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Identity and transfer of male reproductive gland proteins of the dengue vector mosquito, *Aedes aegypti*: potential tools for control of female feeding and reproduction

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Abstract

Male reproductive gland proteins (mRGPs) impact the physiology and/or behavior of mated females in a broad range of organisms. We sought to identify mRGPs of the yellow fever mosquito, *Aedes aegypti*, the primary vector of dengue and yellow fever viruses. Earlier studies with *Ae. aegypti* demonstrated that “matrone” (a partially purified male reproductive accessory gland substance) or male accessory gland fluid injected into virgin female *Ae. aegypti* affect female sexual refractoriness, blood feeding and digestion, flight, ovarian development, and oviposition. Using bioinformatic comparisons to *Drosophila melanogaster* accessory gland proteins and mass spectrometry of proteins from *Ae. aegypti* male accessory glands and ejaculatory ducts (AG/ED) and female reproductive tracts, we identified 63 new putative *Ae. aegypti* mRGPs. Twenty-one of these proteins were found in the reproductive tract of mated females, but not of virgin females, suggesting that they are transferred from males to females during mating. Most of the putative mRGPs fall into the same protein classes as mRGPs in other organisms, although some appear to be evolving rapidly and lack identifiable homologs in *Culex pipiens*, *Anopheles gambiae*, and *D. melanogaster*. Our results identify candidate male-derived molecules that may have an important influence on behavior, survival and reproduction of female mosquitoes.

Keywords

Accessory gland proteins; Gene expression; Reproduction; Mass spectrometry; Sequence comparison

1. Introduction

The yellow fever mosquito, *Aedes aegypti*, is the most important vector of dengue and yellow fever viruses worldwide (Gubler, 1997; Gubler, 2002). An estimated 50 million people are

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afflicted with dengue each year and two thirds of the world's population is now at risk for infection (WHO, 2002). Dengue infections are increasing at high rates globally, with recent major outbreaks reported in Southeast Asia, Mexico, Puerto Rico, and Paraguay (ProMED, 2007b; ProMED, 2007a). Consequently, development of new tools and targets for intervention with the reproductive output of *Ae. aegypti* is of critical public health importance.

Previous studies suggest that proteins produced in the male reproductive accessory glands (mRGPs for male reproductive gland proteins) of *Ae. aegypti* and transferred to females during mating influence reproductive and feeding behavior (reviewed in Clements, 1999a; Klowden, 1999). Studies that have compared phenotypes of mated and unmated females or used direct injection of mosquito male accessory gland homogenate into unmated females have reported post-mating effects on female mosquito behavior including flight (Taylor and Jones, 1969; Jones and Gubbins, 1978; Jones and Gubbins, 1979; Jones, 1981; Chiba et al., 1990; Chiba and Shinkawa, 1992), response to host cues (Lavoipierre, 1958; Judson, 1967; Hartberg, 1971; Klowden and Lea, 1979), oviposition (Gillett, 1955; Leahy and Craig, 1965; Hiss and Fuchs, 1972; Ramalingam and Craig, 1976), fertility and ovarian development (Feyvogel et al., 1968; Klowden and Chambers, 1991; Klowden and Chambers, 1992; Klowden, 1993), blood digestion (Edman, 1970; Downe, 1975), and sexual refractoriness (Fuchs et al., 1968; Fuchs and Hiss, 1970). The stimulus for post-mating effects may not be the same for all mosquitoes (Klowden, 2006).

