAATC-3'	5'-CATCGATAACTTTCGTCGTTTACTG-3'	480 bp	55°C *
AedaeStart1	5'-AGTCCCATATGGTTAATCTACTGA-3'	5'-GACTAATTGGGAGTAAACTAGGAAGC-3'	1763 bp	55°C *
AedaeA/KR	5'-TCGAGTTGATCGTCTGTCTGTA-3'	5'-ATGTGATCTACTGCCAAGCAGC-3'	1167 bp	60°C *
AedaeAR	5'-ATTCGGCACGAGACACAGCTGATA-3'	5'-TACGTCATACGCAACCAAGTACCG-3'	1657 bp	60°C *
AedaeCYP302a1	5'-GACTGGGTATGTGTCCGATCAAGT-3'	5'-TCACAATTACGATTTTCTCGACC-3'	1627 bp	60°C ‡
AedaeCYP315a1	5'-GTCTGTGGAAGAAGCAACAACGGA-3'	5'-GCAAATCTGTTTACTATGTGTCAG-3'	1546 bp	55°C *
AedaeCYP314a1	5'-CGGAGTGAAGTAATTCAGGCGA-3'	5'-TGTACGTTATTGATTTGCTCAGAC-3'	2060 bp	60°C *

* PCR program: 95°C for 2 min, 95°C for 30 sec Tm for 30 sec, 72°C for 30 sec 35 cycles, and 72°C for 7 min

‡ PCR program: 94°C for 2 min, 94°C for 15 sec Tm for 30 sec, 72°C for 90 sec 10 cycles; 94°C for 15 sec Tm for 30 sec, 72°C for 90 to 190 sec (+ 5 sec every cycle) 20 cycles, and 72°C for 7 min

Supplemental Table 2.5. Primers used in real-time PCR analysis of ovary transcript levels before and following a blood meal

Transcript	**Forward primer**	**Reverse primer**	**Amplicon**	**a. a. sequence**
AedaeDBI	5'-GCCAAGGAGGCGTACGTTAAGTTT-3'	5'-TATCCCATCTCGCGCAGTGCTATT-3'	91 bp	A71-3'UTR
AedaeStart1	5'-ACCCCGCAGCCCGTTTTC-3'	5'-GCCTCCGCCGCGACATAAT-3'	134 bp	T108-E151
AedaeA/KR	5'-TGCCGACTATGTGGACACTTGGAA-3'	5'-ATTGCAGACCGGTTTAATGCGAGCGA-3'	139 bp	A140-N185
AedaeAR	5'-GACTTTGACAAACGTGCTATCGGC-3'	5'-AGCACTATTCGAGCTACGTCAACC-3'	150 bp	T141-V189
AedaeCYP302a1	5'-ATCCTACTGTCGAGCCGTTCTGA-3'	5'-CTCCGAGTGTGGCATCTTTGTTC-3	95 bp	S373-G403
AedaeCYP315a1	5'-TTCTGCTCAGTGTGATGCTCGGAA-3'	5'-ATTTCAGGAGGAACGGCCATCAGCTT-3'	139 bp	L179-E223
AedaeCYP314a1	5'-CGTGCATCAAAGAGTCGTACAGGA-3'	5'-CAGCTTTGAGCTGATAACCGGAGA-3'	99 bp	C396-A427

CHAPTER 3

EXPRESSION OF THE CYTOCHROME P450 ENZYME PUTATIVELY CONDUCTING THE 22-HYDROXYLATION REACTION IN *Aedes aegypti* ECDYSTEROIDOGENESIS WITHIN OVARIES DURING A GONOTROPIC CYCLE

1. Introduction

Insect oogenesis is controlled through complex hormonal cascades, parts of which include the coordinated expression of enzymes and proteins involved in the biosynthesis of the ecdysteroid hormones (Hodin and Riddiford, 1998; Buszczak et al., 1999; Freeman et al., 1999; Kozlova and Thummel, 2000; Petryk et al., 2003; Swevers and Iatrou, 2003). The adult ovary has been shown to be the source of ecdysteroids in insect species from many orders (Hagedorn et al., 1975; Bollenbacher et al., 1978; Delbecque et al., 1978; Goltzene et al., 1978; Rubenstein et al., 1982; Zhu et al., 1983; Hoffmann et al., 1992; Lorenz et al., 1995; Romana et al., 1995; Adams et al., 1997; Maniere et al., 2000; Taibi et al., 2003). The necessity for ovarian production of ecdysteroids is exemplified by the observation that the disruption of genes that encode enzymes implicated in ecdysteroid biosynthesis inhibit the completion of *D. melanogaster* ovariole development (Buszczak et al., 1999; Freeman et al., 1999; Petryk et al., 2003).

The characterization of the ecdysteroid biosynthetic pathway has been an active area of research for over three decades, but many presumed steps remain steadfastly elusive (Grieneisen, 1994; Rees, 1995; Gilbert et al., 2002). The involvement of cytochrome P450 enzymes in the biosynthesis of ecdysteroids has been known for some time (Kappler et al., 1988; Kappler et al.,

1989; Grieneisen et al., 1993; Feyereisen, 1999), and recently four cytochrome P450 enzymes responsible for the "terminal hydroxylations" of ecdysteroids have been identified and characterized (Chavez et al., 2000; Warren et al., 2002; Petryk et al., 2003; Gilbert, 2004; Niwa et al., 2004; Warren et al., 2004). The first to be identified was CYP302a1 of *Drosophila melanogaster*, which was accomplished through the analysis of the Halloween mutant, *disembodied* (Chavez et al., 2000). Subsequently, *D. melanogaster* CYP302a1 was determined to conduct the "terminal hydroxylation" at carbon-22 of an ecdysteroid precursor (Warren et al., 2002). An increase in gene transcripts for *D. melanogaster* CYP302a1 correlates with peak ecdysteroid titers throughout development, including oogenesis (Chavez et al., 2000; Warren et al., 2002; Parvy et al., 2005), but such fluctuations in protein levels have not been thoroughly addressed.

A blood meal taken by female *A. aegypti* initiates ovary production of ecdysteroids (Hagedorn et al., 1975; Sieglaff et al., 2005), which serve as the primary hormonal regulator of oogenesis in this mosquito species (Raikhel et al., 2002). Following the blood meal, ovarian ecdysteroid production begins to increase 6 h post blood meal (PBM), peaks 18-24 h PBM, and falls to pre blood feeding levels by 36-48 h PBM, when vitellogenin uptake has ended and chorion formation begins; finally eggs are oviposited by 60-72 h PBM (Hagedorn et al., 1975; Clements, 1992; Sappington and Raikel, 1999; Sieglaff et al., 2005). In a previous study, gene transcript abundance of the putative *A. aegypti* homolog of 22-hydroxylase, *AedaeCYP302a1*, followed the rise and fall of ovarian ecdysteroid production in blood-fed females (Sieglaff et al., 2005), suggesting transcription of this gene may be a means of regulating ovarian ecdysteroid production. Indeed, prothoracicotropic hormone (PTTH) stimulation of ecdysteroid biosynthesis in prothoracic glands (PG) has been shown to require gene transcription (Keightley et al., 1990;

Rybczynski and Gilbert, 1995a), although the necessity for gene transcription, at least in acute stimulation of ecdysteroid biosynthesis by PTTH has not always been observed (Smith and Gilbert, 1989). More evidence has accrued to suggest PTTH stimulation of ecdysteroid biosynthesis is mediated through its promotion of the phosphorylation of ribosomal protein S6 and the subsequent translation of specific proteins (Smith and Gilbert, 1989; Keightley et al., 1990; Rybczynski and Gilbert, 1994; Song and Gilbert, 1995; Rybczynski and Gilbert, 1995a; Gilbert et al., 1997; Song and Gilbert, 1997; Gilbert et al., 2000). There may be up to eight distinct proteins specifically translated following PTTH stimulation (Gilbert et al., 1997).

An observation commonly made for cytochrome P450 enzymes involved in ecdysteroid biosynthesis is their exclusive expression within so-called "ecdysial" tissues (e.g., prothoracic glands and adult ovaries) (Chavez et al., 2000; Warren et al., 2002; Niwa et al., 2004), which is in contrast to that observed for the gene transcripts of *AedaeCYP302a1* (Sieglaff et al., 2005). In this study, the "non-ovarian" tissues expressing *AeadeCYP302a1* also produced a minimal amount of ecdysteroids; albeit, ≤ 8% of that produced by the ovaries PBM (Sieglaff et al., 2005). Importantly, it has been shown that abdomens of ovariectomized *A. aegypti* females can stimulate vitellogenesis (an event coordinated by ecdysteroids produced by the ovary; Raikhel et al., 2002)) following a blood meal (Van Handel and Lea, 1984). In other insects, tissues not classically viewed as the main sources of circulating ecdysteroids (e.g., epidermis, fat body, and Malpighian tubules) have been shown both to produce ecdysteroids and to conduct enzymatic reactions associated with ecdysteroid biosynthesis (Meister et al., 1985; Meister et al., 1987; Delbecque et al., 1990). The presence of gene transcripts, however, does not always signify their inevitable translation, as was observed for 3-dehydroecdysone 3β-reductase of *Spodoptera frugiperta* and CYP306a1 of *D. melanogaster* (Chen et al., 1999; Warren et al., 2004).

To support the role of *A. aegypti* CYP302a1 in ovarian ecdysteroid biosynthesis further, its protein expression levels and localization within developing ovarioles following a blood meal were studied. The significance of *A. aegypti* CYP302a1 localization and expression within ovarioles is discussed in relation to ovarian ecdysteroid production and ovariole developmental events. It was further determined whether the gene transcripts of *A. aegypti* CYP302a1 in non-ovarian tissues are translated into protein as ascribed by the presence of corresponding immunoreactive protein within these tissues.

2. Materials and Methods

2.1 A. aegypti colony

The *A. aegypti* colony was maintained as previously described (Sieglaff et al., 2005). To initiate a gonotropic cycle, females were fed on an anesthetized rat until engorged, and maintained on distilled water until needed.

2.2 Antibody production and affinity purification

A plasmid (pCR-TOPO, Invitrogen) harboring the entire ORF of *A. aegypti CYP302a1* (GenBank accession AY947549) was provided to Proteintech Group Inc. (Chicago, IL.) for production of antiserum and subsequent affinity purification of antibodies specific to AedeaCYP302a1. In brief, the entire ORF of AedeaCYP302a1 was subcloned into the expression vector pET28a, and the modified vector transformed into BL21(DE3) cells. The transformed cells were then induced with 0.5mM IPTG to produce recombinant AedaeCYP302a1 for 3.5 h. The bacterial lysate was then passed over a column to purify >1mg of recombinant AedaeCYP302a1 (rAedeaCYP302a1). Antiserum against rAedeaCYP302a1 was raised in two rabbits, and prior to injection, preimmune blood samples were obtained from each.

One primary injection (1 mg rAedeaCYP302a1/animal in 2 ml both Freund's complete adjuvant and phosphate-buffered saline solution) followed by four antigen boosts (125 µg rAedeaCYP302a1/animal in 500 µl as before but with Freund's incomplete adjuvant) were performed with 2-3 week intervals between injections. Blood was collected 2 weeks after each boost (beginning with the second boost), from which serum was prepared and tested on immunoblots. To decrease the background in immunoblots, serum from the final bleeds of both rabbits were affinity purified using a column to which rAedaeCYP302a1 had been coupled, and the antibodies from one of the rabbits was chosen for all analysis described in this paper.

2.2 Expression of A. aegypti CYP302a1 in body parts and tissues of females following a blood meal

Tissues were dissected from non-blood fed (NBF) and blood-fed females in a physiological saline solution (139 mM NaCl, 3.7 mM KCl, and 1.9 mM $CaCl_2$, 25 mM HEPES, 5 mM Trehalose, 0.6 mM $MgCl_2$, and 1.8 mM $NaHCO_3$), placed directly into a drop of the same saline solution (1µl / tissue), and centrifuged to pellet the tissues. The were then lysed with "Triple Detergent Buffer " (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, protease inhibitor cocktail (Sigma); added at 9µl / tissue or body part and 4µl / ovary pair), incubated on ice for 15 minutes, sonicated with five quick blasts, incubated on ice for an additional 15 minutes, and centrifuged at 10,000x g for 10 minutes. The supernatant was collected and mixed with Laemmli Sample Buffer (Biorad) that contained 5% β-mercaptoethanol at a 1:1 ratio, and the samples were stored at -80ºC until analyzed. Ten tissue or body parts and 12 ovary pairs were processed from females from three separate cohorts.

For SDS-PAGE, the samples were thawed on ice, boiled for 10 minutes, chilled briefly on ice, centrifuged at 14,000 RPM for 2 minutes, the supernatants loaded onto a 10% Tris-HCl

gel (Biorad), and the proteins were separated for ~1.5 h at 130 V (running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS). For the analysis of AedaeCYP302a1 in tissue and body parts, 30 μg of their lysates were added per well. The protein concentrations of the lysates were determined with the Coomassie®Plus Protein Assay Reagent (Pierce). For the analysis of AedaeCYP302a1 expression within ovaries following a blood meal, 1 ovary equivalent was added per well. The separated proteins were blotted onto PVDF membrane (Biorad; transfer buffer: 25 mM Tris, 192 mM glycine, 15% methanol), with the transfer voltage and time kept constant across replicates (130 V for 4 hours). Following the transfer, membranes were briefly rinsed with dH_2O, and then blocked within TBS-T containing 1% Blotting Grade Blocker Non-fat Dry Milk (Biorad), 2% Bovine serum albumin (Sigma), and 1% goat serum (Sigma). Affinity purified anti-AedaeCYP302a1 antibodies were added to the blocking buffer at 32 ng/ml and incubated overnight at 4ºC with gentle rocking. Blots were washed in TBS-T (Tris-Buffered Saline with 0.1% Tween 20; 2x brief, 1x 15 minutes, 3x for 5 minutes), incubated with anti-rabbit antibody conjugated to horseradish peroxidase (1:50,000 dilution, Sigma) for 4 h at room temperature with gentle rocking, and washed as specified above. AedaeCYP302a1 was detected with ECL Advance Western Blotting Detection Kit (Amersham Biosciences) using the GeneGnome imaging system (Syngene Bio Imaging). The molecular weights (MW) and densities of immunoreactive bands were calculated using GeneTools software (Synoptics Limited), and the MWs were based on co-run standards (MagicMark™ Western Standard; Biorad).

Immunoblots for three cohort replicates were analyzed, and each cohort consisted of twelve pooled ovary pairs. The densities of each band were calculated using the GeneTools program (Synoptics Limited), the background signal subtracted from the calculated raw volumes,

and the pixel area analyzed was kept constant across all time points and the three cohort replicates. The densities of the immunoreactive bands were analyzed by ANOVA, and their means separated by Tukey-Kramer HSD test (SAS JMP 5.0.1a, SAS Institute Inc., Cary, N.C.).

2.3 Localization of AedaeCYP302a1 in ovarioles following a blood meal

Ovaries were dissected as previously described and fixed in physiological saline solution containing 4% paraformaldehyde for 1 hour at room temperature with gentle rocking. Ovaries were then washed with phosphate-buffered saline (PBS; 3x 5 minutes), permeabilized with PBS with 0.2% Triton X-100 for 5 minutes at room temperature with gentle rocking, and washed with PBS as previously described. They were then blocked with PBS-T (PBS with 0.1% Tween 20) containing 2% bovine serum albumin and 2% goat serum ("blocking buffer") for 1 hour at room temperature with gentle rocking. Affinity purified anti-AedaeCYP302a1 antibodies were added to the blocking buffer at 0.64 μg/ml and incubated overnight at 4º C with gentle rocking. The ovaries were washed in PBS-T (4x for 5 minutes), incubated within "blocking buffer" with anti-rabbit antibody conjugated to Alexa Fluor® 488 (1:3,000 dilution, Molecular Probes) for 4.5 h at room temperature with gentle rocking, and washed as specified above. Ovaries were mounted on a slide in media (PBS:glycerol 1:1) and examined with a Leica TCS/SP2 confocal microscope. A series of confocal sections were taken from the outer surrounding follicle cells (top) to the middle of the ovariole to assess immunostaining in follicle cells, nurse cells, and the oocyte of ovarioles. For consistency, all confocal settings remained unchanged between samples with the exception of using a 20x lens for the 48 and 60 h PBM times due to the size of the oocyte (i.e., NBF, 6 h, 12 h and 18h PBM oocytes were viewed using a 63x lens). As a control, anti-AedaeCYP302a1 antibodies diluted as above were pre-absorbed to 640 μg rAedaeCYP302a1

(1000-fold excess) overnight at 4º C, and subsequently used in 12, 18 and 48 h PBM ovary treatments.

For the cellular localization experiments, 18 h PBM ovaries were dissected as previously described, incubated in physiological saline solution containing 500 nM MitoTracker® Deep Red 633 for 1 h at 27ºC with gentle rocking, washed with the physiological saline solution (3x 2 minutes), and prepared for immunocytochemistry as described above.

3. Results

3.1 Expression of AedaeCYP302a1 in ovaries following a blood meal, and in various tissues and body parts

The utility and specificity of the anti-AedaeCYP302a1 antibodies were determined by conducting immunoblot analysis against ovary pair lysates prepared from females at peak ovarian ecdysteroid production (18 h PBM) and of rAedaeCYP302a1. The specificity of the antibody is verified by the recognition of immunoreactive bands with the same approximate MWs in both ovary pair lysates and rAedaeCYP302a1 and their absence in blots incubated with pre-immune serum (Figure 3.1A). The purified anti-AedaeCYP302a1 antibodies recognized a prominent band estimated to be 50 kDa, and two additional bands of 100 and 108 kDa in ovary pair lysates. In the original characterization of AedaeCYP302a1, two gene transcripts were cloned and identified (Sieglaff et al., 2005), and the deduced amino acid sequences predicted for these two gene transcripts would result in mature MWs of 56.5 and 52 kDa (correspond to the ORF predicted for GenBank accessions AY947549 and AY947550, respectively). These two MWs are larger than that observed in the current immunoblots; however, SDS-PAGE and subsequent immunoblot analysis provides an approximation of MW. More detailed analysis would be required for a better estimation of MW. The upper immunoreactive bands of the 100

and 108 kDa may represent a doublet of AedaeCYP302a1, or its association with other proteins. These upper immunoreactive bands are quite intriguing for their density appears to be the greatest during the peak of ovary ecdysteroid production in blood-fed females (18 h PBM).

To determine whether an increase in the expression of AedeaCYP302a1 correlates with ovarian ecdysteroid production in blood-fed females, immunoblot analysis was conducted against ovary pair lysates from females before (NBF), the start (6 and 12 h PBM), peak (18 h PBM), and end (48 and 60 h PBM) of ovarian ecdysteroid production. The density of the 50 kDa band increased linearly in the ovary pair lysates following a blood meal, with a significant difference between values of NBF female ovaries and every other time point examined ($p \leq 0.05$; see Figures 3.1B and C). The density of the 50 kDa band was greatest 48 h PBM, but was not significantly different from that calculated for 18 h PBM ($p \leq 0.05$; Figure 3.1C). The upper immunoreactive bands of 100 and 108 kDa were absent in NBF and 6 h PBM ovary pair lysates, and most predominant in the 12, 18 and 48 h PBM ovary pair lysates.

Immunoblot analysis was conducted against the tissue and body part lysates shown in a previous study to contain AedaeCYP302a1 gene transcripts (Sieglaff et al., 2005) to determine whether these same tissues also contain translated protein. The 50 kDa band was observed in every tissue and body part examined both before and following a blood meal (Figure 3.1D). The 100 or 108 kDa immunoreactive bands were only distinguishable in head and ovary pair lysates and only faintly seen in the lysates of other tissue preparations.

