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Expression of genes encoding proteins involved in ecdysteroidogenesis in the female mosquito, *Aedes aegypti*

Douglas H. Sieglaff, Kelli Adams Duncan, Mark R. Brown*

Department of Entomology, University of Georgia, Biological Sciences Bldg., Rm 413, Athens, GA 30602, USA

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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 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 transcript abundance 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.
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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

*Corresponding author. Tel.: +1 706 542 2317;
fax: +1 706 542 2279.
E-mail address: mbrown@bugs.ent.uga.edu (M.R. Brown).

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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, 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^{TM} (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^{TM} (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 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 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 Tables 2 and 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 Tables 2 and 3). Contigs for *CYP315a1* and *CYP314a1* were then formed as described above.

To verify the nucleotide sequence of the derived contigs, cDNAs spanning the open reading frames (ORF) for the genes were amplified from 18 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 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, 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; Perkin-Elmer, 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 s 65 °C for 20 s 72 °C for 20 s for 45 cycles, followed by melting curve analysis (see Supplemental Table 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, NC).

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. 1), AedaeA/KR (Fig. 3), AedaeAR (Fig. 4), AedaeCYP302a1 (22-hydroxylase; Fig. 5), AedaeCYP315a1 (2-hydroxylase; Fig. 6), and AedaeCYP314a1 (20-hydroxylase; Fig. 7). The protein sequence for AedaeStart1a and its relevant homologs (Fig. 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;

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AedaeDBI    1 MSLDQQFNEAAEKVKTFTKRPSDQELLELYALFKQASVGDNTTEKPGMFDLKGKAKWQAW
AnogaDBI    1 ~~MEQKFNESAEKVKSFTKRPSDAELLELYALFKQATVGDNETEKPGMFDLKGKAKWQAW
DromeDBI    1 ~MVSEQFNAAAEKVKSLTKRPSDDEFLQLYALFKQASVGDNDTAKPGLLDLKGKASWEAW
HumanDBI    1 ~MSQAEFDKAAEEVKRLKTQPTDEEMLFIYSHFKQATVGDVNTDRPGLLDLKGKAKWDSW

AedaeDBI   61 SDKKGISQDAAKEAYVKFVEELSAKCL~
AnogaDBI   59 ADRKGTSKEAAMEAYIKMVEELSAKYV~
DromeDBI   60 NKQKGKSSEAAQQEYITFVEGLVAKYA~
HumanDBI   60 NKLKGTSKESAMKTYVEKVDELKKKYGI
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Fig. 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.

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) (Figs. 1–7). 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. 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. 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. 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. 9A). Ecdysteroid production by control preparations of the last two abdominal segments and hindgut (without ovaries) from 18 h PBM females (Fig. 9B) was similar to that of other tissues (Fig. 9A) and ovaries from NBF females or females at 2, 6, 48, and 72 h PBM (Fig. 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. 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.