Post-mating changes in female behavior like those observed with *Ae. aegypti* have been attributed to the effect of specific mRGPs in another Diptera species, *Drosophila melanogaster* (reviewed in Wolfner, 2002; Gillott, 2003; Chapman and Davies, 2004; Wolfner et al., 2005; Wong et al., 2006; Ravi Ram and Wolfner, 2007). These proteins include: (1) simple peptides, including a “sex peptide” (Acp70A) that induces sexual refractoriness, increases the rate of egg production, and changes the feeding behavior and longevity of female *Drosophila* (Chapman et al., 2003; Liu and Kubli, 2003; Wigby and Chapman, 2005; Carvalho et al., 2006); (2) “prohormone-like” peptides, one of which (ovulin/Acp26Aa) stimulates ovulation (Heifetz et al., 2000); (3) large glycoproteins which are important for sperm storage (Neubaum and Wolfner, 1999) and other sperm-binding proteins; and (4) proteolysis regulators (Mueller et al., 2004; Ravi Ram et al., 2006; Mueller et al., 2007). Proteins synthesized in the male reproductive tract also have been found to play a role in regulating female post-mating physiology in a broad representation of other arthropods, including muscoid flies (Nelson et al., 1969), moths (Park et al., 1998), and ticks (Weiss and Kaufman, 2004).

Seminal fluid protein classes are generally conserved across a wide taxonomic range (Mueller et al., 2004; Braswell et al., 2006; Collins et al., 2006; Davies and Chapman, 2006) but several of the proteins themselves evolve more rapidly, on average, than other proteins (Clark et al., 1995; Civetta and Singh, 1999; Civetta and Clark, 2000; Swanson et al., 2001; Swanson and Vacquier, 2002; Clark and Swanson, 2005; Wagstaff and Begun, 2005; Clark et al., 2006; Andrés et al., 2006; Panhuis and Swanson, 2006; Haerty et al., In press). Therefore, it is necessary to identify mRGPs for each species individually. Early work on *Ae. aegypti* mRGPs identified a partially purified protein, named matrone (Craig, 1967), that had a variety of effects on female reproductive and feeding behavior (reviewed in Clements, 1999a; Klowden, 1999; Gillott, 2003). Further work identified a 7.6 kDa peptide that apparently reduced female host-seeking behavior (Lee and Klowden, 1999). These substances have never been fully characterized. In order to discover the specific mRGPs that affect female post-mating responses in *Ae. aegypti*, it is first necessary to identify the suite of mRGPs found in this species.

Here we describe a suite of proteins expressed in the male reproductive glands of *Ae. aegypti* mosquitoes. These proteins were found using bioinformatic and proteomic approaches. We find sequence and protein class similarities with mRGPs in other Diptera, such as *D.*

melanogaster; however, as reported for *Drosophila* male accessory gland proteins (Swanson et al., 2001; Swanson and Vacquier, 2002; Holloway and Begun, 2004; Mueller et al., 2005; Haerty et al., In press), these proteins may be rapidly evolving in *Ae. aegypti* despite their membership in conserved seminal protein classes. We show that *Ae. aegypti* mRGPs include several (21) that are found in the reproductive tracts of female *Ae. aegypti* after mating, but not in the reproductive tracts of virgin females. Many *Ae. aegypti* mRGPs have counterparts in *D. melanogaster* (including 16 previously identified as *D. melanogaster* male accessory gland proteins) and in the genomes of two other medically important mosquitoes, *Culex pipiens* and *Anopheles gambiae*. Identification and exploration of these molecules provides new insights into the mating biology of the yellow fever mosquito and potential new targets for vector population control through intervention in their reproductive output.

2. Methods

2.1 Mosquito rearing

Liverpool strain *Ae. aegypti* were maintained in colony in an incubator set at 27 °C, 70% RH and 14L:10D photoperiod. Males and females were held together in colony cages and offered a 20% sucrose solution. Mosquitoes were offered blood from a human arm (LCH) weekly in accordance with Institutional Review Board approval from Cornell University (FWA00004513). For our experiments, we separated pupae into individual 15 ml vials with 5–10 ml of water; vials were plugged with cotton. Emerged adults were sexed and aged in same-sex cages containing 20% sucrose solution prior to dissection.