3.2 Localization of AedaeCYP302a1 in ovarioles following a blood meal

Each of the paired ovaries of *A. aegypti* comprises approximately 75 ovarioles, and the individual ovarioles possess a germarium and primary follicle consisting of one oocyte and seven nurse cells surrounded by a layer of epithelial (follicle) cells (Clements, 1992). It was the

purpose of the current study to determine whether AedeaCYP302a1 is expressed in the presumed source of ecdysteroids, the ovariole follicle cells (Goltzene et al., 1978; Zhu et al., 1983), and if so, corroborate the localization observed for the transcripts of *D. melanogaster* CYP302a1 (Chavez et al., 2000). AedaeCYP302a1 localized exclusively to ovariole follicle cells (Figure 3.2). In ovarioles of NBF females, a pronounced immunostaining within the germarium was observed, but following the blood meal, this immunostaining decreased (compare Figure 3.2B NBF-middle to 2C 18h-middle). The intensity in follicle cell immunostaining in primary follicles essentially mimicked that observed in immunoblot analysis with the greatest staining observed at 18 h and 48 h PBM (Figure 3.2C). The minimal immunostaining in 18 h PBM ovarioles incubated with antibodies pre-absorbed to rAedaeCYP302a1 attest to the antibody specificity (Figure 3.2C 18h-pre-absorbed; note: a similar decrease in signal intensity was observed in 12 and 48 h PBM ovarioles treated in the same way, results not shown). A feature commonly observed in ovarioles of every time point examined was a decrease or lack of AedaeCYP302a1 immunostaining in certain populations of follicle cells (see middle for Figure 3.2B NBF, 6h, 12h and 2C 48h). This appeared as a more pronounced immunostaining in follicle cells residing above nurse cells, while those surrounding the oocyte displayed less or a complete lack of immunostaining. It should be noted, however, that an exclusion of AedaeCYP302a1 immunostaining in follicle cells surrounding the oocyte was not a consistent feature, and may represent the effect of sample orientation during confocal microscopy analysis (see Figure 3.2C 18h-middle). By 48 and 60 h PBM, the immunostained follicle epithelium appeared to be sloughing-off from the developing oocyte in many of the ovarioles examined, with an obvious demarcation between the follicle epithelium and the oocyte's chorion (see Figures 3.2B 60h-top

and middle), but was not predominant enough to discount the possibility of a sample preparation artifact.

Given that *D. melanogaster* CYP302a1 and 22-hydroxylase activity in *L. migratoria* follicle cells localize to the mitochondria (Rees, 1995; Petryk et al., 2003), it was determined whether AedaeCYP302a1 also localizes to this organelle as ascribed by colocalization with a molecule known to be sequestered within actively respiring mitochondria (MitoTracker® Deep Red 633). The majority of AedaeCYP302a1 immunostaining did not localize to the mitochondria as compared to MitoTracker® Deep Red 633 (Figure 3.3). Within every sample analyzed, a minimal amount of colocalization was observed (Figure 3.3).

4. Discussion

Anautogenous mosquitoes, such as *A. aegypti*, require a blood meal to promote oogenesis, and ecdysteroid hormones produced by the ovaries following the blood meal are key modulators of this developmental event (Dhadialla and Raikhel, 1994; Klowden, 1997; Raikhel et al., 2002). The biosynthesis of ecdysteroids involves the transport and modification of cholesterol to a final bioactive polyhydroxylated steroid (Rees, 1995; Gilbert et al., 2002). Many of the hydroxylation reactions of ecdysteroid biosynthesis are conducted by cytochrome P450 enzymes (Grieneisen et al., 1993), and CYP302a1 represents the first enzyme specifically involved in ecdysteroid biosynthesis to be identified and characterized (Chavez et al., 2000; Warren et al., 2002; Parvy et al., 2005). In a previous study, the abundance of gene transcripts of the *A. aegypti* homolog of CYP302a1 increased in ovaries prior to, peaked, and declined according to ecdysteroid production by this tissue PBM (Sieglaff et al., 2005), suggesting that ecdysteroid production by *A. aegypti* ovaries may be regulated at the transcript level.

The means by which ecdysteroid biosynthesis is regulated, however, does not simply involve the transcription of a defined set of genes encoding biosynthetic enzymes, but may require their translation, possible modification, and other forms of regulation not directly related to the activity of cytochrome P450 enzymes (Gilbert et al., 1997; Gilbert et al., 2002; Parvy et al., 2005). Indeed, a recent study has shown that abundance of gene transcripts encoding several cytochrome P450 enzymes implicated in ecdysteroid biosynthesis, including CYP302a1, do increase along with ecdysteroid production in *D. melanogaster* ring glands (RG), but remain high despite a decline in ecdysteroid production by the RG in the late stage of the 3rd larval instar (Parvy et al., 2005). This suggests that the regulation of ecdysteroid biosynthesis may occur at a level beyond gene transcription. Indeed, there are up to eight distinct proteins specifically translated when PTTH stimulates ecdysteroid biosynthesis in the prothoracic glands of *Manduca sexta* (Gilbert et al., 1997), two of which have been identified as β-tubulin and heat shock protein 70 (Rybczynski and Gilbert, 1995a; Rybczynski and Gilbert, 1995b). It has not yet been determined whether any of the six remaining unknown proteins are cytochrome P450 enzymes.

Whether ovarian ecdysteroid biosynthesis in *A. aegypti* is regulated by the expression of a specific set of proteins that may include cytochrome P450 enzymes has not yet been established. To address this question, the current study was undertaken to determine whether an increase in the expression of AedaeCYP302a1 within ovaries correlates with the rise and fall of ecdysteroid production by ovaries of blood-fed females. Such a correlation between an increase in the protein levels of AedaeCYP302a1 within ovaries and ecdysteroid production by this tissue would further establish the importance of this enzyme in the biosynthesis of ecdysteroids.

Following a blood meal, *A. aegypti* ovaries begin to produce ecdysteroids 6 h PBM, peak 18 -24 h PBM, and begin to decline by 36 h PBM (Hagedorn et al., 1975; Sieglaff et al., 2005). As was observed for the gene transcripts of *AedaeCYP302a1* (Sieglaff et al., 2005), AedaeCYP302a1 protein levels within ovaries increased significantly just before the onset of ovarian ecdysteroid production (i.e., 6 h PBM), and remained high thereafter. The persistence of AedeaCYP302a1 protein in ovaries later in the gonotropic cycle is in contrast to that observed for its gene transcripts, which displayed a rise and fall in abundance that correlated well with ovarian ecdysteroid production in blood-fed females (Sieglaff et al., 2005). A quantitative increase in the gene transcript levels of cytochrome P450 enzymes involved in ecdysteroid biosynthesis has been a suggested regulator of ecdysteroid biosynthesis (Warren et al., 2002; Niwa et al., 2004; Warren et al., 2004; Parvy et al., 2005), but the expression of their respective translated proteins during phases of ecdysteroid biosynthesis in *D. melanogaster* or *Bombyx mori* has not been addressed.

The decline in *AedaeCYP302a1* gene transcripts and a lack of such a decline in translated protein levels of AedaeCYP302a1 may simply be attributed to the persistence of this enzyme once it's translated. The more relevant observation, however, is the decline in ecdysteroid production by ovaries of 48 and 60 h PBM females without a corresponding decrease in AedaeCYP302a1 protein levels. As mentioned above, such a discrepancy between the maintenance of high transcript levels of *D. melanogaster* CYP302a1 and lack of ecdysteroid biosynthesis by the RG has been reported (Parvy et al., 2005), but unlike the study conducted with *A. aegypti* ovaries (Sieglaff et al., 2005), it is assumed that Parvy et al. (2005) analyzed whole body transcript levels rather than that exclusively expressed in the RG themselves (i.e., they do not state that total RNA was obtained specifically from the RG). None-the-less, both the

current study addressing AedaeCYP302a1 protein levels and that conducted by Parvy et al. (2005) suggest that regulation of ecdysteroid biosynthesis does not simply involve the transcription and subsequent translation of biosynthetic enzymes, but likely employs various levels of regulation to fine tune its control. Indeed, the activity of the terminal hydroxylases of which 22-hydroxylase is a member does not change in the prothoracic glands of *M. sexta* despite the developmental fluctuation in ecdysteroid biosynthesis by this tissues (Grieneisen et al., 1993; Grieneisen, 1994). That is, the presence of these terminal hydroxylases does not define the biosynthetic activity of the tissue in which they reside, further substantiating that the regulation of ecdysteroid biosynthesis is beyond the simple expression of biosynthetic enzymes. The rate limiting step of ecdysteroid biosynthesis is thus believed to be the delivery of the ecdysteroid precursor 7-dehydrocholesterol to the mitochondria, and not necessarily its processing by the enzymes that reside there within (Grieneisen et al., 1993; Grieneisen, 1994; Warren and Gilbert, 1996b). This means of regulating ecdysteroid biosynthesis would mimic the suggested rate limiting step of mammalian steroidogenesis, the delivery of the steroid precursor to the mitochrondria (Jefcoate et al., 1992; Stocco, 2001), thus its attractiveness as candidate mechanism. For now, however, no molecule(s) mediating this transport in ecdysteroid biosynthesis has been characterized.

Oogenesis in *A. aegypti* follows a well characterized developmental scheme that begins at adult eclosion when the follicle cells double in size from fewer than 20 to over 200, at which time the ovarioles have reached previtellogenic arrest and are competent for vitellogenin uptake (Sappington and Raikel, 1999). Shortly after a blood meal, the follicle cells separate from the oocyte, mitosis resumes in the follicle cells and ends with an approximate doubling in number (Clements, 1992). The vitellogenic phase begins 4-6 h PBM and is characterized by the initiation

of the rapid uptake of yolk proteins into the developing oocyte, and by 36 h PBM, vitellogenin uptake ends and post vitellogenesis begins. During post-vitellogenesis, follicle cells deposite the chorion, after which the eggs are oviposited 60 -72 h PBM. Throughout various stages of oogenesis, the nurse cells supply the developing oocyte with ribosomal RNA and other developmentally important macromolecules (Sappington and Raikel, 1999), and the follicle cells are the assumed source of ecdysteroids produced by the ovariole based on observations made with other insect species (Goltzene et al., 1978; Zhu et al., 1983). The timing of the above events has been well established by numerous researchers, although the exact point(s) at which nurse and follicle cells begin to degenerate has not been well defined. This is partially attributed to the fact that *A. aegypti* oogenesis has been characterized through ultrastructural studies of ovarioles from females maintained at different temperatures (Anderson and Spielman, 1971; Mathew and Rai, 1975; Lehane and Laurence, 1978; Tadkowski and Jones, 1979). None-the-less, Clements (1992) has attempted to coalesce the various studies into an approximation on the timing of oogenesis events in females maintained at 27°C, which corresponds to follicle cell degeneration at 60-72 h PBM following exochorion deposition. Two forms of evidence that have been published suggest that the follicle cells are still "alive" in the later stages of oogenesis: (1) radioactive tracer incorporation into these follicle cells indicating metabolic activity (Anderson and Spielman, 1973), and (2) chorion deposition takes place 24-60+ h PBM and is promoted by ecdysteroids possibly derived from the follicle cells themselves (Raikel and Lea, 1991; Lin et al., 1993; Edwards et al., 1998). An experiment with propidium iodide (PI), the molecule that addresses membrane integrity and thus cell viability, showed that ovariole follicle cells of 48 h PBM females are still alive, whereas the follicle cells did not exclude PI at 70 h PBM (D. H. Sieglaff, unpublished observations).

The localization of AedaeCYP302a1 protein in ovariole follicle cells and its dynamic expression during the gonotropic cycle lends evidence that these cells are the source of ovariole ecdysteroids in *A. aegypti*. AedaeCYP302a1 localization to ovariole follicle cells agrees with that established for *D. melanogaster* CYP302a1 transcripts (Chavez et al., 2000). Whether the follicle cells are the sole source of ovariole ecdysteroids has not been affirmed for Dipterans, and has come into question because of gene products encoding enzymes involved in ecdysteroid biosynthesis have been localized to both follicle and nurse cells of *D. melanogaster* ovarioles (Buszczak et al., 1999; Petryk et al., 2003; McCall, 2004). Experiments will be conducted with follicle cells isolated away from *A. aegypti* ovarioles as was done with *N. cinerea* and *L. migratoria* (Goltzene et al., 1978; Zhu et al., 1983) to better establish these cells as the source of ecdysteroids.

Ovariole follicle cells residing above nurse cells appeared to stain more heavily for AedeaCYP302a1 immunoreactivity in many samples examined. This AedaeCYP302a1 expression suggests that these follicle cells are the primary source of ecdysteroids, and that there may be communication between the follicle and nurse cells either promoting or establishing AedaeCYP302a1 expression in this population of follicle cells. Such a defined localization within a subset of follicle cells was not surprising for such expression gradients are well established features of *D. melanogaster* oogenesis (Deng and Bownes, 1997; Deng and Bownes, 1998; Bryant et al., 1999; Buszczak et al., 1999; Tzolovsky et al., 1999). Moreover, gene transcripts that encode two cytochrome P450 enzymes conducting other "terminal hydroxylase" reactions of ecdysteroid biosynthesis are also restricted to specific follicle cell populations in *D. melanogaster* ovarioles (Warren et al., 2002; Petryk et al., 2003; Niwa et al., 2004; Warren et al., 2004). The gene transcripts encoding 2-hydroxylase (CYP315a1) and 25-hydroxylase

(CYP306a1) are enriched in follicle cells surrounding the oocyte (Warren et al., 2002; Niwa et al., 2004; Warren et al., 2004), whereas gene transcripts encoding 22-hydroxylase (CYP302a1) and 20-hydroxylase (CYP314a1) are enriched in follicle cells surrounding the nurse cells (Chavez et al., 2000; Petryk et al., 2003). The nurse cells themselves contain gene transcripts for some of these cytochrome P450 enzymes (Warren et al., 2002; Warren et al., 2004), the reason for which has not been explored. The transcripts for *D. melanogaster* 22-hydroxylase (CYP302a1), however, did not display an obvious expression within ovariole nurse cells (Chavez et al., 2000). Before this study, the expression of a cytochrome P450 enzyme specifically involved in ecdysteroid biosynthesis at the protein level within a developing ovariole has not been reported. Further, a thorough analysis of the protein level expression of such a cytochrome P450 enzyme as it relates to a tissue's ecdysteroid production has never been directly addressed.

Immunocytochemical analysis showed that AedaeCYP302a1 protein was present in the ovarioles throughout the gonotropic cycle. The presence of AedaeCYP302a1 at later times of oogenesis does not conclusively attest that this enzyme is functional, and the immunoreactive protein may be degraded and localized within lysomes or vacuoles of degenerated follicle epithelium (Lehane and Laurence, 1978). The follicle cells of *A. aegypti* ovaries remain structurally intact throughout the later stages of oogenesis (Lehane and Laurence, 1978), and AedaeCYP302a1 staining was observed in sloughed follicle epithelium of the primary follicles of ovarioles from 48 and 60 h PBM females. Whether AedaeCYP302a1 is still functional in these later stages of oogenesis will have to be determined with further studies that address the enzymatic capability during the rise and fall of ecdysteroid production by this tissue (Smith and Mitchell, 1986; Kappler et al., 1988). As suggested previously, the enzymatic activity of a

terminal hydroxylase involved in ecdysteroid biosynthesis does not, however, directly correlate to a tissue's ecdysteroid production (Grieneisen et al., 1993; Grieneisen, 1994).

22-hydroxylase activity in both prothoracic glands and ovaries has been localized to subcellular fractions enriched in mitochrondria (Kappler et al., 1988; Grieneisen et al., 1993; Rees, 1995), and *D. melanogaster* 22-hydroxylase (CYP302a1) was later shown to localize to mitochondria when expressed in S2 cells (Petryk et al., 2003). To establish better AedaeCYP302a1 as the putative 22-hydroxylase of ovarian ecdysteroid biosynthesis, colocalization of the immunostainined protein with a fluorophore sequestered by mitochondria was attempted. The colocolization experiments show that AedeaCYP302a1 does not localize exclusively to the mitochondria, which disagrees with 22-hydroxylase activity in other insects and more specifically confocal microscopic analysis conducted with S2 cells expressing *D. melanogaster* CYP302a1 (Kappler et al., 1989; Rees, 1995; Petryk et al., 2003). The lack of colocalization with MitoTracker® Deep Red 633 suggests that AedaeCYP302a1 may have a microsomal localization (endoplasmic reticulum), or the anti-AedaeCYP302a1 antibodies may be recognizing a cytosolic version of the translated *A. aegypti* CYP302a1 for the antibodies were raised against the precursor version of AedaeCYP302a1 with the N-terminal targeting sequence, thus the anti-AedaeCYP302a1 antibodies may be recognizing the precursor version prior to mitochondrial import (Matocha and Waterman, 1985). Given that the immunostaining was substantially diminished in ovarioles incubated with anti-AedaeCYP302a1 antibodies that had been pre-absorbed to rAedaeCYP302a1 protein attests to the specificity of the antibody. We cannot discount the possibility that the anti-AedaeCYP302a1 antibodies are recognizing cytochrome P450s in addition to AedeaCYP302a1 since the antibodies are polyclonal, and

conserved motifs shared among all cytochrome P450 enzymes (Werck-Reichhart and Feyereisen, 2000).

In the current study, AedeaCYP302a1 was observed in every tissue examined (head, thorax, abdominal pelt, midgut, Malpighian tubules and hindgut, and ovaries) both before and following a blood meal, which agrees with presence of its gene transcripts within these tissues observed in the previous study (Sieglaff et al., 2005). Tissues other than those classically viewed as "ecdysial" (e.g., fat body, Malpighian tubules, gut, epidermis) have not only shown the ability to produce or release ecdysteroids (Delbecque et al., 1990), but more importantly, have been shown to conduct the terminal hydroxylations of ecdysteroid biosynthesis suggesting the presence of these enzymes within these tissues (Meister et al., 1985; Delbecque et al., 1986; Meister et al., 1987). More specifically, 22-hydroxylase activity has been observed in numerous tissues in *L. migratoria*, but greater enzymatic capacity was observed within the prothoracic glands and ovarian follicle cells (Haag et al., 1988). The ovaries of *A. aegypti* are the primary source of ecdysteroids following a blood meal, producing at least 12 times more ecdysteroids than the other tissue examined within this study (i.e., head, thorax, abdominal pelt, and midgut) (Sieglaff et al., 2005). The presence of AedaeCYP302a1 and low levels of ecdysteroid produced by the other tissues further corroborates the hypothesis that the expression of terminal hydroxlases of ecdysteroid biosynthesis is not solely an acutely regulated process (Grieneisen et al., 1993; Grieneisen, 1994), and that delivery of 7-dehydrocholesterol to mitochondria represents the rate limiting step (Grieneisen et al., 1993; Warren et al., 1996a). The protein(s) mediating the delivery of 7-dehydrocholesterol to the mitochondria may display an exclusive or enhanced expression within primary ecdysteroid producing tissues such as ovaries.