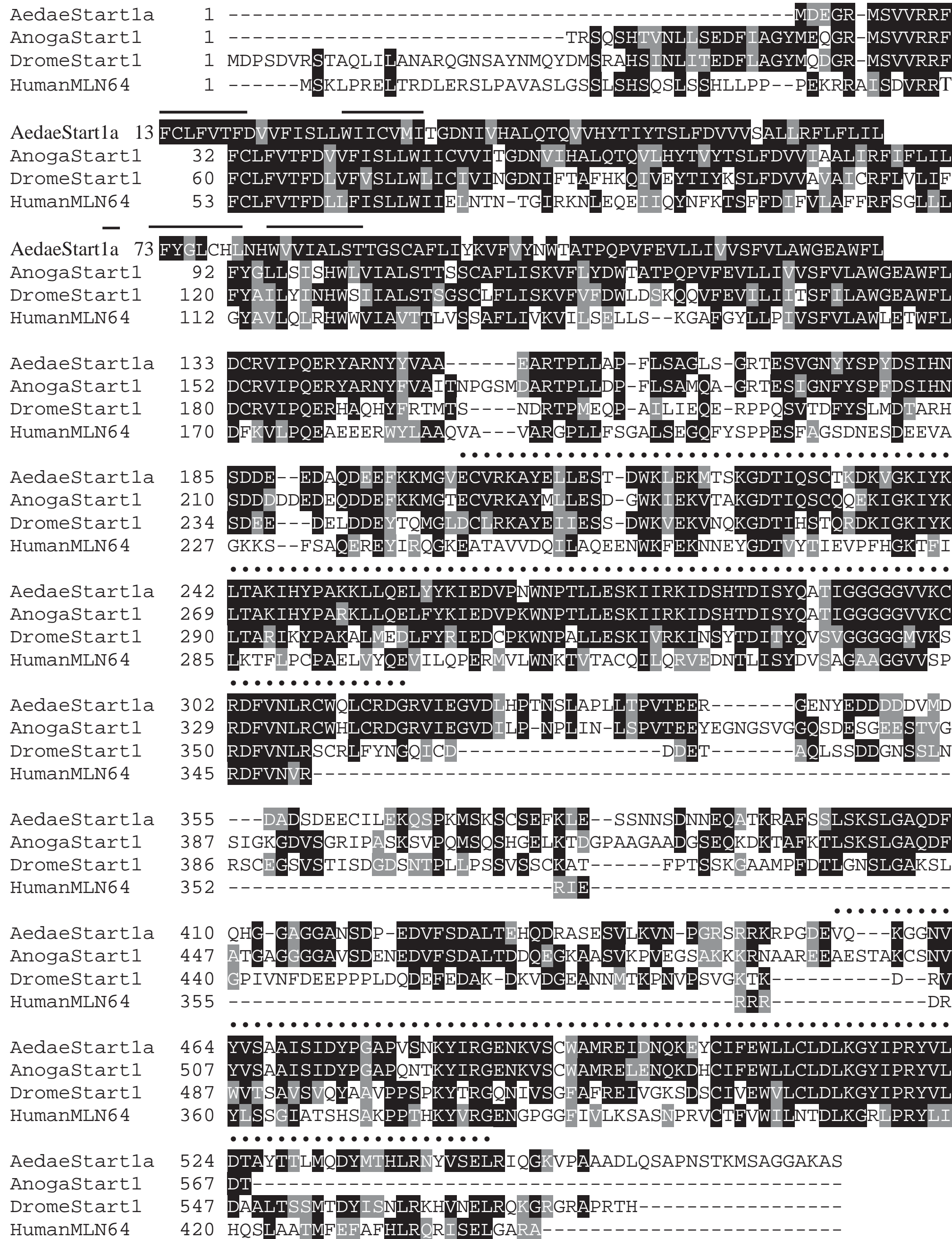

Fig. 2. Protein sequence of the *A. aegypti* StAR-related lipid transfer domain containing protein, AedaeStart1, translated from a 2049 bp cDNA (GenBank AY947545) with a single ORF of 574 amino acids and predicted to have a pI of 5.24 and a MW of 64680.4 Da. Sequence alignment of AedaeStart1a with the 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.

All seven genes displayed changes in transcript abundance in ovaries following a blood meal (Fig. 10). Only *AedaeCYP302a1* and *AedaeCYP315a1* had statistically greater transcript numbers at the peak of ecdysteroid production, 18 h PBM, with 9- and 12-fold increases, respectively (Fig. 10C), in comparison to those in NBF ovaries (Tukey-Kramer HSD $p \leqslant 0.05$). *AedaeStart1* (Fig. 10A) and *AedaeA/KR* (Fig. 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. 10B) and *AedaeDBI*, five-fold greater in 18 h PBM ovaries (Fig. 10A), than in NBF ovaries, but the differences were not statistically significant. There was a 262-fold