2.2 Detection of *Ae. aegypti* homologs of *D. melanogaster* male accessory gland proteins using bioinformatics

As an initial method to identify *Ae. aegypti* male mRGPs, we used a bioinformatic approach. Even though primary sequences of reproductive proteins are often rapidly evolving (Swanson et al., 2001; Swanson and Vacquier, 2002; Holloway and Begun, 2004; Mueller et al., 2005; Andrés et al., 2006; Haerty et al., In press), the fact that some appear to be conserved (e.g., *Drosophila* sex peptide has activity in a moth (Fan et al., 1999; Nagalakshmi et al., 2004) made it important to begin with sequence comparisons to identify *Ae. aegypti* homologs. We compared the amino acid sequences of 112 predicted or known *D. melanogaster* accessory gland proteins (Acps; Ravi Ram and Wolfner, 2007) to translated *Ae. aegypti* transcripts (AaegL1.1 Gene build transcripts and supplemental transcripts) and genomic supercontigs using tblastn through the *Ae. aegypti* Vectorbase web site (<http://aaegypti.vectorbase.org/index.php>). Top hits from *Ae. aegypti* were then compared back to a predicted *D. melanogaster* gene database (tblastn to version 5.2 translated annotated genes; <http://flybase.bio.indiana.edu/>) to determine whether the original *D. melanogaster* Acp was the top hit. An *Ae. aegypti* gene was considered a homolog to a *D. melanogaster* Acp if the two were reciprocal best BLAST hits with e-values $< 1 \times 10^{-3}$ and had identities $\geq 30\%$. The *Ae. aegypti* proteins they encoded were examined for predicted secretion signal sequences using SignalP 3.0 (www.cbs.dtu.dk/services/SignalP/).

2.3 Protein extraction, separation, and identification

In order to avoid the possibility of rapidly-evolving genes being missed, we also identified *Ae. aegypti* mRGPs directly, by using mass spectrometry. Mass spectrometry was conducted on protein from male reproductive glands (i.e. accessory gland (AG) and ejaculatory duct (ED)) samples pooled from 20 virgin males, and on the lower reproductive tract (including bursa in seminalis and spermathecae) of 20 virgin or 20 mated females (all individuals were 7–10 days post-eclosion). Mated females were obtained by placing 2–5 virgin females in an 8-L cage with an excess of males (~40–60 males/cage). Cages were observed continuously for mating pairs. Pairs were allowed to mate for 10–20 sec, which represents the mean duration of mating

observed for this species (Spielman et al., 1967; Ackey and Jones, 1968; Ponlawat and Harrington, unpublished data). Pairs were then aspirated out of the cage and frozen at -20°C until dissection. AG/EDs of virgin males were dissected by first chilling the whole body on ice and then removing the legs. The AG/ED was removed from each male by carefully pulling the last abdominal segment away from the remainder of the body. Lower reproductive tracts from virgin or mated females were dissected out by carefully pulling the last abdominal segment away from the remainder of the body and then removing tissues surrounding the bursa and spermathecae. Reproductive tracts were dissected in MOPS buffer (80mM NaCl, 10mM KCl, 1 mM CaCl₂, 0.2 mM MgCl₂, 10 mM MOPS) and then placed in 40 µL 10% Dulbecco's PBS with protease inhibitors (Roche). Samples were centrifuged at ~11,000 × g for 30min at 4°C to separate soluble molecules from cells and tissue fragments. Supernatants (soluble fraction) were removed and placed in new tubes. The pellets were resuspended in 20 µL 10% DPBS with protease inhibitors (Roche) and 20 µL 2x SDS sample buffer (125mM Tris-HCl pH6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.001% Bromophenol blue) was added to the supernatant and pellet samples. Proteins were separated on a one-dimensional 5-15% gradient SDS-polyacrylamide gel and stained with SimplyBlue™ SafeStain (Invitrogen) for visualization of the bands (according to the manufacturer's instructions; Fig. 1). We submitted proteins from three independent male AG/ED supernatant samples, two independent mated female reproductive tract supernatant samples, and one virgin female reproductive tract supernatant sample for mass spectrometry analysis.