The near ubiquitous expression of AedaeCYP302a1 protein also hints at the possibility that it has substrates other than the ecdysteroid precursor(s) ascribed to its *D. melanogaster* homolog, CYP302a1 (Kappler et al., 1988; Warren et al., 2002). Cytochrome P450 enzymes can act on a great variety of substrates, and in some instances, multiple substrates (Poulos, 1995; Mansuy, 1998; Scott and Wen, 2001). Multiple functional cytochrome P450s in insects are classical associated with catabolism of exogenous substrates such as insecticides (Scott and Wen, 2001; Li et al., 2004), but have also been observed in cytochrome P450s known to act on endogenous substrates (Sutherland et al., 1998). AedeaCYP302a1 enzymatic activity will have to be addressed in further studies, for our assumption that AedaeCYP302a1 represents the *A. aegypti* 22-hydroxylase of ecdysteroid biosynthesis is based on sequence homology with *D. melanogaster* CYP302a1 (56.5% identity and 67.7% similarity), and a single amino acid change in a cytochrome P450 can alter its substrate specificity (Feyereisen, 1999).

The regulation of ecdysteroid biosynthesis has been an active area of study for insect endocrinologists for nearly half a century. With the advent of molecular biology techniques, mutant analysis and more recently genomics many of the component enzymes and proteins directly involved in ecdysteroid biosynthesis are being identified and characterized. Anautogenous mosquitoes are a great model for studying ecdysteroid biosynthesis, for this pathway can be initiated *in vivo* by providing adult female mosquitoes a blood meal. Moreover, with the completion of the *Anopheles gambiae* genome and employment of microarray technology that specifically encompasses gene transcripts that encode classes of enzymes involved in ecdysteroid biosynthesis (David et al., 2005), many of the enzymes alluding researchers for the past three decades may more readily be identified. These "blood thirsty"

mosquitoes also make good models for studying oogenesis for again the blood meal marks the beginning of development of the primary follicle.

5. References

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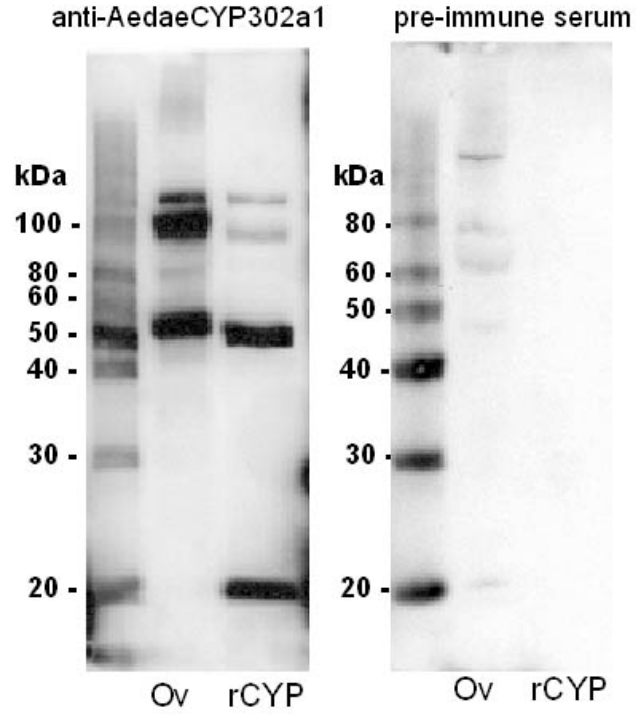
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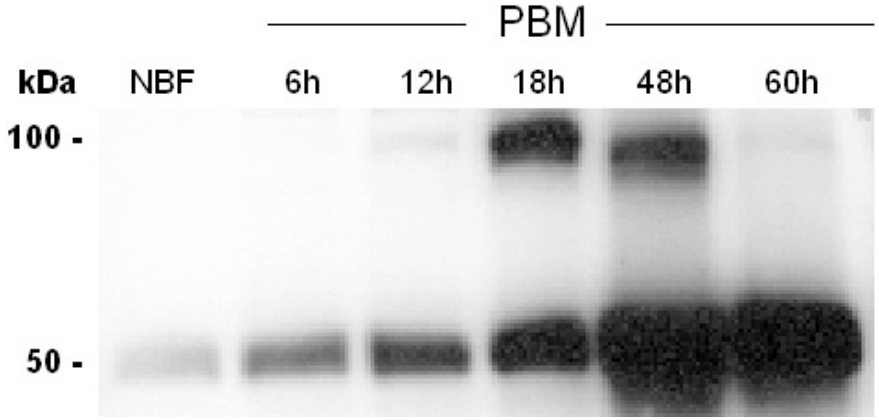
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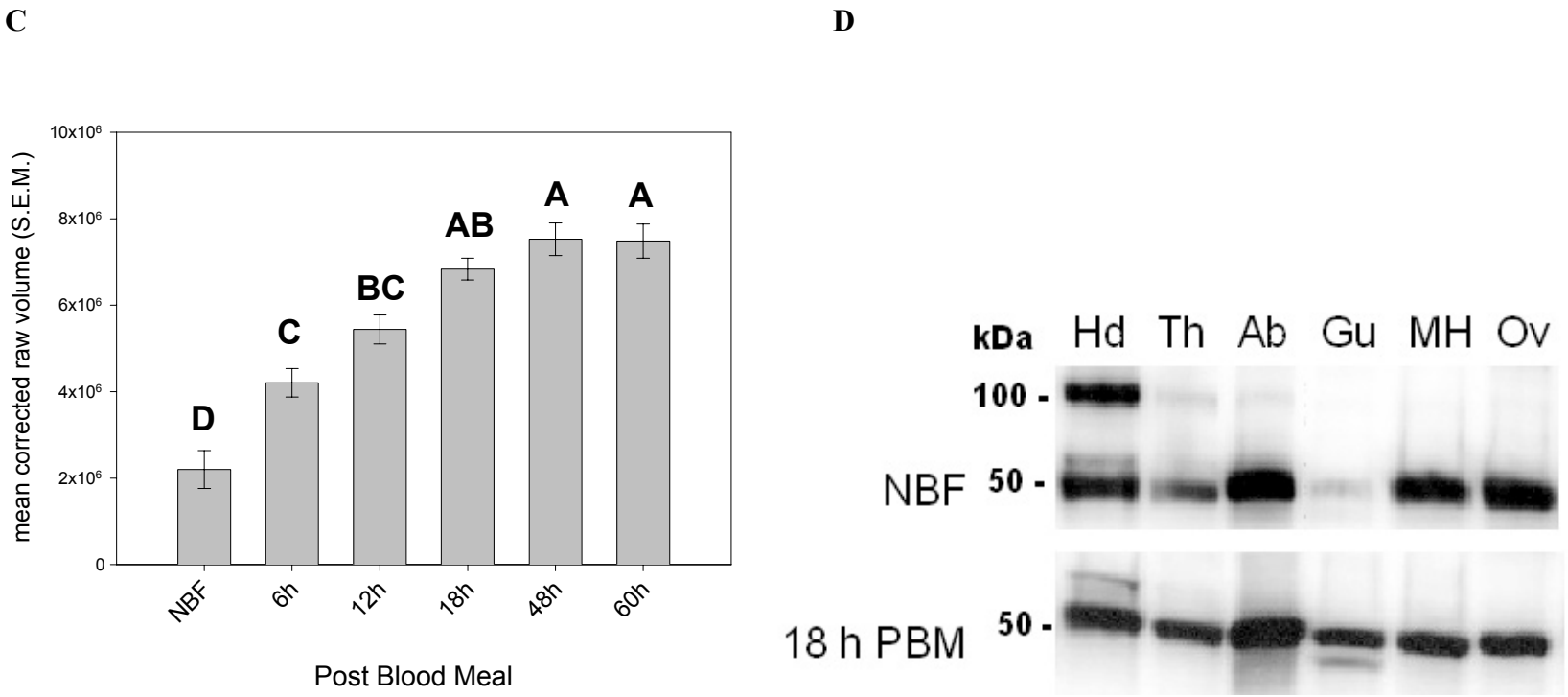

Figure 3.1. Immunoblots of tissue lysates of female *Aedes aegytpi*. (**A**) Ovaries from *A. aegypti* females 18 h post blood meal (PBM) (Ov, 0.5 ovary pair equivalent), and recombinant AedaeCYP302a1 (rCYP, 75 ng). (**B**) Representative immunoblot displaying the expression of AedaeCYP302a1 in ovaries from females at different time points following a blood meal (0.5 ovary pair equivalents/ well). (**C**) Densitometry analysis of AedaeCYP320a1 expression in ovaries from females of three separate cohorts following a blood meal (bars with the same letter are not significantly different; Tukey-Kramer, $p \leq 0.05$). (**D**) Tissue distribution of AedaeCYP302a1 in

non-blood fed (NBF) 18 h PBM females, each lane loaded with 30 µg of protein: Hd = head, Th = thorax, Ab = abdominal pelt, Gu = midgut, MH = Malpighian tubules and hindgut, Ov = ovaries.

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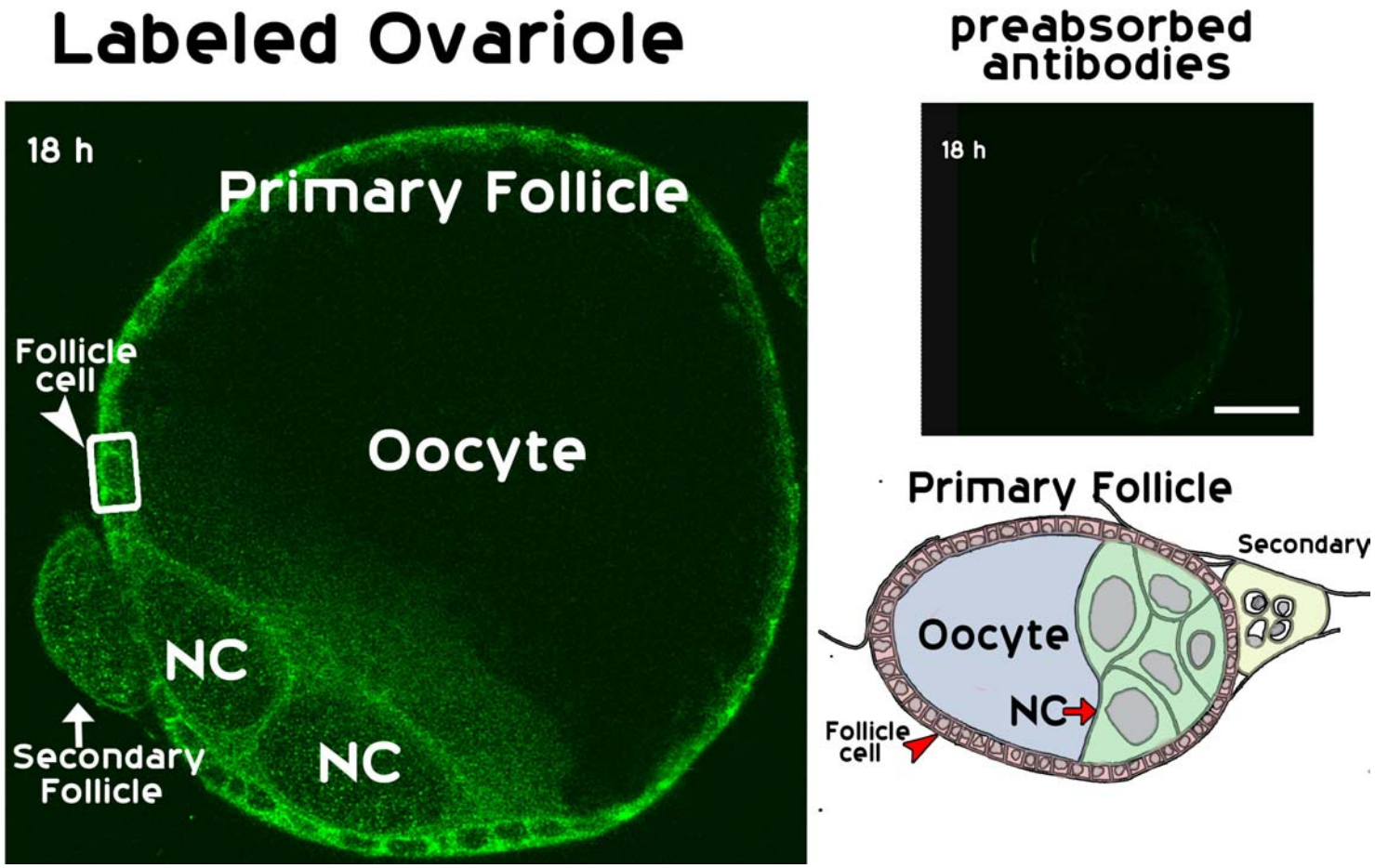
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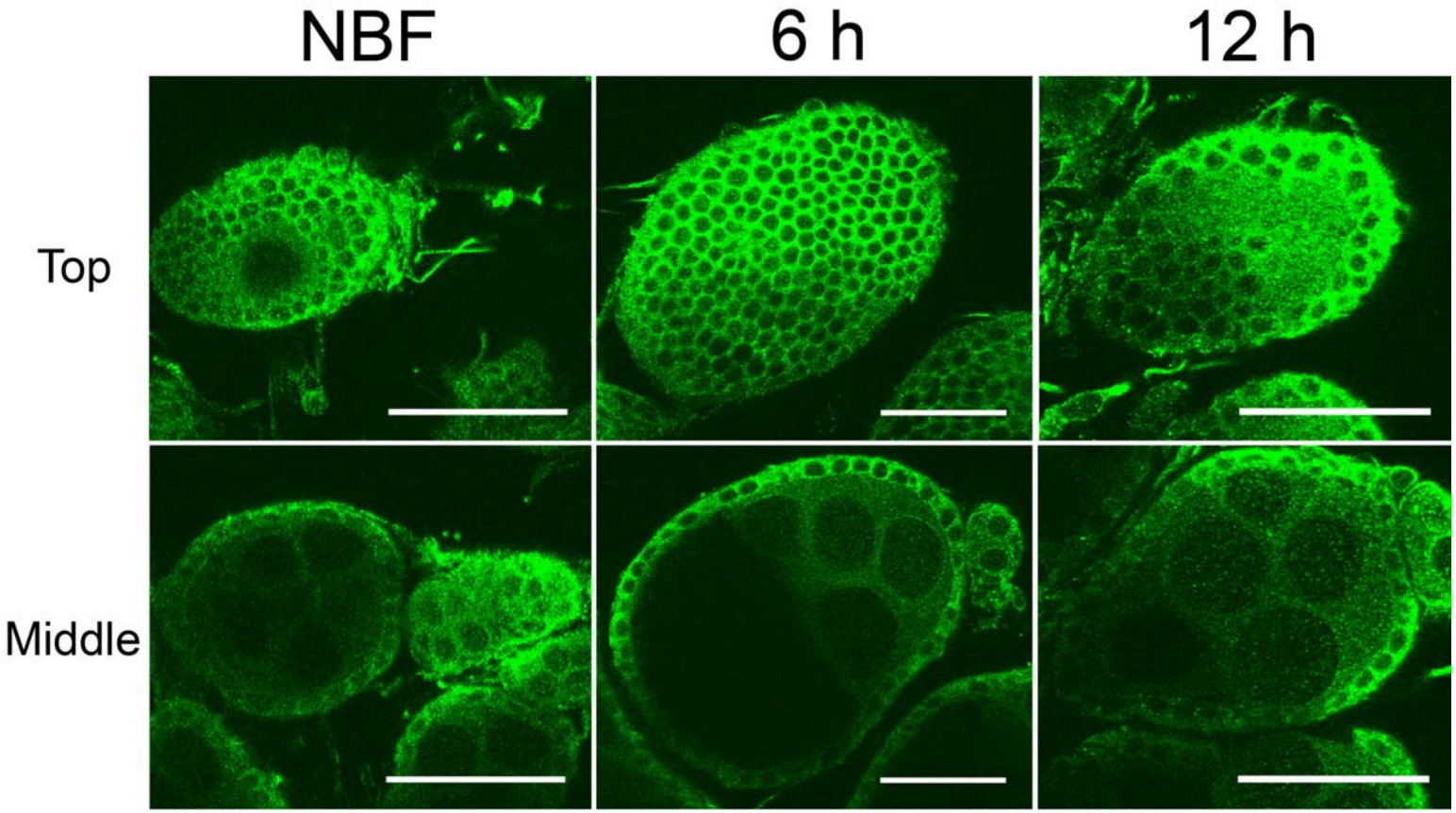

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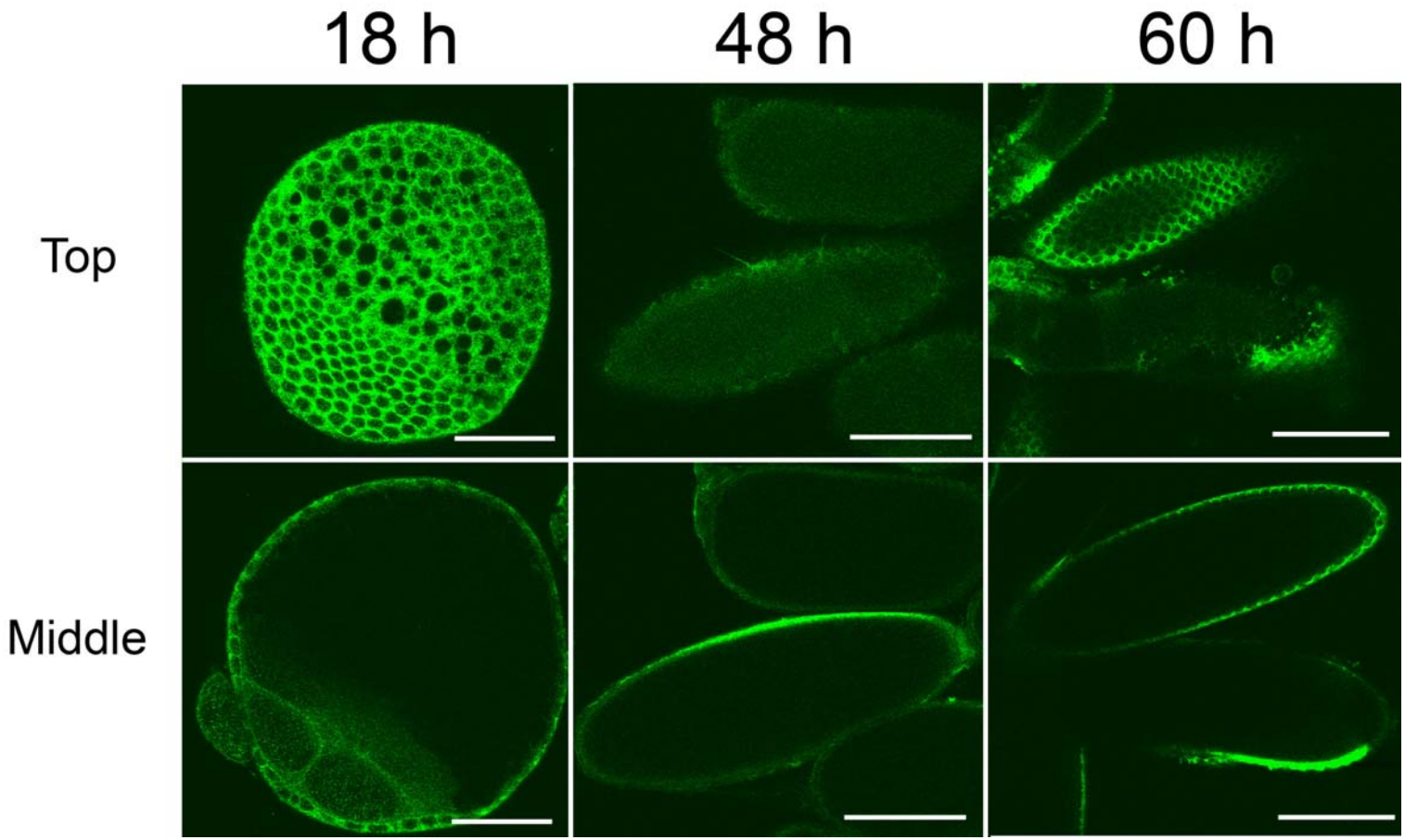

Figure 3.2. Immunocytochemistry of AedaeCYP302a1 in ovaries of NBF females and those of different time points PBM: 6, 12, 18, 48 and 60 h PBM. Cross sections of sample ovarioles for each time point are presented from the perspective of the ovarioles surface

(top) and its middle to display the ovariole cell populations(s) immunostained for AedaeCYP302a1. (**A**) Immunostained 18 h PBM ovariole displaying the oocyte, nurse cells (NC), follicle cells, and the primary and secondary follicles, and a cartoon rendition of the different cell populations of the ovarioles; 18 h PBM ovarioles treated with pre-absorbed AedaeCYP302a1 antibodies (preabsorbed antibodies), (**B**) NBF, 6 and 12 h PBM ovarioles, and (**C**) 18, 48 and 60 h PBM ovarioles. The scale bars = 50 μm for NBF, 6 h, 12 h, and 18 h PBM samples; and 150 μm for 48 h and 60 h PBM samples.