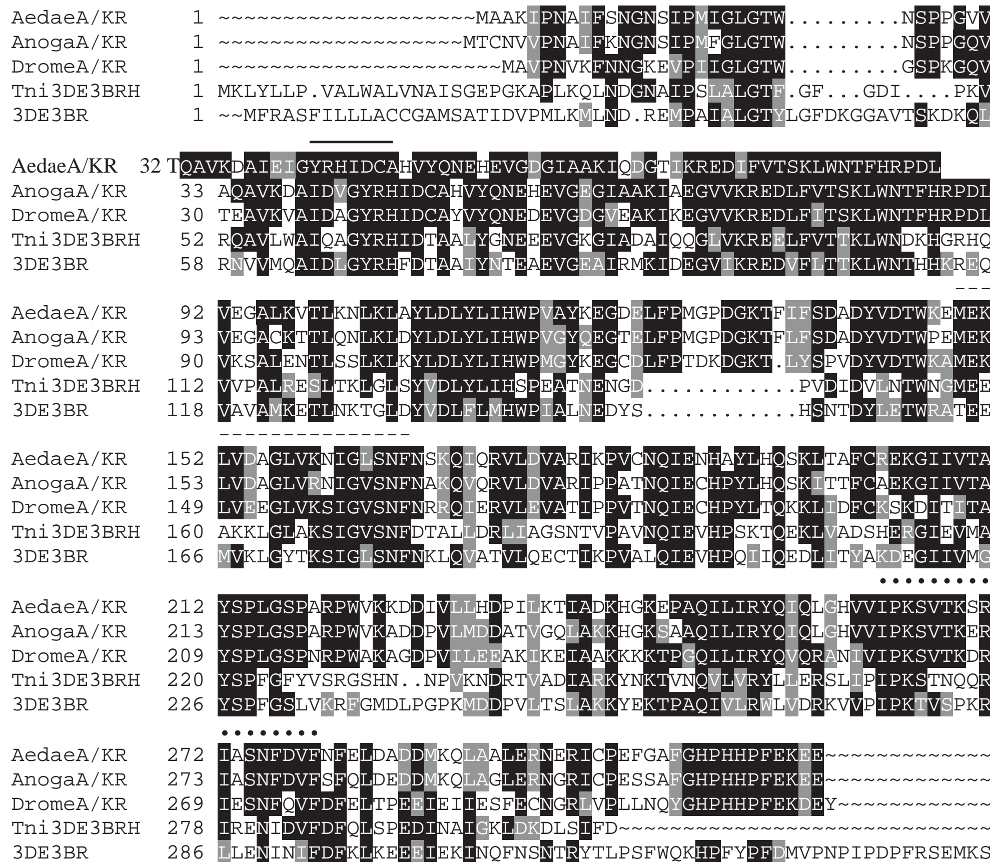

Fig. 3. Protein sequence of the *A. aegypti* aldo/keto reductase, AedaeA/KR, translated from a 1277 bp cDNA (GenBank AY947547) with a single ORF of 318 amino acids and predicted to have a pI of 6.4 and a MW of 35789.1 Da. Sequence alignment of AedaeA/KR with the dipteran homologues of *A. gambiae*, AnogaA/KR (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, Tni3DE3BRH (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].

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 $p \leqslant 0.05$), 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,

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AedaeAR    1 ~~~~~~~MWRNAGLLTVWQWGRQRVSRFASSSSGGSIPKRICIVGAGPAGFYAAQYILKH
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
HumanAR  360 IGYKSRPVDPSVPFDSKLGVIPNVEGRVM............DV.....PGLYCSGWVKRG

AedaeAR  403 PTGVILTTMNNSFGVADTICKDFNNNVIRVKEDKPG.......LDTNGKRVVSWEGWKLI
AnogaAR  402 PTGVILTTMNNSFGVADLVCRDFNSNTIRLNGSRPG.......LELAGRPYVSWNGWKAI
DromeAR  384 PTGVIVTTMNGAFAVAKTICDDINTNALDTSSVKPG.......YDADGKRVVTWDGWQRI
HumanAR  403 PTGVIATTMTDSFLTGQMLLQDLKAGLLP.SGPRPGYAAIQALLSSRGVRPVSFSDWEKL

AedaeAR  456 DAEECRRGETKGKPREKIVKIDEMLSIADGK~~
AnogaAR  455 DSEEVRLGQAQGKPREKLTRIETMLQIASNASD
DromeAR  437 NDFESAAGKAKGKPREKIVSIEEMLRVAGV~~~
HumanAR  462 DAEEVARGQGTGKPREKLVDPQEMLRLLGH~~~
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Fig. 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.