In-gel digestion, tryptic peptide extractions, and Nano-LC-MS/MS were conducted by the Cornell University Life Sciences Core Proteomics and Mass Spectrometry facility following the methods reported by (Morris et al., 2007). Briefly, protein bands were excised and subjected to in-gel digestion and tryptic peptide extraction. The nanoLC was carried out using an LC Packings Ultimate integrated capillary HPLC system equipped with a Switchos valve switching unit (Dionex, Sunnyvale, CA), which was connected in-line to a hybrid triple quadrupole linear ion trap mass spectrometer, 4000 Q Trap from ABI/MDS Sciex (Framingham, MA) equipped with Micro Ion Spray Head ion source. The MS/MS data generated from nanoLC/ESI-based IDA analysis were submitted for database searching using ProteinPilot™ software 1.0 (Applied Biosystems, Foster City, CA) with paragon algorithm against the *Ae. aegypti* predicted peptide (AaegL1.1 Gene Build) fasta databases (peptides and supplemental peptides, <http://aaegypti.vectorbase.org/index.php>). Allowances were made for one missed trypsin cleavage, the carbobamidomethyl modification of cysteine, and a methionine oxidation for Rapid Protein ID searching without additional modifications and amino acid substitutions. The protein identifications are based on total ProtScore >1.3 for each protein representing > 95% statistical significance. Using the primary sequences of the proteins identified in these searches, we tested for predicted secretion signal sequences using SignalP 3.0 (www.cbs.dtu.dk/services/SignalP/) and for predicted protein domains using SMART (<http://smart.embl-heidelberg.de/>).

2.4 RNA extraction and reverse transcription PCR (RT-PCR)

To determine the expression patterns of the genes we identified, reverse transcription PCR was conducted using RNA from male AG/ED (from ~40 males per sample), male whole body without AG/ED (15 males per sample), and male or female whole body samples (15 males or females per sample). All individuals used were 1-4 days post-eclosion. For female whole body samples, specimens were chilled on ice, their legs were removed, and bodies were ground in Trizol (Invitrogen). For samples from male *Ae. aegypti*, animals were chilled on ice, their legs were removed, and their bodies then dissected in 0.7% NaCl in 0.1% DEPC. AG/EDs were removed from bodies as described above in section 2.3 by removing surrounding exoskeleton and other tissue, and placed in Trizol on ice. Once AG/EDs from ~40 males had been collected,

they were ground for RNA extraction. The remainder of the body (including the testes) from each male was pooled and ground in Trizol for RNA extraction.

RNA was extracted from the ground tissue using Trizol (Invitrogen) following the manufacturer's instructions. Extracted RNA was treated with RNase-free DNase I (Roche) to remove DNA. 500 ng of RNA was used to synthesize first-strand cDNA using Superscript II RNase H Reverse Transcriptase (Invitrogen). PCR was conducted on the first-strand cDNA using Invitrogen Platinum PCR Supermix and 0.4 μ M gene-specific primers (Supplementary Table 1). To determine gene expression patterns, the PCR cycles used were: 94°C 2 min; 40 cycles of 94°C 30 sec, 55°C 30 sec, 72°C 45 sec; followed by 10 min at 72°C. PCR was conducted on two independent samples for each tissue type (labeled "1" and "2" in Fig. 2). A ribosomal protein gene (S17; Nene et al., 2007) was used as a control.

2.5 Identification of *D. melanogaster*, *Anopheles gambiae*, and *Culex pipiens* homologs of putative *Ae. aegypti* male mRGPs

We used the reciprocal BLAST approach with the criteria described in section 2.2 to identify *An. gambiae* (using AgamP3.3 gene build transcript database) and *Cx. pipiens* (using BROAD, JCVI, and Vectorbase CpipJ1.0_5 gene build CDSs databases) homologs to the putative *Ae. aegypti* mRGP genes identified through bioinformatics and *D. melanogaster*, *An. gambiae*, and *Cx. pipiens* homologs of the putative *Ae. aegypti* mRGP genes identified through mass spectrometry. Expression patterns for the homologs were investigated using transcript array data (available in online databases) for *D. melanogaster* (www.flyatlas.org; (Chintapalli et al., 2007) and *An. gambiae* (www.angaged.bio.uci.edu; (Dissanayake et al., 2006).