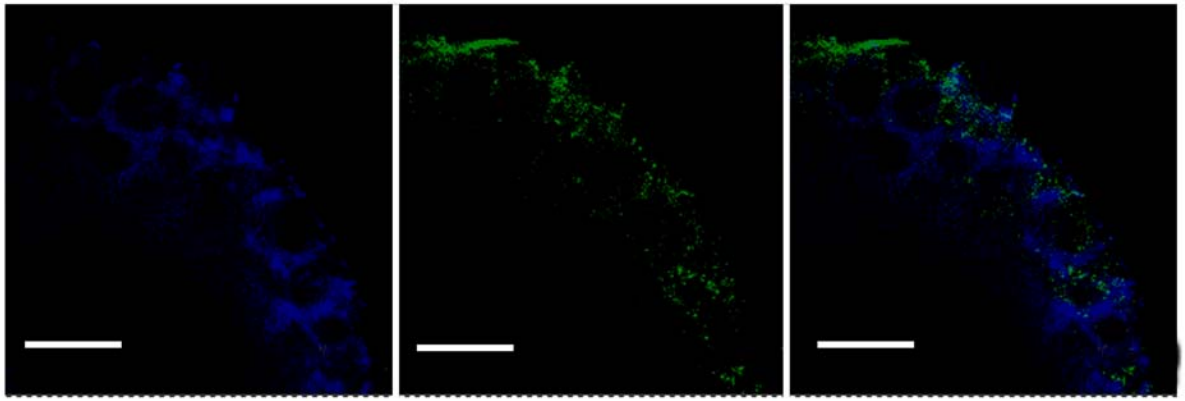

Figure 3.3. Localization of AedaeCYP302a1 and mitochondria in follicle cells of 18 h PBM females. Ovaries from 18 h PBM were treated with MitoTracker® Deep Red 633, and then with anti-AedaeCYP302a1 antibodies as specified in the materials and methods. The three panels from left to right indicate MitoTracker® Deep Red 633, AedaeCYP302a1, and merge. The scale bars = 15 μm.

CHAPTER 4

EXPRESSION AND LOCALIZATION OF START1 IMMUNOREACTIVITY IN THE OVARIES OF THE YELLOW FEVER MOSQUITO, *Aedes aegypti*, DURING A GONOTROPIC CYCLE

1. Introduction

The precursors for ecdysteroid hormones in insects are sterols (e.g., cholesterol), which must be received from their diet because insects are unable to synthesize sterols *de novo* (Clayton, 1964; Kircher, 1982). The extra- or intracellular source of cholesterol used in insect ecdysteroid biosynthesis in either prothoracic glands or ovaries is not known, but cholesterol used in mammalian steroidogenesis can be derived from three separate sources: (1) exogenous cholesterol transported to the cell via lipoproteins, (2) the plasma membrane, or (3) intracellular *de novo* synthesis (Grummer and Carroll, 1988; Liscum and Underwood, 1995; Thomson, 1998; Azhar et al., 2003). Given the lack of *de novo* cholesterol synthesis in insects, its source for ecdysteroid biosynthesis must either come from hemolymph lipoproteins or the plasma membrane. It has been shown in lepidopteran insects that cholesterol absorbed by the midgut is loaded into lipoproteins and transported to peripheral tissues for internalization (Chino and Gilbert, 1971; Jouni et al., 2002), and such lipoprotein delivery to the ovaries of two members in this order has been reported (Kawooya and Law, 1988; Kawooya et al., 1988; Jouni et al., 2003).

Following a blood meal taken by *A. aegypti* whole body ecdysteroid titers rise (Greenplate et al., 1985; Borovsky et al., 1986), and the ovary is considered the primary source of these ecdysteroids (Hagedorn et al., 1975; Sieglaff et al., 2005). Based on the observations

made with the cockroach *Nauphoeta cinerea* and the locust *Locusta migratoria* (Goltzene et al., 1978; Zhu et al., 1983), the epithelial follicle cells that surround the oocyte and nurse cells of *A. aegypti* ovarioles are hypothesized to be the site of ecdysteroid biosynthesis. The source of cholesterol for *A. aegypti* ovary ecdysteroid biosynthesis is not known, but may be obtained from either a receptor or non-receptor mediated processes, as shown to function in mammalian steroidogenesis (Azhar et al., 2003). The means by which cholesterol is delivered and internalized by the follicle cells of *A. aegypti* is also unknown, but as suggested with studies conducted with the ovaries of lepidopteran insects, lipoprotein may fulfill this role. An increase in both transcripts and protein levels of the *Aedes aegypti* lipoprotein, lipophorin, and transcripts of its ovary specific receptor (AaLpRov) correlate well with the rise and fall of ecdysteroid levels in blood-fed females (Capurro Mde et al., 1994; Sun et al., 2000; Cheon et al., 2001), supporting the hypothesis that cholesterol transported by lipophorin is the source used for *A. aegypti* ovary ecdysteroid biosynthesis. Indeed, lipophorin begins to accumulate within *A. aegypti* ovaries 6-12 h post blood meal (PBM) (Sun et al., 2000), the same period at which ovary ecdysteroid production begins (Hagedorn et al., 1975; Sieglaff et al., 2005). Recombinant AaLpRov is capable of binding lipophorin; however, *in situ* hybridization studies suggest that gene transcripts of AaLpRov are expressed in the oocyte and nurse cells and not the follicle cells, and thus may not have a role in the acquisition of cholesterol by ovariole follicle cells (Cheon et al., 2001).

Ecdysteroid biosynthesis begins with the modification of cholesterol to 7-dehydrocholesterol by the microsomal cytochrome P450 enzyme, 7, 8-dehydrogenase (Grieneisen et al., 1993; Grieneisen, 1994). The means by which cholesterol is delivered to the endoplasmic reticulum (ER) is not known but it is not considered to be the rate limiting step of

ecdysteroid biosynthesis (Grieneisen et al., 1993; Grieneisen, 1994; Warren and Gilbert, 1996b). Instead, the subsequent delivery of 7-dehydrocholesterol to the inner mitochondrial matrix (IMM) for enzymatic processing is proposed to be the regulated step (Grieneisen et al., 1993; Warren and Gilbert, 1996b). In mammalian steroidogenesis, the delivery of cholesterol to the IMM is considered the rate limiting step (Jefcoate et al., 1992), and various mediators of this process have been proposed, such as the diazepam-binding inhibitor (DBI), sterol-carrier protein 2 (SCP-2), and the steroidogenic acute regulatory protein (StAR) (Jefcoate et al., 1992; Papadopoulos, 1993; Schroeder et al., 1996; Christenson and Strauss, 2001). Homologs of DBI and SCP-2 have been identified in insects (Snyder and Feyereisen, 1993; Kolmer et al., 1994; Matsumoto et al., 2001; Krebs and Lan, 2003; Sieglaff et al., 2005). A distinct homolog of StAR has not yet been identified in insects (Gilbert et al., 2002), instead two proteins containing a domain homologous to StAR have been identified (Tabunoki et al., 2002; Roth et al., 2004). Evidence for DBI and SCP-2 functioning in ecdysteroid biosynthesis was obtained when it was observed that disrupted prothoracic glands incubated with antibodies to DBI decreased ecdysteroid biosynthesis by this tissue (Snyder and Van Antwerpen, 1998), and recombinant SCP-2 was shown to bind cholesterol (Krebs and Lan, 2003). No additional evidence linking these two proteins to ecdysteroid biosynthesis has yet been provided.

The StAR protein has accumulated the most evidence that it performs the rate limiting step of mammalian steroidogenesis, the transfer of cholesterol to the IMM (Clark et al., 1994; Arakane et al., 1996; Arakane et al., 1998; Bose et al., 2002). The START domain ("StAR-related Lipid Transfer Proteins") within the StAR protein (comprises 3/4 of the StAR protein) is present in different proteins from plants to animals (Bauer et al., 2000; Tsujishita and Hurley, 2000; Soccio and Breslow, 2003; Strauss et al., 2003; Schrick et al., 2004). Various functions

have been assigned to these proteins ranging from lipid and sterol metabolism, transcription factors, to signal transduction (Ponting and Aravind, 1999; Tsujishita and Hurley, 2000; Soccio and Breslow, 2003; Schrick et al., 2004). For insects, two proteins with the START domain have been identified and characterized, one implicated in carotenoid binding (Tabunoki et al., 2002; Tsuchida et al., 2004) and the other ecdysteroid biosynthesis (Roth et al., 2004). The later one, *Start1*, was identified in the fruitfly *Drosophila melanogaster* and displays significant sequence identity to MLN64 ("Metastatic Lymph Nodes 64"), a protein that was originally isolated during a screen for genes upregulated in human breast cancer cells (Moog-Lutz et al., 1997). MLN64 has an amino terminal region with four transmembrane domains and the carboxyl region has the START domain. The four transmembrane domains of MLN64 traverse the plasma membrane of endosomal vesicles leaving the START domain on the cytosolic surface (Alpy et al., 2001; Zhang et al., 2002; Alpy et al., 2005). Importantly, when the START domain of MLN64 is expressed within heterologous cell systems, it is capable of stimulating steroid biosynthesis (Watari et al., 1997; Bose et al., 2000; Tuckey et al., 2004). The current hypothesis for MLN64 action is that the transmembrane domain derives cholesterol from the endosomal membrane and transfers the cholesterol to the cytoplasmic START domain, which in turn transfers the cholesterol to acceptor membranes such as the mitochondria (Alpy et al., 2005).

Start1 in *D. melanogaster* also possesses four transmembrane domains in its amino terminus, and the START domain in the carboxyl terminus is split in two by a novel "insert coding region" (Roth et al., 2004). *D. melanogaster* Start1 is hypothesized to be involved in ecdysteroid biosynthesis because of its enhanced transcript expression within known ecdysial tissues, prothoracic glands of larvae and adult ovaries (Roth et al., 2004). The expression of *D. melanogaster* Start1 transcripts also correlate with ecdysone peaks during larval development

(Roth et al., 2004), but there is no direct evidence that *D. melanogaster* Start1 functions in ecdysteroid biosynthesis,

Two homologs of *D. melanogaster* Start1 were recently identified as cDNAs from the ovaries of the yellow fever mosquito, *Aedes aegypti*, AedaeStart1a and b, and analysis that addresses both forms of AedaeStart1s showed that transcript levels peaked within ovaries when ecdysteroid production in blood-fed females is highest (Hagedorn et al., 1975; Sieglaff et al., 2005). The AedaeStart1s are also predicted to have four transmembrane domains in their amino termini, and the START domains are also split by an "insert coding region" in their carboxy termini (Sieglaff et al., 2005). In this report, the expression and localization of Start1 protein in *A. aegypti* ovarioles, and its correlation to ecdysteroid production by this tissue was assessed. The information attained in this study may lend further support for the possible role of insect Start1s in ecdysteroid biosynthesis.

2. Materials and Methods

2.1 A. aegypti colony

The *A. aegypti* colony was maintained as previously described (Sieglaff et al., 2005). To initiate a gonotropic cycle, females were fed on an anesthetized rat until engorged and maintained on distilled water until needed.

2.2 Start1 antiserum

Start1 antiserum and pre-immune serum were kindly provided by Guenther Roth (Freie Universitaet Berlin, Germany). The antiserum was made by inoculating a rabbit with a portion of the *D. melanogaster* Start1 protein (accession AAR19767) that encompasses the START domain (M228-G572) but excludes the four transmembrane domains (see Roth et al., 2004). This antigen

region corresponds to the region of Y179-G549 of AedaeStart1a (Figure 4.1). Upon arrival, the antiserum and pre-immune serum were aliquoted and stored at -80ºC until used.

2.2 Immunoblots

Preparation of ovary pair lysates, SDS-PAGE and transfer of proteins to PVDF membranes are detailed in the materials and methods section of Chapter 3. In brief, the ovary pair lysates were added at 3 ovary pair equivalents/ well for SDS-PAGE. Following the transfer, membranes were rinsed with dH_2O, and then treated with a blocking buffer (TBS-T contained 1% Blotting Grade Blocker Non-fat Dry Milk (Biorad), 2% bovine serum albumin (Sigma), and 1% goat serum (Sigma)). Start1 antiserum was added to the blocking buffer at a 1:20,000 dilution and incubated overnight at 4º C with gentle rocking, and control blots were incubated with pre-immune serum at the same dilution. Blots were washed in TBS-T (Tris-buffered saline with 0.1% Tween 20; 2x quick, 1x 15 minutes, 3x for 5 minutes), incubated in blocking buffer containing anti-rabbit antibody conjugated to horseradish peroxidase (1:50,000 dilution, Sigma) for 4 h at room temperature with gentle rocking, and washed as specified above. Immunoreactive proteins were detected with ECL Advance Western Blotting Detection Kit (Amersham Biosciences) and the GeneGnome imaging system (Syngene Bio Imaging). The molecular weights of immunoreactive bands were estimated with GeneTools (Synoptics Limited) and based on simultaneously run standards (Magic Mark®; Biorad).

Immunoblots for three cohort replicates were analyzed, and each cohort consisted of twelve pooled ovary pairs. The densities of each band were calculated using the GeneTools program (Synoptics Limited), the background signal subtracted from the calculated raw volumes, and the pixel area analyzed was kept constant across all time points and the three cohort

replicates. The density of immunoreactive bands were analyzed by ANOVA, and their means separated by Tukey-Kramer HSD test (SAS JMP 5.0.1a, SAS Institute Inc., Cary, N.C.).

2.3 Immunocytochemistry

Ovaries were dissected and prepared as specified in the materials and methods section of Chapter 3. In brief, ovaries were treated with Start1 antiserum at a 1:3,000 dilution in blocking buffer (PBS-T containing 2% bovine serum albumin (Sigma) and 2% goat serum (Sigma)) and incubated overnight at 4º C with gentle rocking, and control ovaries were incubated with pre-immune serum at the same dilution. Ovaries were washed in blocking buffer (4x for 5 minutes), incubated in the same blocking buffer containing anti-rabbit antibody conjugated to Alexa Fluor® 488 (1:3,000 dilution, Molecular Probes) for 4.5 h at room temperature with gentle rocking and washed as specified above. Ovaries were mounted on a slide in media (PBS:glycerol 1:1) and examined with a Leica TCS/SP2 confocal microscope. A series of confocal sections were taken from the outer surrounding follicle cells (top) to the middle of the ovariole to assess immunostaining in follicle cells, nurse cells, and the oocyte of ovarioles. For consistency, all confocal settings remained unchanged between samples with the exception of using a 20x lens for the 48 and 60 h PBM time points due to the size of the oocyte (i.e., NBF, 6 h, 12 h and 18h PBM oocytes were viewed using a 63x lens). Three separate cohort replicates were analyzed.

For the cellular localization experiments, 12 h PBM ovaries were dissected as previously described, incubated in physiological saline solution containing 500 nM MitoTracker® Deep Red 633 for 1 h at 27ºC with gentle rocking, washed with the physiological saline solution (3x 2 minutes), and prepared for immunocytochemistry as described above.

3. Results

3.1 Immnunoblots analysis of Start1 expression in ovaries during a gonotropic cycle

The utility and specificity of the Start1 antiserum for immunoblot analysis of *A. aegypti* ovaries was determined by conducting immunoblot analysis against ovary pair lysates prepared from females at peak PBM ovarian ecdysteroid production (18 h PBM). The Start1 antiserum recognized a prominent band estimated to be 55 kDa in ovary pair lysates (Figure 4.2A). The specificity of the antiserum is established by the absence of any immunoreactive bands within the same range of molecular weights (MW) in immunoblots incubated with pre-immune serum.

To determine whether an increase in the expression of Start1 correlates with ovarian ecdysteroid production in blood-fed *A. aegypti*, immunoblot analysis was conducted against ovary pair lysates from three separate cohorts of females before (NBF), the initiation (6 and 12 h PBM), peak (18 h PBM), and end (48 and 60 h PBM) of ovarian ecdysteroid production. Analysis of the immunoblots determined that the density of the 55 kDa band did not change significantly in ovary pair lysates of females during a gonotropic cycle, and reached it lowest density in the 48 h PBM ovary pair lysates ($p \geq 0.05$; see Figure 4.2B and C). Start1 immunoreactive bands were most evident in NBF, 6, 12 and 18h PBM samples, and in the later samples of 48 and 60 h PBM bands were hardly visible by the naked eye. The background of all immunoblots was quite high, and thus may have obscured analysis. The imagining system software, however, detected products at the predicted size in the 48 and 60 h PBM ovary pair lysates.

3.2 Immunocytochemistry of Start1 in ovarioles during a gonotropic cycle

Each of the paired ovaries of *A. aegypti* is comprised of approximately 75 ovarioles, and the individual ovarioles possess a germarium and primary follicle consisting of one oocyte and seven nurse cells surrounded by a layer of epithelial (follicle) cells (Clements, 1992). It was the

purpose of the current study to determine whether AedaeStart1 is expressed in the presumed source of ecdysteroids, the follicle cells (Goltzene et al., 1978; Zhu et al., 1983), or if its localization agrees with localization of *Start1* transcripts in *D. melanogaster* in nurse cell (Roth et al., 2004). Start1 immunostaining was observed in both ovariole follicle and nurse cells (Figure 4.3), with nurse cell immunostaining becoming more pronounced following a blood meal (see Figure 4.3 middle 6h, 12h and 18h). The near absence of immunostaining in ovarioles incubated with pre-immune serum attests to the specificity of the anti-Start1 serum (Figure 4.3 12h-pre-immune sera; note: a similar absence of signal was observed in ovarioles from every time point examined, not shown). A pronounced Start1 immunostaining in follicle cells residing above nurse cells was observed in every time point examined (see Figure 4.3 middle NBF, 6h, 12h). It should be noted, however, that an exclusion of Start1 immunostaining in follicle cells surrounding the oocyte was not a consistent feature, and may represent the effect of sample orientation during confocal microscopy analysis (see the follicle cells stained along side the oocyte in Figure 4.3 18h-middle).

Start1 immunostaining was punctate in appearance, being distributed throughout the cytosol of ovariole follicle cells (Figure 4.4). At the resolution examined, no definition to a cellular localization could be discerned.