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.

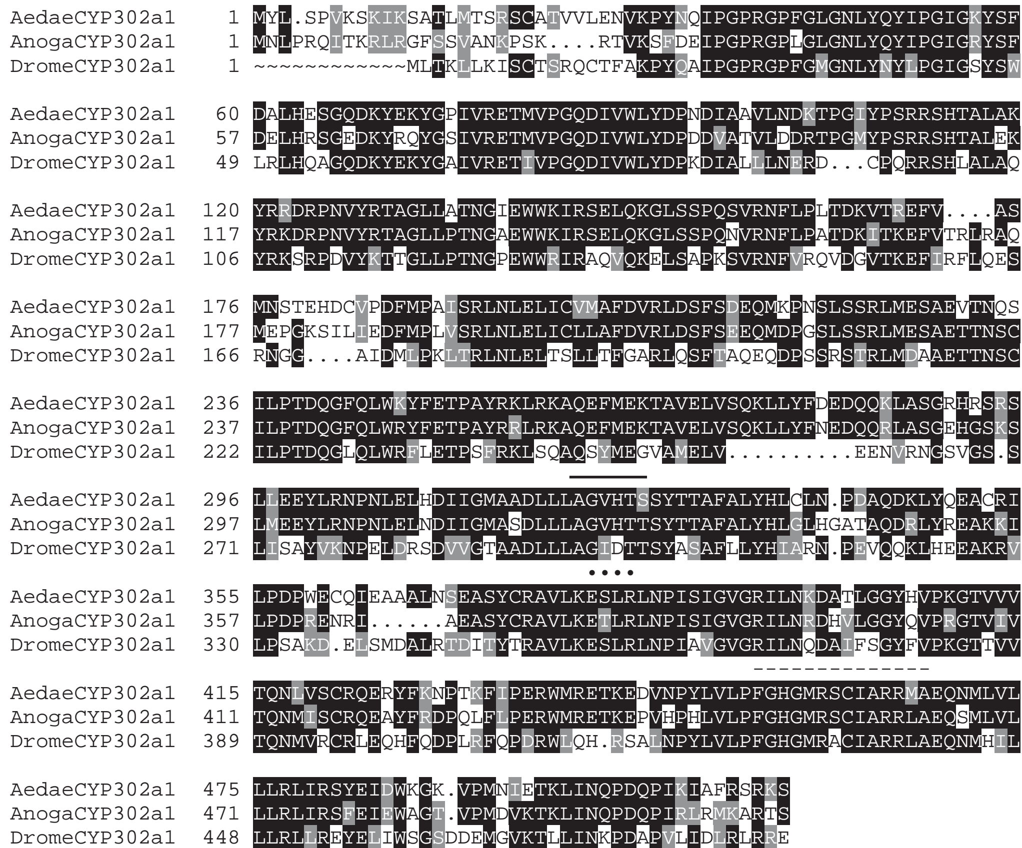

Fig. 5. Protein sequence of the *A. aegypti* 22-hydroxylase, AedaeCYP302a1, translated from a 1726 bp cDNA (GenBank AY947549) with a single ORF of 515 amino acids and predicted to have a pI of 8.9 and a MW of 58816.7 Da. Sequence alignment of AedaeCYP302a1 with the dipteran orthologues of *A. gambiae*, AnogaCYP302a1 (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).