3. Results and discussion

3.1 Identification of putative *Ae. aegypti* mRGPs

Using bioinformatic comparisons ("B" in ID method, Table 1), we were able to identify *Ae. aegypti* homologs for 22 of the 112 putative or known *D. melanogaster* Acps. Sixteen of the 22 *Ae. aegypti* genes encoded proteins with a predicted secretion signal sequence (Table 1), suggesting that they can be secreted from cells and thus be available for transfer to the female during mating. Using nano-LC-MS/MS on proteins from male AG/ED supernatant samples ("P" in ID method, Table 1), we identified 53 distinct proteins having predicted signal sequences. Six of these 53 overlap with ones found using bioinformatics. Hereafter, we consider these 63 proteins (10 identified through bioinformatics, 47 identified through proteomics, 6 identified through both methods) as putative *Ae. aegypti* mRGPs.

The predicted molecular weight of the putative mRGPs (based on amino acid sequences) ranged from 9 to 114 kD. The observed molecular weights of the proteins identified through proteomics (based on mobility on a 1D-SDS polyacrylamide gel) matched closely with their predicted weights (Table 1), with two exceptions whose predicted sizes were much smaller than the sizes observed on the gel (Supp_Aedes004104 and Supp_Aedes009007; Table 1.). This discrepancy could result from a mis-annotation of these two genes, especially given the large intron size frequently seen in *Ae. aegypti* (Nene et al., 2007). Although we detected 15 and 2 proteins in the 30 and 60 kDa range, respectively, which are reported to be the size of the main protein bands in the partially-purified matrone (Fuchs et al., 1969), we cannot be certain that any correspond to matrone's active fraction since the active component is unknown.

3.2 Putative mRGPs are detectable in female reproductive tract after mating

Twenty-one (40%) of the 53 putative mRGPs were found in the mated female sample but not in the virgin female sample using nano-LC-MS/MS. Seven additional putative mRGPs were found in both the virgin and mated female samples. Although these latter 7 proteins are found

in the reproductive tract of virgin females, they all have predicted secretion signals so that the male may transfer them to the female during mating and therefore we still consider them putative mRGPs. Also, it is important to note that absence of a particular protein from a tissue sample does not necessarily mean that the protein is not present in the tissue; it could simply reflect protein levels that are too low to be detected given the sensitivity of our LC-MS/MS analyses.

3.3 Expression patterns of putative *Ae. aegypti* mRGPs

We examined expression patterns of genes encoding 34 putative *Ae. aegypti* mRGPs (5 identified through bioinformatics, 24 identified through proteomics, 5 identified through both methods). The genes identified through proteomics that we chose to examine through RT-PCR were those that encoded proteins found both in the male AG/ED and in the reproductive tract of mated females (and, in some cases, also in the reproductive tract of virgin females). All of the genes examined were expressed in the AG/ED (Table 1). Of the 34 genes we tested, 5 are expressed exclusively in the AG/ED, yet the proteins they encode were found in the mated female reproductive tract, providing strong evidence that these proteins are transferred to females during mating. Six show AG/ED-enriched expression, and two show male-enriched expression (Fig. 2). The remaining 23 are expressed at equal levels in all tissues tested. Although these genes are expressed in tissues other than the male reproductive glands, we still consider them putative mRGPs since they are expressed in the male reproductive glands and encode proteins with predicted signal sequences, suggesting that they may be transferred to females during mating.