4. Discussion:

A blood meal taken by female *A. aegypti* initiates ovarian production of ecdysteroids (Hagedorn et al., 1975; Sieglaff et al., 2005), and these hormones in turn coordinate the events of oogenesis resulting in a mature egg to be fertilized and deposited (Klowden, 1997; Raikhel et al., 2002). Though the entire biosynthetic pathway of ecdysteroid biosynthesis is not known, the use of molecular biology techniques, genomics, and analysis of *D. melanogaster* mutants displaying

anomalies in ecdysteroid biosynthesis are allowing the identification of many of the components of this biosynthetic pathway (e.g., Chen et al., 1999; Freeman et al., 1999; Gilbert, 2004). Based on the amino acid sequences published for component members of the ecdysteroid biosynthetic pathway, seven genes putatively involved in ecdysteroid biosynthesis in *A. aegypti* were recently identified and characterized (Sieglaff et al., 2005). The transcript levels for *AedaeStart1* peaked at the same time as ovary ecdysteroid production in blood-fed females (Sieglaff et al., 2005). The *D. melanogaster* Start1, a putative sterol transfer protein has been hypothesized to mediate the transfer of sterols to and from the ER and/or IMM during ecdysteroid biosynthesis (Roth et al., 2004). This assertion is based primarily on its enhanced expression within ecdysial tissues, prothoracic glands and ovaries, and its sequence similarity to the mammalian protein MLN64, which has acquired evidence for its function in endosomal cholesterol trafficking and steroidogenesis (Watari et al., 1997; Bose et al., 2000; Bose et al., 2000b; Alpy et al., 2001; Zhang et al., 2002; Uribe et al., 2003; Alpy et al., 2005).

The primary purpose of the current study was to determine whether there is any correlation between the expression of AedaeStart1 protein in *A. aegypti* ovarioles and ovarian ecdysteroid production after a female takes a blood meal. To this end, immunoblot and immunocytochemical analyses were undertaken to assess AedaeStart1 expression within ovaries during the gonotropic cycle, and localize its expression to specific cell populations of the developing ovariole. It can not be predicted whether the anti-Start1 serum would recognize AedaeStart1a and b differently, and thus no distinction between these two predicted forms of AedaeStart1s is made in the discussion below. The results obtained in the current study are discussed in relation to AedeaStart1's possible role in ecdysteroid biosynthesis, and is based on observations made for its mammalian homolog, MLN64.

Given that the Start1 antiserum was raised against a heterologous Start1, it was important to first determine whether it could be used in analyzing AedaeStart1 expression in *A. aegypti* tissues. The deduced amino acid sequences of AedaeStart1a and b predict 65 and 68 kDa proteins, respectively (Sieglaff et al., 2005), which are both larger than the ~55 kDa immunoreactive band observed in immunoblots (Figure 4.2A). Interestingly, if the so-called "insert coding region" is removed from AedaeStart1a and b, 49 and 52 kDa proteins are predicted, respectively. The "insert coding region" of *D. melanogaster* Start1 is expressed at the protein level as indicated by immunocytochemical analysis conducted against ring glands using antibodies raised exclusively against the "insert coding region" of Start1 (Roth et al., 2004). Immunocytochemical analysis does not conclusively attest that the "insert coding region" is retained in the *D. melanogaster* Start1 protein, however, but simply confirms "insert coding region" immunoreactivity within the tissue. The possibility that the "insert coding region" is removed from AedaeStart1 either during its translation or more significantly as a mechanism of activation is very intriguing, but this of course would require greater detailed analysis. The significance and function of the "insert coding region" is difficult to predict, and is confounded by the observation that the START domain of MLN64 is hypothesized to transport a single cholesterol molecule (Tsujishita and Hurley, 2000). Moreover, like the 55 kDa immunoreactive band observed in *A. aegypti* ovary pair lysates (Figure 4.2A), MLN64 was estimated to be 54-55 kDa proteins on immunoblots of mitochondria preparations from human placenta and rat hepatocytes recombinantly expressing MLN64 (Uribe et al., 2003; Ren et al., 2004). Other researchers have reported a 50 kDa protein on immunoblots of placental homogenates and monkey kidney COS-1 cells recombinantly expressing MLN64 (Watari et al., 1997; Bose et al., 2000a). SDS-PAGE and subsequent immunoblotting is only an approximation of MW, but the

similarity in MWs observed in immunoblots of *A. aegypti* ovary pair lysates and the aforementioned mammalian MLN64 studies adds more mystery to the "insert coding region" dilemma.

As determined through immunoblot analysis, AedaeStart1 protein levels did not increase or change significantly in *A. aegypti* ovaries during the gonotropic cycle, nor did its levels correspond to ovarian ecdysteroid production in blood-fed females (Figure 4.2B and C; Sieglaff et al., 2005). Thus, if AedaeStart1 is to be a regulator of the initiation of ecdysteroid biosynthesis through its transport of sterols, its "activation" does not occur through an increase in either its gene transcripts (Sieglaff et al., 2005) nor its translated protein in the early stages of ovarian ecdysteroid production in blood-fed females (i.e., 6 - 12 h PBM). According to Roth et al. (2004), *D. melanogaster* Start1 transcripts are highest during peak ecdysteroid titers during embryogenesis and in ovaries in stage 10 egg chambers, thus they suggest that this temporal expression is a means of regulating ecdysteroid biosynthesis. They further suggest that Start1 transcript levels are positively regulated by ecdysteroids themselves, based on the depression in Start1 transcripts in the ring glands of a *D. melanogaster* mutant with reduced ecdysone titers (Roth et al., 2004).

Start1 immunostaining within *A. aegypti* ovarioles displayed a characteristic punctuate as has been observed for MLN64 (Moog-Lutz et al., 1997; Alpy et al., 2001; Zhang et al., 2002), suggesting *A. aegypti* Start1's localization to endosomes. On closer assessment of Start1 immunostaining in ovariole follicle cells, the punctata were distributed throughout the cytosol and displayed an enhancement in staining near the plasma membrane (PM) (Figure 4.4). The enhancement in immunostaining near the PM and its localization to punctata throughout the cytosol suggest that AedaeStart1 may be involved in trafficking of cholesterol from endocytosis

of lipophorin at the PM to its delivery to organelles within the cytosol via endosomes as ascribed for its mammalian homolog MLN64 (Alpy et al., 2001; Zhang et al., 2002).

Start1 immunostaining within nurse cells increased in blood-fed females (compare Figure 4.3 middle NBF to 6 h, 12 h or 18 h), suggesting that this increase in levels of Aedaestart1 protein within ovariole nurse cells is regulated. The lack of a significant fluctuation in AedaeStart1 protein levels during a gonotropic cycle observed in immunoblots is likely the result of analyzing whole ovary pair lysates which would obscure nurse cell specific expression of AedaeStart1. AedaeStart1 immunostaining within ovariole nurse cells (esp., following a blood meal) was not surprising for gene transcripts of *Start1* localize to ovariole nurse cells in *D. melanogaster* (Roth et al., 2004). Interestingly, gene transcripts encoding another protein hypothesized to be involved in ecdysteroid biosynthesis, adrenodoxin reductase, also localize to ovariole nurse cells in *D. melanogaster* (Freeman et al., 1999). Furthermore, gene transcripts encoding three cytochrome P450 enzymes known to be involved in ecdysteroid biosynthesis are observed in both follicle and nurse cells of *D. melanogaster* ovarioles (Warren et al., 2002; Petryk et al., 2003; Warren et al., 2004), as opposed to the exclusive follicle cell localization of *D. melanogaster* and *A. aegypti* CYP302a1 (Chapter 3; Chavez et al., 2000). Whether the nurse cells are involved in ecdysteroid biosynthesis, as is suggested by localization of various components of its biosynthetic pathway, will require further research for it has been shown previously that the follicle cells are the source of ovarian ecdysteroids in two insects (Goltzene et al., 1978; Zhu et al., 1983).

The rate limiting step of ecdysteroid biosynthesis is hypothesized to be the transport of 7-dehydrocholsterol to the IMM for subsequent enzymatic processing (Grieneisen et al., 1993; Grieneisen, 1994; Warren and Gilbert, 1996b). It is possible that AedaeStart1 may function as

the transporter of 7-dehydrocholesterol to the IMM, and this hypothesis is derived primarily from experiments conducted with MLN64 or its START domain (Strauss et al., 2000; Soccio and Breslow, 2003; Strauss et al., 2003; Uribe et al., 2003). Support for this assertion comes from the observation that MLN64 can mediate the transfer of cholesterol from sterol-rich liposomes to liver mitochondrial membranes (Zhang et al., 2002). Also, the START domain of StAR has been shown to mediate the transfer of cholesterol from liposomes to both mitochondrial and microsomal membranes of various tissues (Kallen et al., 1998). The START domain of MLN64 has also been shown to stimulate steroid biosynthesis by placental mitochondrial preparations (Tuckey et al., 2004), and immunoblot analysis of this same tissue displayed MLN64 immunoreactivity on the outer mitochondrial membrane (Uribe et al., 2003). Therefore, the possibility that AedaeStart1 is functioning in endosomal delivery of cholesterol to acceptor membranes can be hypothesized. The ability to hypothesize functions for the insect Start1s based on the observations made for MLN64 or the START domain of StAR is difficult, however, given that the START domain of the insect Start1s are split in two by the "insert coding region," which is expressed at the protein level in *D. melanogaster* ring glands (Roth et al., 2004).

Intracellular cholesterol movement is a complex process that involves trafficking to different organelles and sites of interaction (Jefcoate et al., 1992; Liscum and Underwood, 1995; Thomson, 1998), a process that is not known for insects. Since insects can not synthesize cholesterol *de novo*, the acquisition and processing of cholesterol may be a simpler process than that observed in mammals. A more lucid picture of intracellular cholesterol movement in insects may be attained through the use of microscopic techniques and fluorescently labeled cholesterol molecules or its derivatives (Schroeder et al., 2001). Given that the transport of an early ecdysteroid intermediate(s) may represent the rate limiting step of ecdysteroid biosynthesis

(Grieneisen et al., 1993; Warren and Gilbert, 1996b), the mosquito ovary represents an ideal candidate to study this rate limiting movement because one can follow the inception of ecdysteroid biosynthesis after providing a blood meal to adult females. Using the ovaries of anautogenous mosquitoes with the *Anopheles gambiae* genome (Holt et al., 2002) and soon to be completed *A. aegypti* genome (Severson et al., 2004) the potential players in the initiation of ecdysteroid biosynthesis may more readily be identified.

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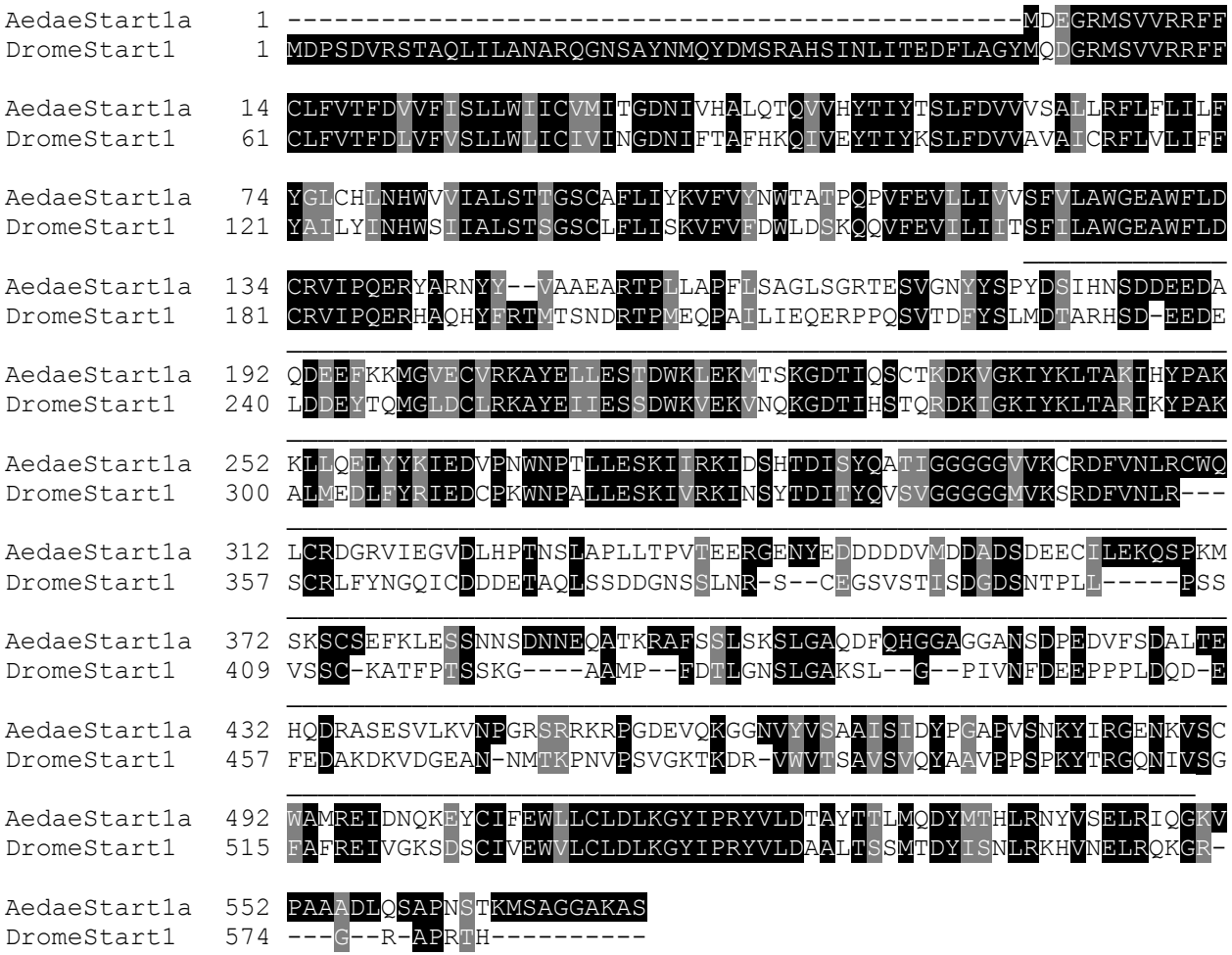


Figure 4.1. Protein sequence alignment of *A. aegypti* StAR-related lipid transfer domain containing protein, AedaeStart1a (GenBank AY947545), with *D. melanogaster* Start1, DromeStart1 (GenBank AAF47232). The region used for antigen production is denoted by a solid over bar. Sequences were aligned using the program CLUSTAL W 1.74 (http://www.ch.embnet.org/software/ClustalW.html).

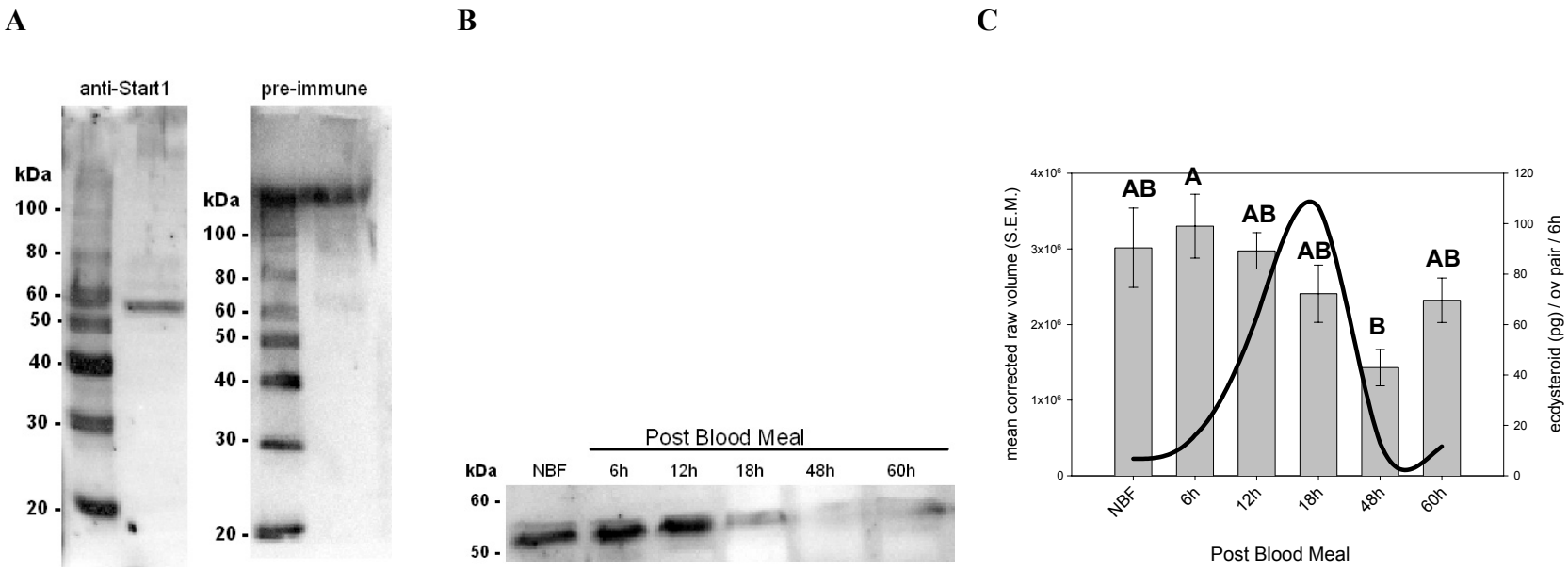
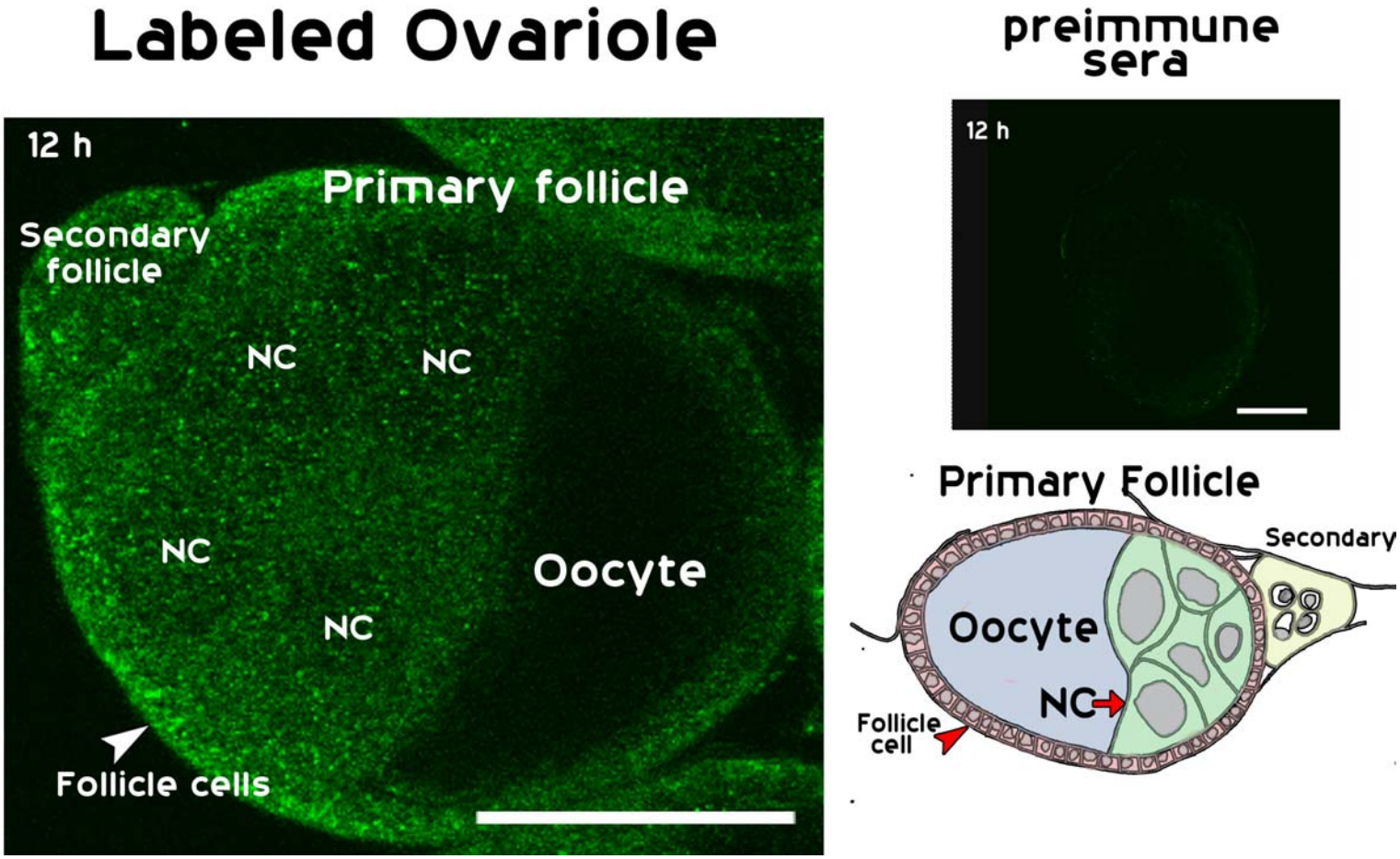
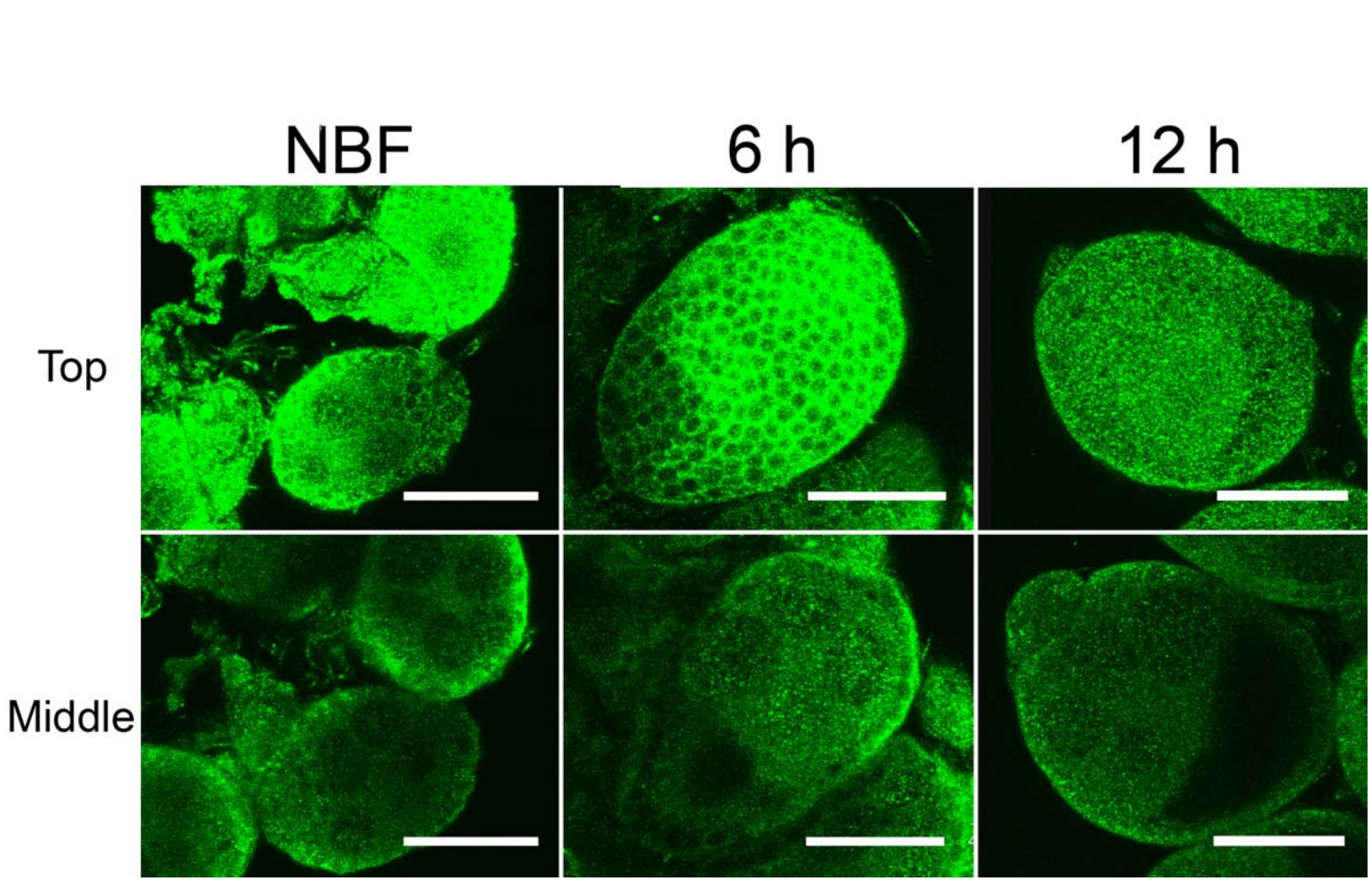