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 usable 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, 1996; 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

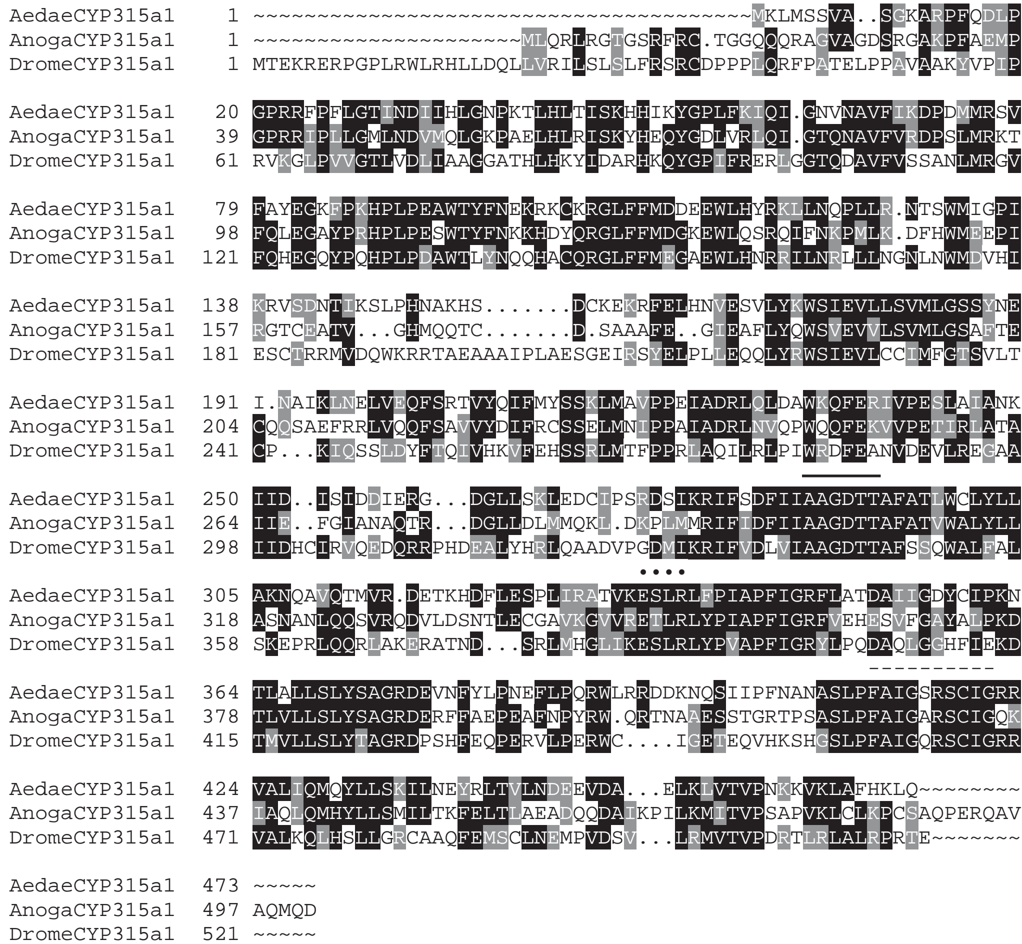

Fig. 6. Protein sequence of the *A. aegypti* 2-hydroxylase, AedaeCYP315a1, translated from a 1576 bp cDNA (GenBank AY947551) with a single ORF of 472 amino acids and predicted to have a pI of 9.17 and a MW of 54320.27 Da. Sequence alignment of AedaeCYP315a1 with the dipteran homologues of *A. gambiae*, 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."

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). 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).

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, 2002; Zhang et al., 2002; Roth et al., 2004). Given the high sequence identity and similarity between human MLN64 and the insect Start1 s (Fig. 2), it is likely that the insect Start1 s

Fig. 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 6.59 and 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."