3.4 *D. melanogaster*, *An. gambiae*, and *Cx. pipiens* homologs of putative *Ae. aegypti* mRGPs

Forty-seven (75%) of the 63 putative *Ae. aegypti* mRGPs have a *Cx. pipiens* homolog (Table 1). Forty (63%) of the 63 putative *Ae. aegypti* mRGPs have an *An. gambiae* homolog. Thirty-three of the 40 homologs are expressed in male *An. gambiae* (Table 1). Six of these 33 (in bold in Table 1) are among the putative *An. gambiae* mRGPs identified by Dottorini et al. (2007). Since a large percentage of the putative *Ae. aegypti* mRGPs have homologs in *Cx. pipiens* and *An. gambiae*, it is likely that future functional characterization of *Ae. aegypti* mRGPs may provide insights into the function of their homologs in other species of mosquito.

Thirty-eight (60%) of the 63 putative *Ae. aegypti* mRGPs have homologs in the *D. melanogaster* genome (these 38 genes include the 16 we identified through bioinformatics comparison with *D. melanogaster* Acps; Table 1). Thirty-two of the 38 *D. melanogaster* homologs are expressed in the *D. melanogaster* male reproductive accessory gland (www.flyatlas.org; (Chintapalli et al., 2007)). These 32 *D. melanogaster* genes include 7 that have not previously been reported as Acps but have upregulated expression in *D. melanogaster* male AGs relative to other tissues (in bold in Table 1). These sequence similarities to *D. melanogaster* genes may aid us in identifying *Ae. aegypti* mRGPs with functional importance in the future and may aid the *Drosophila* community in identifying new mRGPs.

Interestingly, the putative *Ae. aegypti* mRGP genes that have AG/ED-specific or male- or AG/ED-enriched expression (using RT-PCR) show evidence of evolving more rapidly than the mRGPs that are expressed in multiple tissues. For the mRGP genes with AG/ED-specific or male- or AG/ED-enriched expression, we found homologs for only 46% (6/13), 23% (3/13), and 23% (3/13) in *Cx. pipiens*, *An. gambiae*, and *D. melanogaster*, respectively. In contrast, for the mRGP genes expressed equally in all tissues, we found homologs for 90% (19/21), 90% (19/21), and 81% (17/21) in *Cx. pipiens*, *An. gambiae*, and *D. melanogaster*, respectively.

3.5 Characterization of putative *Ae. aegypti* mRGPs

3.5.A *Ae. aegypti* mRGP protein classes—One striking feature of male mRGPs is the conservation of protein classes across a wide range of organisms (Mueller et al., 2004; Clark and Swanson, 2005; Braswell et al., 2006; Collins et al., 2006; Davies and Chapman, 2006; Poiani, 2006; Dottorini et al., 2007). The 63 putative *Ae. aegypti* mRGPs we identified in this study (Table 1) are consistent with this general pattern. Like the mRGPs of other species, the suite of putative *Ae. aegypti* mRGPs we identified is rich in proteolysis regulators: 25% (16/63) are predicted proteases and 11% (7/63) are predicted protease inhibitors. Other protein classes that we identified in the putative *Ae. aegypti* mRGPs have previously been found in male mRGPs of other insects including: chaperones, CRISPs (cysteine-rich secreted proteins), defensins, lectins, lipases, oxidoreductases, RNAses (Mueller et al., 2004; Collins et al., 2006; Walker et al., 2006), and small peptides (Table 1).

In contrast to the conservation of mRGP protein classes, the sequences of many individual mRGPs have been reported to be rapidly evolving (Swanson et al., 2001; Holloway and Begun, 2004; Mueller et al., 2005; Haerty et al., In press). This pattern is also reflected in our suite of putative *Ae. aegypti* mRGPs: 12 (19%) of the 63 putative mRGPs lack homologs identifiable by BLAST in all three of the species that we tested (*Cx. pipiens*, *An. gambiae*, and *D. melanogaster*) and 8 (13%) contain no conserved protein domains (6 of these two sets of genes are overlapping; Table 1). This pattern is similar to that found within the *Drosophila* genus: in a comparison across 12 *Drosophila* species Haerty et al. (In press) found far fewer homologs of *D. melanogaster* mRGPs than of non-mRGPs.