Figure 4.2. Immunoblots of female A. *aegytpi* ovary pair lysates. (**A**) Ovaries from *A. aegypti* females 18 h PBM (18h PBM ov, 3 ovary pair equivalent). (**B**) Expression of Start1 in ovaries from females at different time points following a blood meal (3 ovary pair equivalents/ well). (**C**) Densitometry analysis of Start1 expression in ovaries from females of three separate cohorts following a blood meal (bars with the same letter are not significantly different; Tukey-Kramer, $p \leq 0.05$). A representation of PBM ovary ecdysteroid production is superimposed over the bar graph for descriptive purposes.

A


Labeled Ovariole
12 h
Primary follicle
Secondary follicle
NC
NC
NC
NC
Oocyte
Follicle cells
preimmune sera
12 h
Primary Follicle
Secondary
Oocyte
NC
Follicle cell

B



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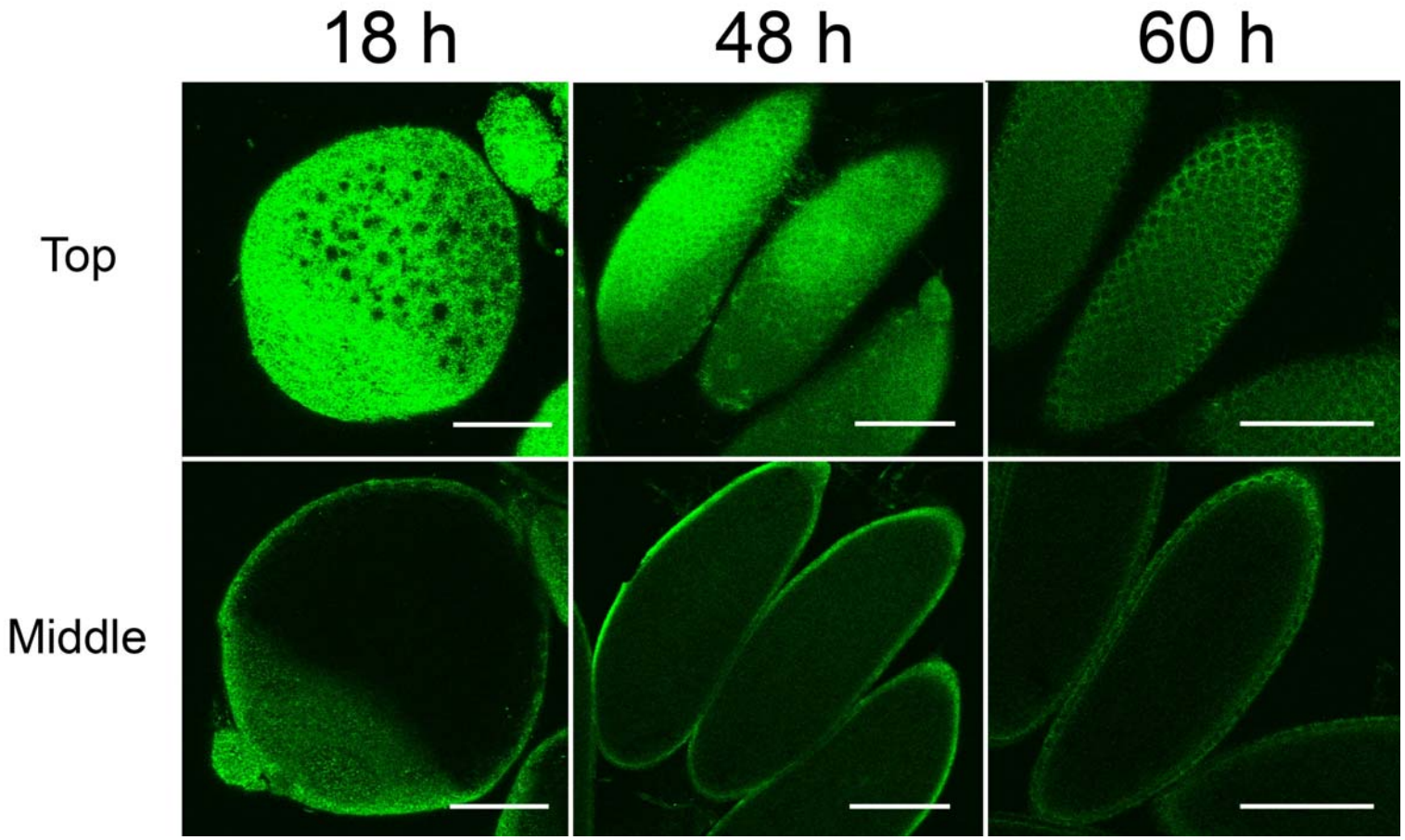

Figure 4.3. Immunocytochemistry of Start1 in ovaries of NBF females and those of different time points PBM: 6, 12, 18, 48 and 60 h PBM. Cross sections of sample ovarioles for each time point are presented from the perspective of the ovarioles surface (top) and its middle to display the ovariole cell populations(s) immunostained for AedaeStart1. (**A**) Start1 immunostained 12 h PBM ovariole

displaying the oocyte, nurse cells (NC), follicle cells, and the primary and secondary follicles, and a cartoon rendition of the different cell populations of the ovarioles; 12 h PBM ovarioles treated with pre-immune sera. Start1 immunostained (**B**) NBF, 6 and 12 h PBM ovarioles, (**C**) 18, 48 and 60 h PBM ovarioles. Scale bars = 50 μm in NBF, 6 h, 12 h, and 18 h PBM samples; 150 μm in 48 h and 60 h PBM samples.

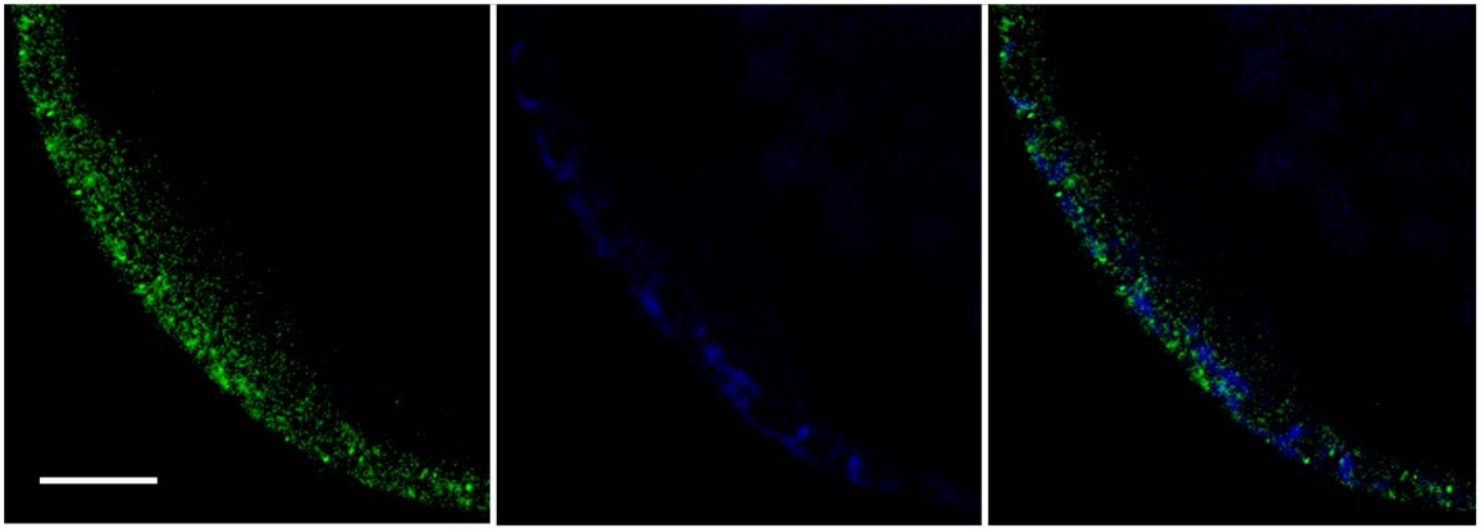

Figure 4.4. Localization of Start1 immunoreactivity and mitochondria in follicle cells of 12 h PBM females. Ovaries from 12 h PBM were treated with MitoTracker® Deep Red 633, and then with anti-Start1 antibodies as specified in the materials and methods. The three panels from left to right indicate Start1 immunoreactivity, MitoTracker® Deep Red 633, and merge. The scale bar = 15 μm.

CHAPTER 5

CONCLUSIONS

As described within this dissertation, a blood meal taken by *Aedes aegypti* initiates ovarian production of ecdysteroids, the primary hormonal regulator of ovary development (Klowden, 1997; Raikhel et al., 2002), and the capacity of ovarian ecdysteroid production is likely regulated at the gene transcript and translational levels. Following a blood meal, ovarian ecdysteroid production begins 6 h post blood meal (PBM), peaks at 18 h PBM, and falls to pre-blood feeding levels by 48 hours PBM. Ovary transcript abundance for two genes encoding cytochrome P450 enzymes putatively conducting the "terminal hydroxylation" reactions at 22-C and 2-C, *AedaeCYP302a1* and *AedaeCYP315a1*, respectively, followed the rise and fall of ovarian ecdysteroid production after a blood meal. The gene transcript abundance of *AedaeStart1*, *AedaeA/KR* and *AedaeAR* also peaked in ovaries 18 h PBM, but the increases were not as a significant as the aforementioned cytochrome P450s. These results suggest that ovarian ecdysteroid production following a blood meal is the result of increasing the abundance of specific components of the ecdysteroid biosynthetic pathway. Indeed, such a correlation between gene transcript abundance and whole body ecdysteroid titers has been observed for specific components of the ecdysteroid biosynthetic pathway in *Bombyx mori* and *Drosophila melanogaster* (Chavez et al., 2000; Warren et al., 2002; Niwa et al., 2004; Roth et al., 2004; Warren et al., 2004).

The observations that ovarian ecdysteroid production in *A. aegypti* can be stimulated *in vitro* with heterologous insulins and cAMP analogs (Shapiro, 1983; Brown et al., 1995; Graf et

al., 1997; Riehle and Brown, 1999), allows the opportunity to address further whether ovarian ecdysteroid production stimulated by these agents is the result of an increase in transcript abundance of any of the characterized genes. Bovine insulin and the cAMP analogs pCPT-cAMP stimulated ovarian ecdysteroid production *in vitro* 4 and 6-fold, respectively. The increase in ovarian ecdysteroid production *in vitro*, however, did not correlate with an increase in transcript abundance of any of the characterized genes. The difference between the *in vitro* and *in vivo* state may be related to various effects: *in vitro* stimulation of ovarian ecdysteroid production involves the (1) translation of specific proteins involved in ecdysteroid biosynthesis, (2) activation of such proteins through post-translational modification like phosphorylation, or (4) the ovaries are not synthesizing ecdysteroids but simply releasing them from pre-existing stores. Of note is that the endogenous gonadotropin, Ovary Ecdysteroidogenic Hormone I (OEH I), has not been tested *in vitro*, and thus the increase in transcript abundance observed *in vivo* may be elicited by OEH I. To address whether protein translation is the means by which ovarian ecdysteroid production is regulated *in vitro* can be accomplished with various experimental approaches : (1) determine whether the protein synthesis inhibitors such as cyloheximide or puromycin disrupt the ability of bovine insulin or pCPT-cAMP to stimulate ovarian ecdysteroid production, (2) provide the ovaries radiolabeled amino acids such as [^{35}S] and conduct chase experiments to assess the incorportation of [^{35}S] into new proteins following bovine insulin or pCPT-cAMP stimulation, or (3) determine whether stimulation of ovarian ecdysteroid production by bovine insulin or pCPT-cAMP corresponds with an increase in expression levels of proteins specifically involved in ecdysteroid biosynthesis (e.g., AedaeCYP302a1). To determine whether phosphorylation of specific proteins is the means by which bovine insulin or pCPT-cAMP stimulate ovarian ecdysteroid production *in vitro* is more involved and would

require the ability to isolate the protein of interest and determine its state of phosphorylation before and after stimulation. The isolation of the specific protein is suggested because simply going by location on an immunoblot is not adequate given that many of the proteins involved in ecdysteroid biosynthesis may have similar molecular weights as ascribed by similar migration rates in SDS-PAGE (e.g., cytochrome P450 enzymes). The use of immunoprecipitation and subsequent immunoblotting with antibodies directed against phosphorylated amino acids may be a means by which the isolation and assessment of phosphorylation state of specific proteins can be addressed. Finally, to determine whether bovine insulin or pCPT-cAMP simply stimulates the release of ecdysteroids from pre-existing stores, one could determine the concentration of ecdysteroids within the ovary before and after stimulation.

To address further the means by which the capacity of ovaries to produce ecdysteroids in blood-fed *A. aegypti* increases, two proteins representing separate control points in ecdysteroid biosynthesis, enzyme modification, AedaeCYP302a1, and sterol transfer, AedaeStart1, were studied in ovaries during a gonotropic cycle. The enzyme representing the putative 22-hydroxylase of ecdysteroid biosynthesis, AedaeCYP302a1, increased in abundance within ovaries during the gonotropic cycle, but unlike its gene transcripts, remained high despite the lack of ovarian ecdysteroid production later in the gonotropic cycle. The maintenance of high levels of AedaeCYP302a1 in ovaries and the lack of ovarian ecdysteroid production suggests that biosynthesis of ecdysteroids in *A. aegypti* ovaries is regulated beyond the translation of biosynthetic enzymes, and likely involves additional points of regulation such as substrate availability. Whether the availability of substrate is the limiting factor for ovarian ecdysteroid biosynthesis could be addressed either directly by providing substrate (ecdysteroid precursors) to ovaries of the later gonotropic stages, or indirectly by determining the enzymatic capacity of

AedaeCYP302a1 in ovaries of these later stages. The protein that may mediate substrate availability through intracellular transfer of sterols, Aedeastart1, did not change significantly in ovaries during the gonotropic cycle, and was also present in ovaries at later stages of the gonotropic cycle. Whether AedeaStart1 has a role in intracellular transport of sterols for ecdysteroid biosynthesis, however, is quite speculative and based primarily on its sequence homology with the mammalian cholesterol transfer protein, MLN64. The fact that AedaeStart1 and its *D. melanogaster* ortholog, Start1, possess an "insert coding region" splitting the putative sterol transfer domain (START) in half confounds the presumed functional homology between the insect Start1s and MLN64. The ability of AedaeStart1 to bind precursor ecdysteroids and its intracellular movement may provide some indication of its role in ecdysteroid biosynthesis. The presence of AedaeStart1 and AedeaCYP302a1 in ovaries late into the gonotropic cycle when ovaries are no longer producing significant quantities of ecdysteroids suggest that neither of these proteins is the "key" regulator of ovarian ecdysteroid biosynthesis.