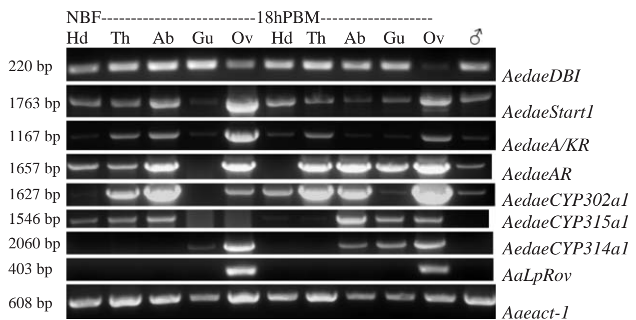

Fig. 8. RT-PCR analysis of the tissue distribution of the seven gene transcripts characterized in the current study along with tissue specific and loading controls, mosquito ovarian lipophorin receptor (AaL-pRov) and *A. aegypti* actin (Aaeact-1), respectively. Hd = head, Th = thorax, Ab = abdomen, Gu = gut, Ov ovary pair, ♂ = male *A. aegypti*.

are involved in the endosomal trafficking of cholesterol to acceptor membranes, such as the mitochondria, in the same way as MLN64 (Bose et al., 2000; 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 *AedaeStart1 s* represent two different genes, splice variants, or different alleles. In prior and later

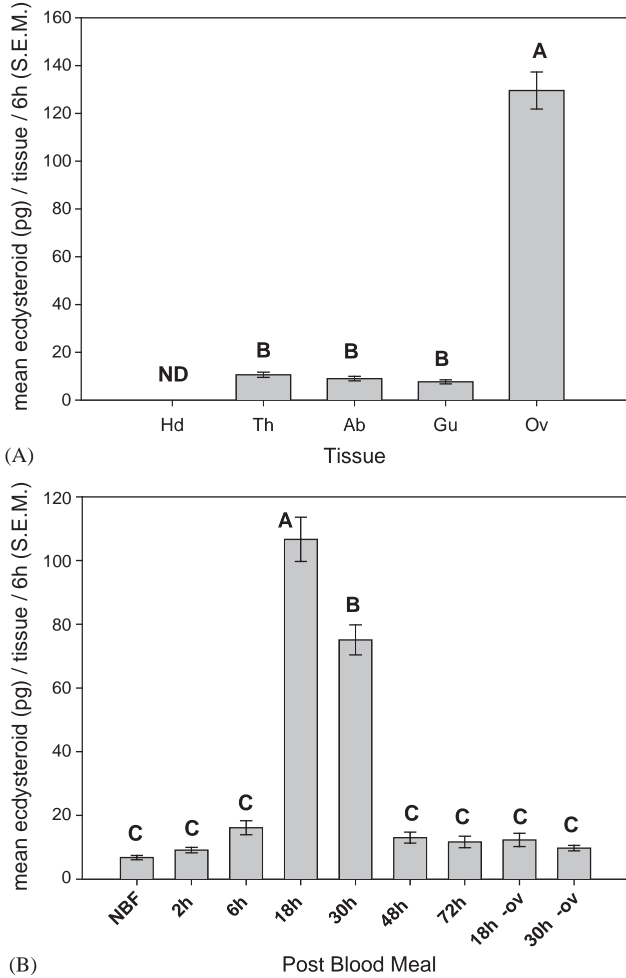

Fig. 9. (A) Ecdysteroid production by various tissue and body parts of *A. aegypti* females 18 h PBM as assessed by RIA. Hd = head, Th = thorax, Ab = abdomen, Gu = gut, and Ov = ovary pair. Amounts are presented as ecdysteroid (pg) produced by a single tissue equivalent in 6 h (error bars = S.E.M.), 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 6 h (error bars = S.E.M.). Bars with the same letter are not significantly different (Tukey-Kramer HSD, $p \leqslant 0.05$).