3.5.B Proteolysis regulation—Proteolysis-regulating mRGPs are known or suggested to affect important biological processes including sperm motility (mammals: Robert et al., 1997), degradation of semenogelins (mammals: Robert et al., 1997; Malm et al., 2000), processing of proteins that regulate female post-mating reproductive changes such as ovulation and sperm storage (insects: Ravi Ram et al., 2006), and viability and defense against microbial infections (insects: Mueller et al., 2007). All seven protease inhibitors we identified include predicted serine protease inhibitor (serpin) domains. Two of these proteins include trypsin-inhibitor-like domains, one includes a pacifastin inhibitor domain (AAEL000551; Simonet et al., 2002), and one includes a Kazal-type serpin domain (Supp_Aedes004111; Friedrich et al., 1993). The predicted proteases we identified in *Ae. aegypti* fall into four classes: there are 7 predicted serine proteases, 7 metalloproteases, 1 aspartyl protease, and 1 cysteine protease.

One of the proteases we identified (Supp_Aedes002636) is a predicted angiotensin-converting enzyme (ACE). ACEs are found in the testes and male reproductive glands (as well as other tissues) of insects and mammals (Isaac et al., 1999; Leung and Sernia, 2003; Rylett et al., 2007) and have dramatic effects on the reproductive biology of both groups of organisms (Hagaman et al., 1998; Isaac et al., 1999; Ekbote et al., 2003; Kondoh et al., 2005). Of particular interest, the effect of male-derived ACEs on female fecundity has been investigated in the mosquito, *Anopheles stephensi*. Female *An. stephensi* mated to males fed ACE inhibitors laid less than 20% of the eggs laid by females mated to control males (Isaac et al., 1999). Several lines of evidence suggest that ACEs effect on insect reproduction is due, at least in part, to their role in peptide hormone processing (Isaac et al., 1999). Although the effect of inhibiting male-derived ACEs on female fecundity has not been investigated in *Ae. aegypti*, the results from *An. stephensi* suggest that Supp_Aedes002636 is a candidate to test for a role in the post-mating increase in female egg production observed in *Ae. aegypti* (Feyvogel et al., 1968; Klowden and Chambers, 1991; Klowden and Chambers, 1992; Klowden, 1993).

3.5.C Protein folding—Eight of the putative mRGPs are predicted chaperones or other proteins involved in protein folding (“chaperones” and “protein folding” in Table 1). In addition

to a potential protein-folding role, some of these proteins may have other roles of importance to female reproductive physiology. For example, AAEL000641, which is homologous to the *D. melanogaster* Acp CG6988 (Walker et al., 2006), is a predicted protein disulfide isomerase (PDI) containing a thioredoxin domain. Thioredoxins play a role in redox reactions (Arner and Holmgren, 2000) and thus the mRGP may protect sperm from oxidative damage, as proposed for a *D. melanogaster* Acp (Mueller et al., 2004).

3.5.D Sperm interaction—Six of the putative mRGPs are in predicted protein classes that are known or predicted to interact with sperm in other species. AAEL009239 is a predicted CRISP. Proteins in this class are found in the seminal fluids of mammals (Cohen et al., 2001) and *D. melanogaster* (Mueller et al., 2004) and in the egg jelly of the frog, *Xenopus laevis* (Olson et al., 2001). A *D. melanogaster* CRISP (CG17575) participates in regulating the release of sperm from storage (Ravi Ram and Wolfner, In press). In mammals, there is evidence suggesting that seminal fluid CRISPs are necessary for gamete fusion (Cohen et al., 2001). In *Xenopus*, a CRISP-like protein in the egg jelly acts as a sperm attractant (Olson et al., 2001). AAEL001121 and AAEL10596 are predicted lectins, a family of proteins which have been found to influence gamete interactions during fertilization (Romero et al., 1997) and regulate sperm binding to the female oviduct (Suarez et al., 1998) in mammals and mediate the release of sperm from female sperm storage organs in *D. melanogaster* (Ravi Ram and Wolfner, In press).