Confocal microscopy allowed a more detailed assessment of the localization and expression of both AedaeCYP302a1 and AedaeStart1. AedaeCYP302a1 localized to ovariole follicle cells, the presumed source of ovary ecdysteroids (Goltzene et al., 1978; Zhu et al., 1983), and AedaeCYP302a1 immunostaining increased in follicle cells following a blood meal. Unlike *D. melanogaster* CYP302a1 (Petryk et al., 2003), AedaeCYP302a1 did not localize exclusively to the mitochondria. AedaeStart1 was observed in both nurse and follicle cells of developing ovarioles, and AedaeStart1 immunostaining increased in nurse cells following a blood meal. Whether *A. aegypti* ovariole follicle are the source of ecdysteroids has still not been determined experimentally, but could be accomplished by isolating the follicle cells from the rest of the ovariole and then the ability of these isolated follicle to produce ecdysteroid *in vitro* assessed.

Additional analysis can also be undertaken to determine the subcellular location of both proteins, such as electron microscopy and differential centrifugation (esp., density gradient ultracentrifugation).

An observation made for both the gene transcripts characterized in this dissertation and AedaeCYP302a1 protein is that they are expressed within tissues peripheral to the primary source of ecdysteroids in adult female *A. aegypti*, the ovary, and that these tissues (thorax, abdominal pelt, and gut) produced minimal amount of ecdysteroids (≤ 8% that of ovaries from 18 h PBM females). Orthologs of these genes in other insect species also display a wide tissue distribution at the transcript level (DBI, Start1, A/KR, AR, and CYP314a1) or as described by enzymatic activity (25-hydroxylase, 22-hydroxylase, 2-hydroxylase) (Meister et al., 1985; Meister et al., 1987; Haag et al., 1988; Snyder and Feyereisen, 1993; Kolmer et al., 1994; Chen et al., 1999; Freeman et al., 1999; Petryk et al., 2003; Roth et al., 2004). The question arises of how and why the ovary is capable of producing significantly greater quantities of ecdysteroids compared to the peripheral tissues despite the shared expression of the gene transcripts and AedeaCYP302a1 protein? From these results it can be assumed that the gene transcripts and proteins studied within this dissertation do not represent the limiting factor(s) that define a tissue's capacity to synthesize ecdysteroids. Does this "missing link" between the ovaries and peripheral tissue's represent the much searched for key regulator of ecdysteroid biosynthesis? Is the key regulator a transcription factor, intracellular sterol transfer protein, biosynthetic enzyme, or a yet undefined mediator of steroidogenesis? With the development of molecular techniques such as microarray technology (David et al., 2005) and the availability of the genomes of *Anopheles gambiae* (Holt et al., 2002) and soon to be completed *A. aegypti* (Severson et al., 2004), the discovery of this key regulator(s) may be more readily attained.

Future studies should address the functional properties of the seven gene transcripts identified in Chapter 2 because the "homology" with other characterized proteins is based solely on the percent identity or similarity between the amino acid sequences deduced from the nucleotide sequences of the *A. aegypti* gene transcripts and their respective homologs in other species. First and foremost the enzymatic activity of the three cytochrome P450 enzymes (AedaeCYP302a1, AedaeCYP315a1 and AedaeCYP314a1) needs to be determined. Transforming plasmid clones of these cytochrome P450 enzymes into a heterologous expression system such as *Drosophila* S2 cells, providing these transformed cells with ecdysteroid precursors as substrate, and assessing catalysis of these substrates is the most direct method of determining substrate specificity for these enzymes. This method of analysis was used to determine the substrate specificity of the *Drosophila melanogaster* cytochrome P450 enzymes, CYP302a1, CYP315a1, CYP314a1 and CYP306a1 (Warren et al., 2002; Petryk et al., 2003; Warren et al., 2004). Likewise, the functions for the other gene products, AedaeDBI, AedaeStart1, AedaeA/KR could also be addressed through their expression in heterologous cell systems. Following their transformation into a heterologous cell system, the capability of AedaeDBI and AedaeStart1 to bind cholesterol or other ecdysteroid precursors and the enzymatic activity of AedeaA/KR could be assessed. The capacity of the two putative "sterol transfer" proteins, AedaeDBI and AedaeStart1, to promote ecdysteroid biosynthesis would require experiments beyond simply addressing their capacity to bind cholesterol. Experimental protocols that addressed the ability of the mammalian homologs DBI and MLN64 to promote steroid production when transformed into heterologous cell systems could be adopted in studies addressing the functionality of the two insect "sterol transfer" proteins (see citations in Chapters 2 and 4). Furthermore, it must be determined whether these two "sterol transfer" proteins are

involved exclusively in ecdysteroid biosynthesis or more generally in intracellular cholesterol trafficking.

The information reported within this dissertation represents the beginning of the study of ecdysteroid biosynthesis in *A. aegypti* ovaries. The characterization of gene transcripts that encode seven proteins putatively involved in this biosynthetic process provided the first glimpse of the means by which the ovaries of blood-fed females produce such large quantities of ecdysteroids. The non-dogmatic observations mentioned above and discussed throughout the dissertation clearly indicate that the regulation of this biosynthetic process is complex and certainly requires much further study. The information provided in this dissertation may help advance the understanding of the biosynthesis of the hormone responsible for the development and reproductive success of the most abundant metazoan life forms on earth, the arthropods.

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APPENDIX A

ECDYSTEROID PRODUCTION BY *Aedes aegypti* OVARIES FOLLOWING *IN VITRO* STIMULATION BY BOVINE INSULIN AND A CAMP ANALOG: EFFECTS ON GENE TRANSCRIPTS ENCODING PROTEINS PUTATIVELY INVOLVED IN OVARY ECDYSTEROIDOGENESIS

1. Introduction

Regulation of insect ecdysteroidogenesis by peptide hormones occurs at three distinct levels: gene transcription, translation and post-translational modifications, such as phosphorylation of regulatory proteins (Smith and Gilbert, 1989; Keightley et al., 1990; Rybczynski and Gilbert, 1994; Song and Gilbert, 1995; Song and Gilbert, 1997; Gilbert et al., 2000; Smith et al., 2003). The influence of these three regulatory levels were deduced from the observation that various inhibitors of these processes decreased or abolished ecdysteroid production by *Manduca sexta* prothoracic glands stimulated by the prothoracicotropic hormone (PTTH) (Smith and Gilbert, 1989; Keightley et al., 1990; Rybczynski and Gilbert, 1994; Song and Gilbert, 1995; Rybczynski and Gilbert, 1995a; Gilbert et al., 1997; Smith et al., 2003). PTTH and other peptide hormones stimulate ecdysteroid biosynthesis through either a G-protein coupled receptor (GPCR) / cAMP-dependent pathway or receptor tyrosine kinase (RTK) / insulin pathway, both of which have been shown to promote ovarian ecdysteroidogenesis in *Aedes aegypti* (Shapiro, 1983; Graf et al., 1997; Riehle and Brown, 1999) and the blowfly *Phormia regina* (Maniere et al., 2000; Maniere et al., 2002; Maniere et al., 2004).

The purpose of the current study was to determine whether ecdysteroid production by *A. aegypti* ovaries stimulated by a cAMP analog or bovine insulin is associated with an increase in gene transcript abundance of the genes characterized in chapter 2.

2. Materials and Methods

2.1 Insects

Aedes aegypti (UGAL strain) were maintained as previously described (see Chapter 2; Sieglaff et al., 2005).

2.2 In vitro stimulation of isolated ovaries

Modified procedures for *in vitro* tissue ecdysteroid production and the ecdysteroid radioimmunoassay (RIA) were followed (Sappington et al., 1997; Riehle and Brown, 1999). Ovary pairs with the last two abdominal segments and a small portion of the hindgut were dissected from females (4 or 5 days post eclosion) in a buffered medium (1.9 mM $CaCl_2$, 3.7 mM KCl, 139 mM NaCl, 25mM HEPES, 5 mM Trehalose, 0.6 mM $MgCl_2$, 1.8 mM $NaHCO_3$). As previously shown, the ovaries are the primary source of ecdysteroids produced *in vitro* (Sieglaff et al., 2005). For each experiment, triplicates of four ovary pairs were placed in 60 µl of the buffered medium in a polypropylene tube lid and incubated for 8 h at 27° C. Ovary pairs were stimulated with 100 µM pCPT-cAMP (Sigma), which is in the range used to stimulate *P. regina* ovarian ecdysteroid production *in vitro* (Maniere et al., 2000), or with 17 µM bovine insulin (Sigma), which was shown to be the optimum concentration to stimulate *A. aegypti* ovarian ecdysteroid production *in vitro* in a previous study (Riehle and Brown, 1999). As controls, ovaries were incubated in buffered medium alone as a control for the pCPT-cAMP treatment or buffered medium supplemented with an equivalent molarity of HCl for the bovine insulin treatment. Each experiment was replicated with ovaries from females from six separate cohorts.

After incubation, 50 µl of incubation medium was collected, stored at -80° C, and later thawed and subjected to RIA as previously described (Sieglaff et al., 2005). For gene transcript analysis, ovary pairs were dissected away from the other tissue, treatments pooled (n = 12/ replicate), placed directly into RNAlater™ (Sigma), allowed to incubate at 4° C overnight, and then stored at -80° C until processed.

2.3 Real-time PCR analysis of gene transcripts

Total RNA was extracted from the ovary pairs using the RNeasy mini kit (Qiagen), treated with Dnase-I (Qiagen), and cDNA synthesized from total RNA (1 µg) with the Advantage® RT-for-PCR kit (BD Biosciences Clontech) and oligo(dT) as the primer. Thus, all the treatments were standardized with equivalent total RNA input.

Real-time PCR was conducted on a Rotor-Gene RG-3000 (Corbett Research) using the program: 95°C for 3 min, 95°C for 20 sec 65°C for 20 sec 72°C for 20 sec for 45 cycles, followed by melting curve analysis. For the analysis of gene transcript abundance, template cDNA-specific master mixes were prepared with IQ™ SYBR® Green Supermix (Biorad), nanopure H_20, and template cDNA at a standard volume so that each PCR contained 20 ng total RNA input. The cDNA-specific master mixes were then split into three separate aliquots, after which three primer sets were added at 100 nmol final concentration/PCR. The three different primer sets corresponded to two target genes (TG) and *A. aegypti* S7 ribosomal protein gene (AaS7; GenBank **AY380336**). The total volume for each PCR was 10 µl, and each template cDNA reaction had four internal replicates to address pipetting error. The gene specific primers used in the real-time PCR are specified in chapter 2 (supplemental Table 2.5). The primers for AaS7 amplified a 107 base pair product and are forward 5'-

TCAGTGTACAAGAAGCTGACCGGA-3 and reverse 5'-TTCCGCGCGCGCTCACTTATTAGATT-3' (corresponds to amino acids S173-3'UTR).

Real-time PCR data for each gene was analyzed with the RotoGene 4.6 software (Corbett Research), and samples that did not display a product as defined by its melting curve were excluded from further analysis. Melting curve analysis confirmed the amplification of a single product in the real-time PCR. Transcript abundance of the TGs were normalized against that of AaS7 using an equation that takes both the critical threshold and PCR amplification efficiency into account (Liu and Saint, 2002):

$(Rn/AMP^{Ct}, TG) / (Rn/AMP^{Ct}, AaS7) = Ro_{TG}/ Ro_{AaS7}$

where Rn = the global threshold, AMP = amplification efficiency, Ct = the critical threshold, and Ro = initial reporter fluorescence (an indicator of the initial number of transcript copies in the sample).

2.4 Statistical analysis

Ecdysteroid production by isolated ovaries and the real-time PCR results for each gene were analyzed by Student's t-test, with the comparison made between the results obtained with the media alone and the media supplemented with pCTP-cAMP or bovine insulin (SAS JMP 5.0.1a, SAS Institute Inc., Cary, N.C.).

3. Results and Discussion

3.1 Ecdysteroid production by ovaries stimulated in vitro

Ovaries incubated with 100 μM pCPT-cAMP produced 42.1 ± 2.9 pg ecdysteroid / ovary pair/ 8h, which represented a 6 fold increase over media alone (6.9 ± 0.8 pg; see Figure A.1), and those incubated with 17 μM bovine insulin, 40.2 ± 2.5 pg ecdysteroid / ovary pair/ 8h, which represented a 4 fold increase over media + HCl (9.3 ± 0.9 pg). Ecdysteroid quantities produced

after stimulation by either agent were slightly higher than that reported for 8-bromo cAMP by Shapiro (1983) and bovine insulin by Riehle and Brown (1999). In the current study ecdysteroid production is reported per ovary pair, whereas Shapiro (1983) reports ecdysteroid production per ten ovary pairs and Riehle and Brown (1999) per four ovary pairs. Thus, the ecdysteroid production received in the current study is slightly above the two previous studies if ecdysteroid production reported in the previous studies is calculated on the bases of single ovary pairs. The minimal difference in ecdysteroid production between the current and previous studies is likely related to the use of different anti-ecdysteroid serums in RIA analysis among the three studies.

3.1 Real-time PCR analysis of transcript abundance following in vitro stimulation

The experimental results were obtained from six separate cohorts of females to increase the sample size in an attempt to receive a more accurate assessment through statistical analysis. Importantly, the transcript abundance and PCR amplification efficiency of AaS7 (represented as Ro_{AaS7}) did not change significantly between treatments, thus validating the use of its transcripts as a "normalizer" in real-time PCR analysis ($p \leq 0.05$; see Figure A.2).

Figure A.3 A-G display the results of the real-time PCR for *A. aegypti* diazepam-binding inhibitor (AedaeDBI), StAR-related lipid transfer domain containing protein (AedaeStart1), aldo/keto reductase (AedaeA/KR), adrenodoxin reductase (AedeaAR), 22-hydroxylase - CYP302a1 (AedeaCYP302a1), 2-hydroxylase - CYP315a1 (AedeaCYP315a1), and 20-hydroxylase - CYP314a1 (AedeaCYP314a1). None of the genes analyzed displayed a significant increase or decrease in transcript abundance following *in vitro* stimulation ($p \leq 0.05$). There were notable results, reduction of *AedaeDBI* transcript abundance in the 17 μM bovine insulin treatment ($p = 0.07$), whereas transcript abundance of *AedaeA/KR* increased with this treatment ($p = 0.13$).

In many statistical analysis, however, there were at least one and possible two normalized Ros that represented "outliers" in that they were well beyond the 95% confidence limits defined in the results of the student t-test. In every case, when the "outlier" normalized Ros were removed from analysis, the difference between the control and treatments lessened. Most importantly, the standard deviations of the control and treatment were more comparable. The results of the removal of such outliers are presented in graphs just below their associated graphs that comprise the analysis of all six cohort replicates. The modified analysis were done against the 100 μM pCPT-cAMP treatments of AedeaCYP302a1 (Figure A.3E) and AedaeCYP315a1 (Figure A.3F), and the 17 μM bovine insulin treatments of AedaeA/KR (Figure A.3C) and AedaeCYP302a1 (Figure A.3E).

With the lack of overwhelming evidence as to a correlation between ovarian ecdysteroid production and an increase in transcript abundance for any gene addressed in the current study suggests that the regulation of ovarian ecdysteroidogenesis in *A. aegypti* occurs at either the translational or post translational level. The observation that the transcripts of many of these genes analyzed in the current study do increase significantly in ovaries of blood-fed females (Chapter 2; Sieglaff et al., 2005), however, suggests that we are not mimicking the *in vivo* state within our *in vitro* assay. For example, transcript abundance of *AedaeCYP302a1* reached significant levels in ovaries at the onset of ovarian ecdysteroid production PBM (6 h PBM) (Sieglaff et al., 2005), and such a positive correlation between AedaeCYP302a1 transcript abundance and ovarian ecdysteroid production was not observed *in vitro*. Such a discrepancy between the *in vivo* and *in vitro* results requires further study, not to mention the need to study the impact of Ovary Ecdysteroidogenic Hormone I (the natural gonotrophic hormone) stimulated ovarian ecdysteroid production on the abundance of these gene transcripts. See chapter 2 for

discussion on transcript abundance following a blood meal and the roles that the proteins encoded by these gene transcripts may play in ovarian ecdysteroidogenesis.

4. References

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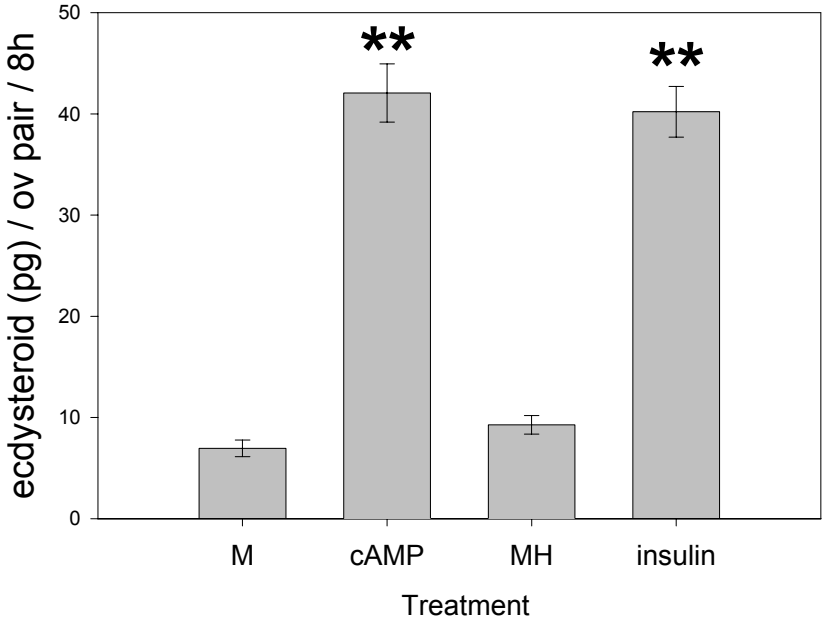


Figure A.1. Ecdysteroid production by ovary pairs incubated within buffered medium (M), M with 100 μM pCPT-cAMP (cAMP), M with HCl (MH), and M with 17 μM bovine insulin as assessed by RIA. Amounts are presented as ecdysteroid (pg) produced per ovary pair in 8 h (error bars = S.E.M.). The "**" denote high significance difference between control buffered medium and treatments (student t-test, $p \leq 0.001$).