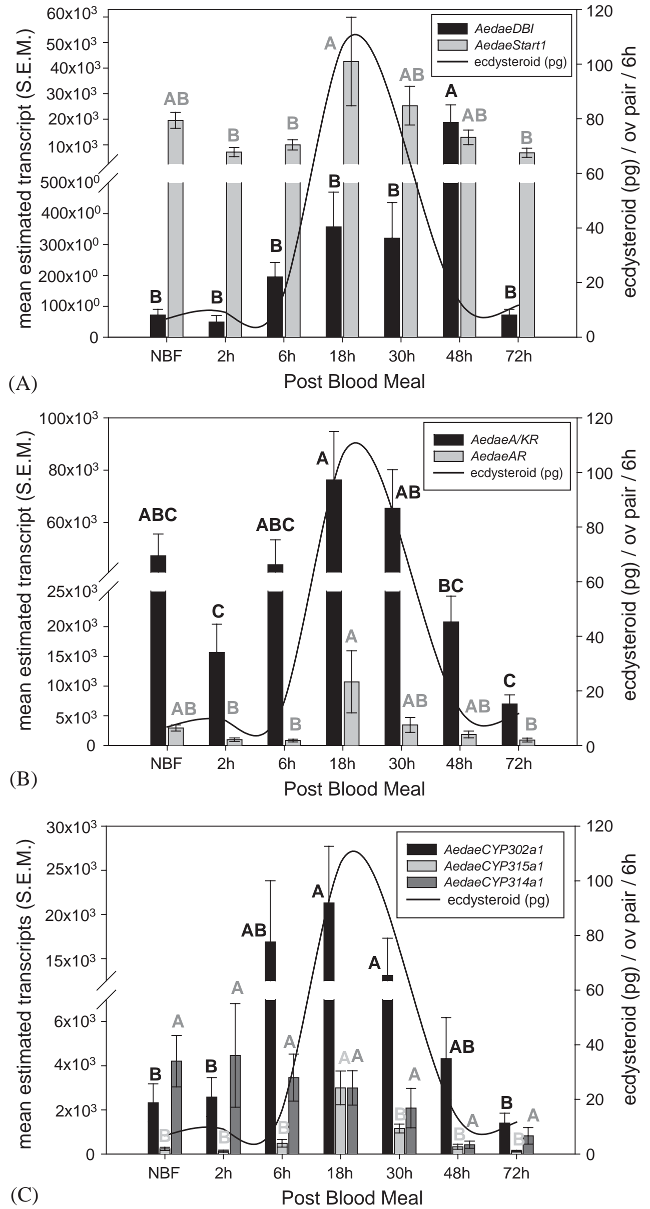

Fig. 10. Real-time PCR analysis of gene transcripts in ovaries in non-blood fed (NBF) and following a blood meal (PBM). The graphs display mean estimated transcript copies ± S.E.M per ovary pair for (A) *AedaeDBI* and *AedaeStart1*, (B) *AedaeA/KR* and *AedaeAR*, and (C) *AedaeCYP302a1*, *AedaeCYP315a1* and *AedaeCYP314a1*. Bars with the same letter are not significantly different (Tukey-Kramer HSD, $p \leqslant 0.05$). A smoothed line representing PBM ovary ecdysteroid production is superimposed over each graph for descriptive purposes (*note*: ecdysteroid measurements from current study).

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. 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, 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. 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, 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. 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. 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. 4), as shown for *D. melanogaster* AR (Freeman et al., 1999). *AedaeAR* transcript abundance was highest in 18 h PBM ovaries (Fig. 10B), the peak of in vitro ecdysteroid production. Gene transcripts for AedaeAR were observed in males and every tissue/body part of females (Fig. 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 ecdysteroiodogenesis.

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., 1996). 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 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. 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 4) but displayed different ECOR-I digestion patterns. The ORF of one clone encodes a protein with 515 amino acid residues (Fig. 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. 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. 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. 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. 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. 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, 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. 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., 1996), 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., 1996).

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; Rybczynski and Gilbert, 1995; Song and Gilbert, 1995, 1996, 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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Appendix A. Supplementary data

The online version of this article contains additional supplementary data. Please visit doi:10.1016/j.ibmb.2005.01.011

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