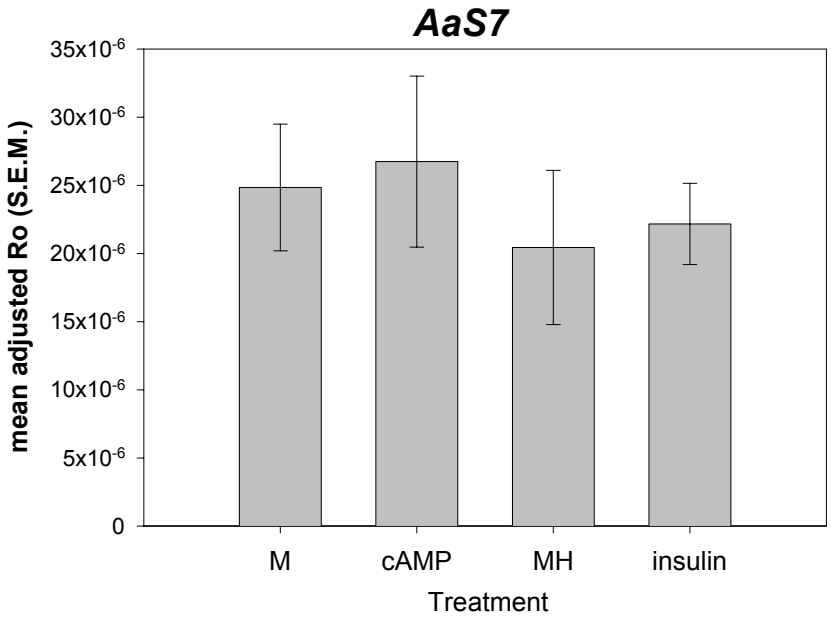
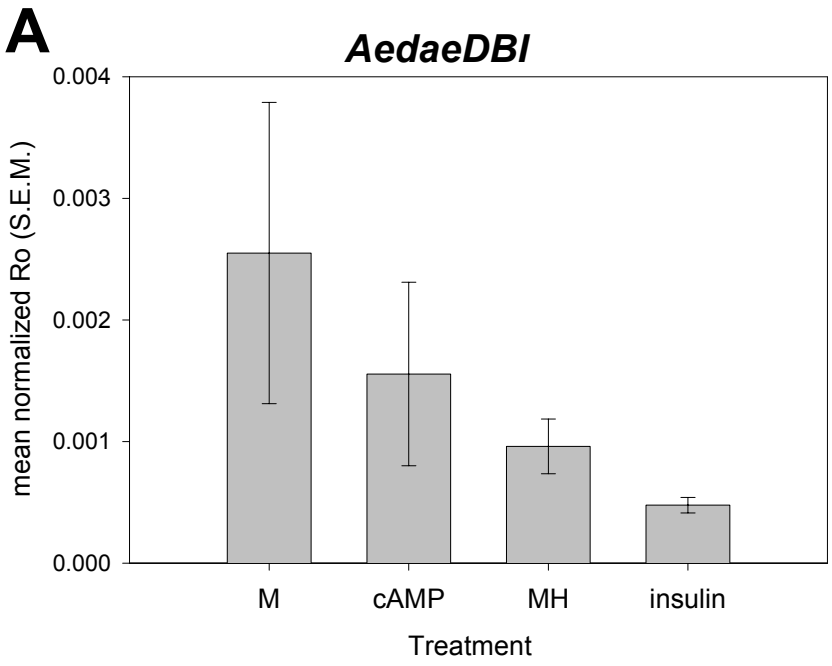
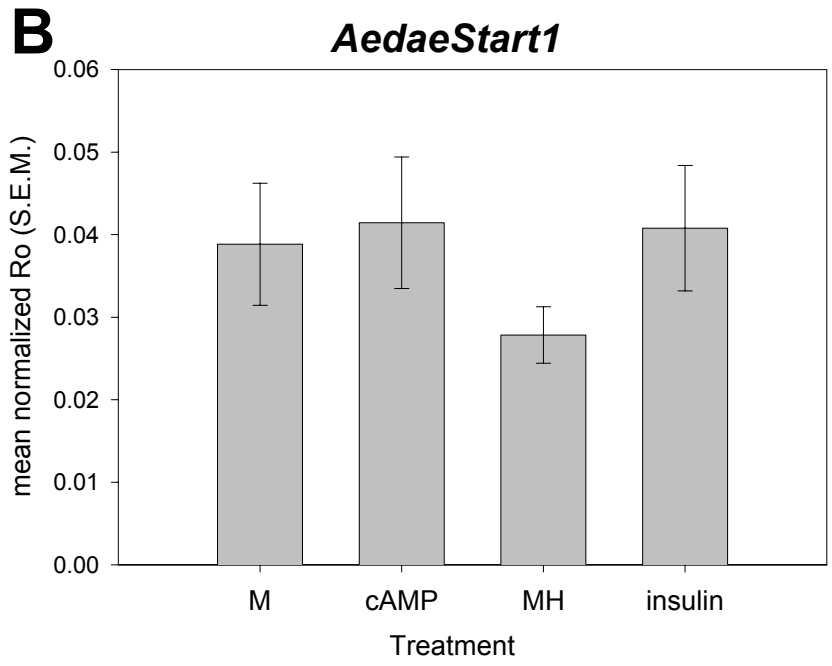
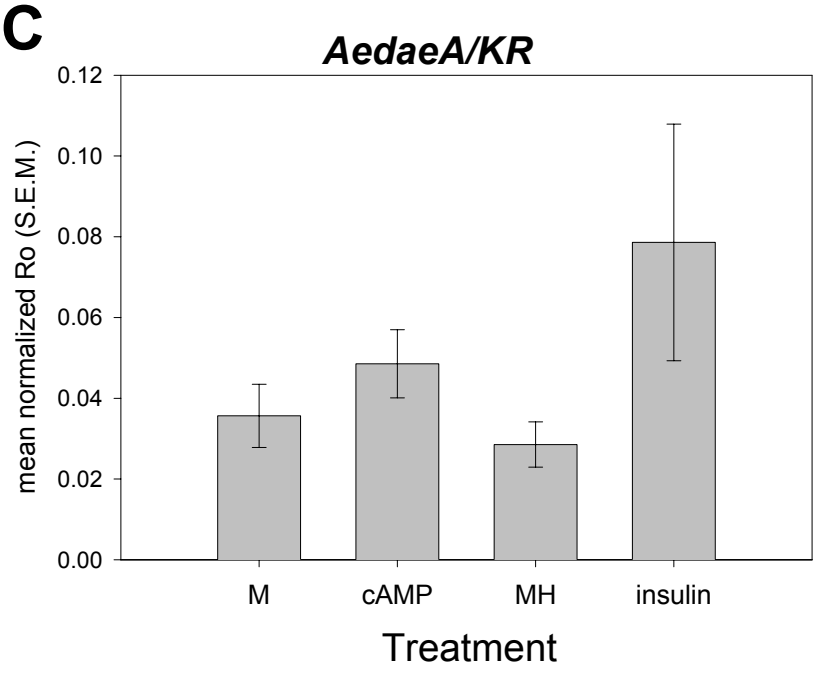


Figure A.2. Mean adjusted Ro of *A. aegypti* ribosomal protein S7 (*AaS7*). The adjusted Ro is a measure of the transcript abundance and PCR amplification efficiency of a given amplicon. The graph displays that neither pCPT-cAMP or bovine insulin had a significant effect on the adjusted Ro of *AaS7* (error bars = S.E.M.).


A
AedaeDBI
0.004
0.003
0.002
0.001
0.000
mean normalized Ro (S.E.M.)
M
cAMP
MH
insulin
Treatment


B
AedaeStart1
0.06
0.05
0.04
0.03
0.02
0.01
0.00
mean normalized Ro (S.E.M.)
M
cAMP
MH
insulin
Treatment

C


AedaeA/KR
mean normalized Ro (S.E.M.)
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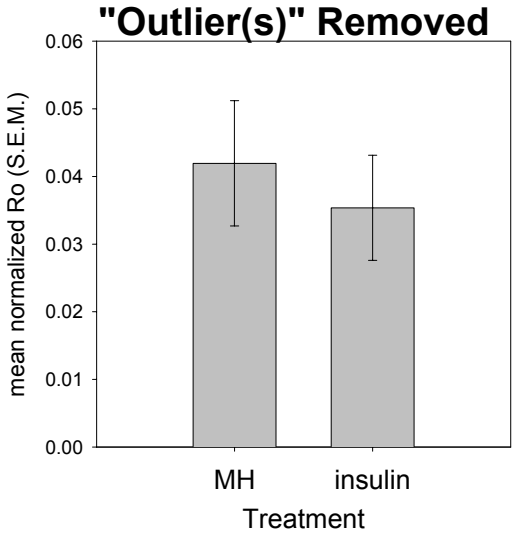
0.10
0.08
0.06
0.04
0.02
0.00
M
cAMP
MH
insulin
Treatment


"Outlier(s)" Removed
mean normalized Ro (S.E.M.)
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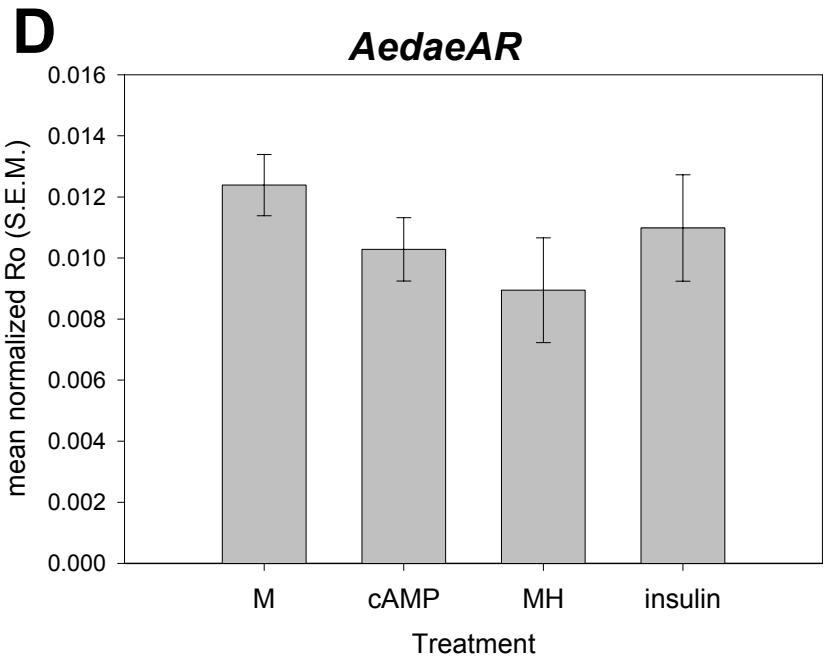
0.05
0.04
0.03
0.02
0.01
0.00
MH
insulin
Treatment


D
AedaeAR
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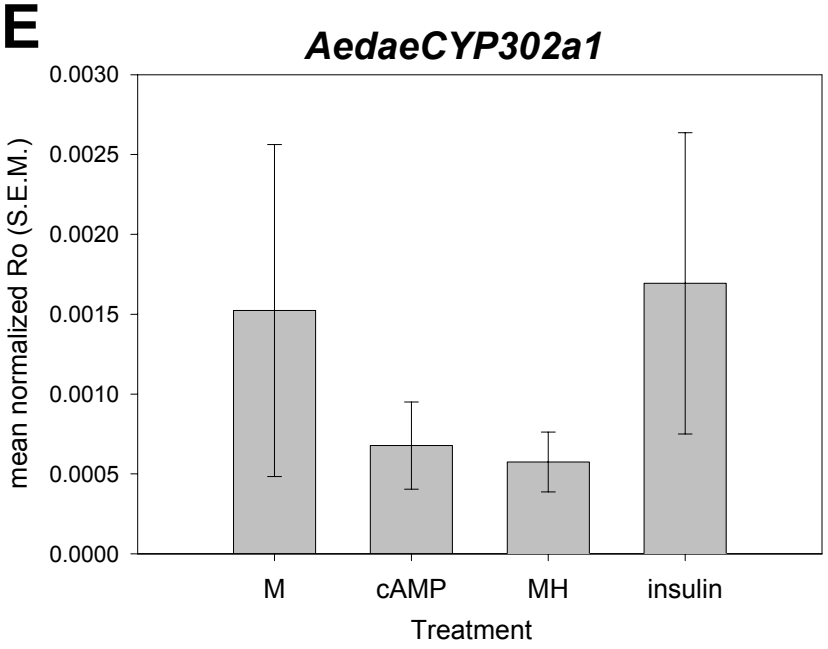
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
mean normalized Ro (S.E.M.)
M
cAMP
MH
insulin
Treatment

E


AedaeCYP302a1
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0.0010
0.0005
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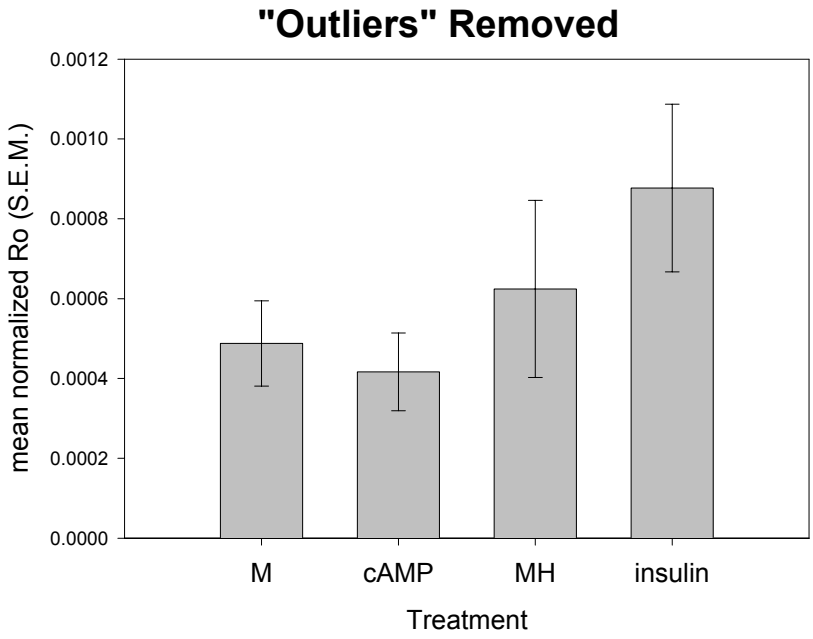
cAMP
MH
insulin
Treatment


"Outliers" Removed
mean normalized Ro (S.E.M.)
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0.0010
0.0008
0.0006
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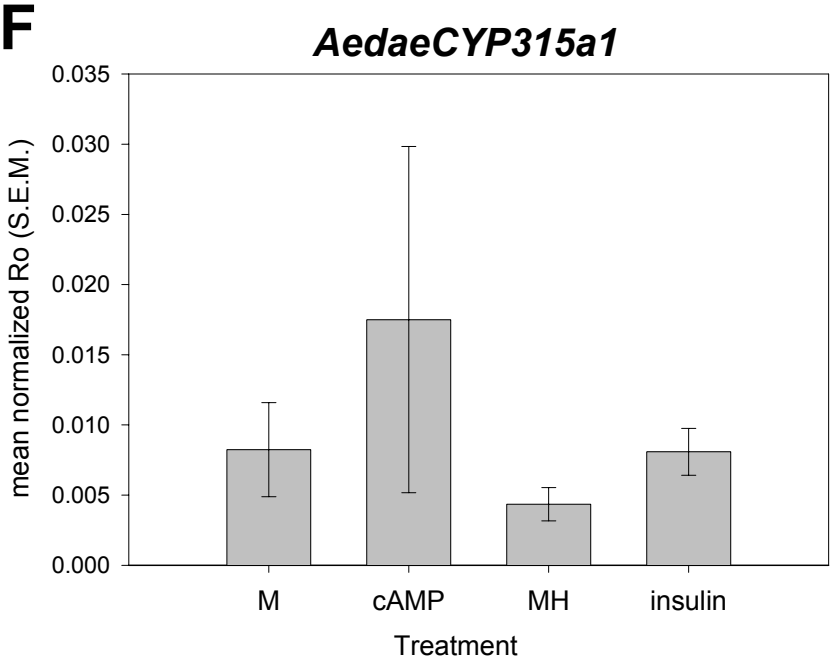
0.0002
0.0000
M
cAMP
MH
insulin
Treatment

F


AedaeCYP315a1
mean normalized Ro (S.E.M.)
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0.010
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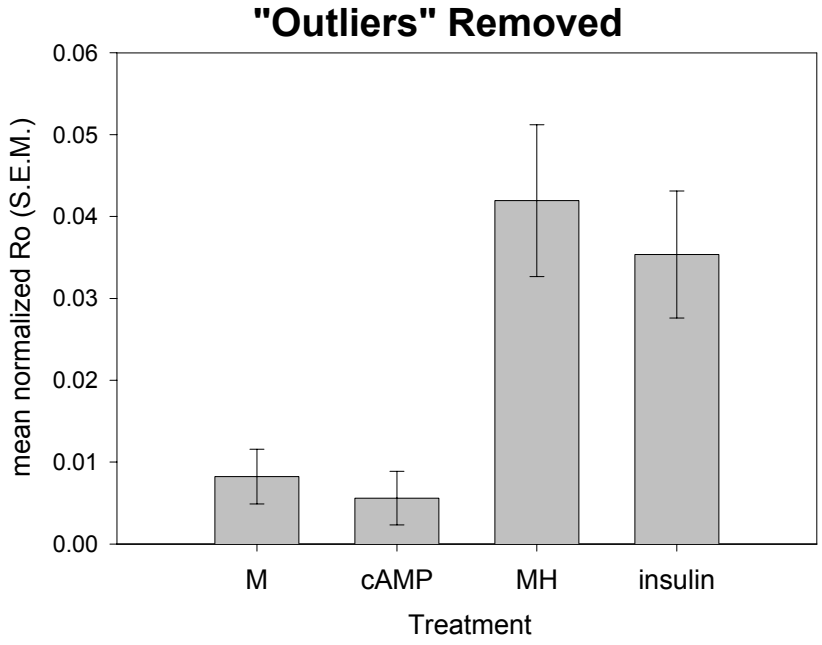
M
cAMP
MH
insulin
Treatment


"Outliers" Removed
mean normalized Ro (S.E.M.)
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0.05
0.04
0.03
0.02
0.01
0.00
M
cAMP
MH
insulin
Treatment

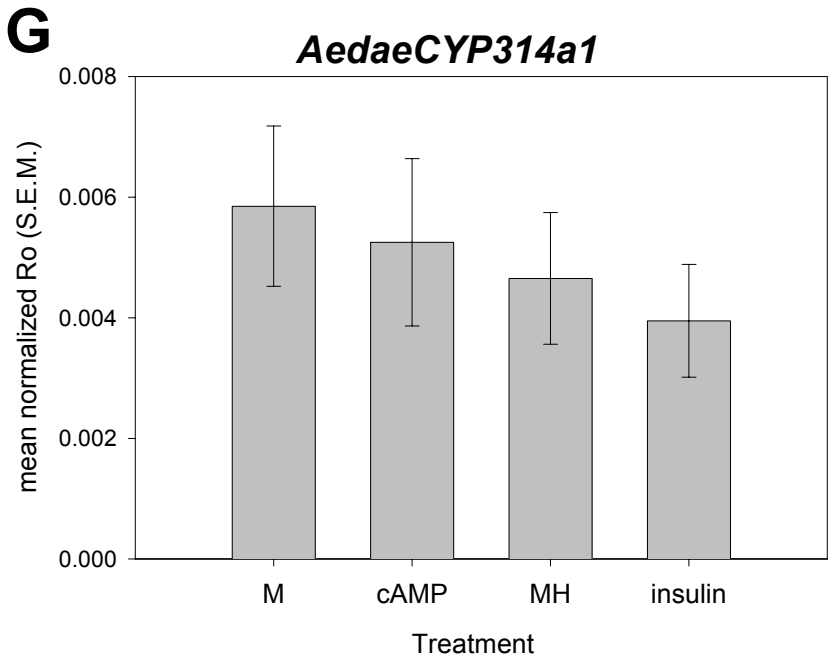

Figure A.3. Mean normalized Ro of (**A**) *AedaeDBI*, (**B**) *AedaeStart1*, (**C**) *AedaeA/KR* and when "outliers removed," (**D**) *AedaeAR*, (**E**) *AedaeCYP302a1* and when "outliers removed," (**F**) *AedaeCYP315a1* and when "outliers removed," and (**G**) *AedaeCYP314a1*. The adjusted Ro of each of the "Target Genes" were normalized against the adjusted Ro of *AaS7* of its respective replicate (error bars = S.E.M.).