3.5.E Defense response and toxicity—The process of mating has the potential to introduce pathogens into the female reproductive tract that could adversely affect both female and male reproductive success. In *D. melanogaster*, several Acps have been found to have either direct antimicrobial activity or to modulate a female's ability to fight infection (Samakovlis et al., 1991; Lung et al., 2001; Mueller et al., 2007). Several putative *Ae. aegypti* mRGPs also appear likely to play a role in immune response. One (AAEL015458/15639/Supp_Aedes05593) is a predicted transferrin that is up-regulated in response to bacterial infection (Yoshiga et al., 1997). Transferrin is an iron-binding protein and may fight pathogens by depriving them of iron, as has been proposed for vertebrate lactoferrin (Kontoghiorghes and Weinberg, 1995). In the ovaries of female *Ae. aegypti*, transferrin is processed into smaller peptides, which have been proposed to be active in immune response (Harizanova et al., 2005).

AAEL009861 has a predicted defensin domain. Defensins are antimicrobial peptides that act against Gram-negative and Gram-positive bacteria, fungi, and viruses. Four other putative *Ae. aegypti* RGPs have homologs in other species or belong to protein classes that are predicted or known to be involved in defense responses against pathogens. The *An. gambiae* homologs of the two predicted *Ae. aegypti* hydrolases, AAEL001964 and AAEL002023, both are processed in response to bacterial challenges and may play a role in proliferation or differentiation of haemocyte cells after infection (Shi and Paskewitz, 2004). AAEL011758 and AAEL013279 are predicted cyclophilins. The involvement of cyclophilins in immune response has been documented in *D. melanogaster* (Guedes et al., 2005) and mammals (Arora et al., 2005; Barik, 2006) and has been proposed in *Xenopus laevis*, where a cyclophilin is found in skin secretions (Miele et al., 2003).

3.5.F Iron regulation and blood clotting—Since female *Ae. aegypti* require a blood meal for egg production (Clements, 1999b), and blood digestion is accelerated by mating (Downe, 1975), it is particularly interesting that three of the putative *Ae. aegypti* mRGPs are predicted to play a role in either iron regulation or blood clotting. In addition to its potential immunity-related function described in section 3.5.E, insect transferrin (AAEL015458/15639/Supp_Aedes05593) helps to regulate iron levels and to transport iron into developing oocytes (Nichol et al., 2002). Iron regulation is especially important in blood-feeding animals since

iron is consumed in large amounts and can be toxic when ingested in excess (Nichol et al., 2002). To our knowledge, transferrin has only been reported from the male reproductive tissues of one other insect, the tsetse fly (*Glossina morsitans morsitans*; (Strickler-Dinglasan et al., 2006), which also feeds on blood. Notably, the *D. melanogaster* homolog of *Ae. aegypti* transferrin is not expressed in male reproductive accessory glands (Table 1). It is thus plausible that in blood-feeding insects like *Ae. aegypti* and *G. morsitans morsitans* there has been selection for transfer of transferrin from males to females during mating, perhaps to assist in blood utilization to enhance egg production. Similarly, the two putative mRGPs with protein domains involved in clotting of vertebrate blood (AAEL001713: fibrinogen/fibronectin; AAEL008274: von Willebrand factor) may also aid in rapid coagulation of the blood in the female's gut into a bolus after ingestion (Lehane, 1991). We do not know if *Ae. aegypti* mRGPs enter the gut of the mated female, but in *D. melanogaster*, of the 16 mRGPs examined, 12 enter the hemolymph following mating (Monsma et al., 1990; Ravi Ram et al., 2005; Aigaki et al., 1991; Ravi Ram and Wolfner, unpublished data; Meikle et al., 1990; Lung and Wolfner, 1999; Coleman et al., 1995). Therefore, it is also possible that mRGPs transferred to female mosquitoes could play a role in partitioning or binding digestive products after they are absorbed through the midgut.

3.5.G Novel proteins—Eight of the putative *Ae. aegypti* mRGPs did not fall into any predicted protein class found in seminal fluid or tissues of other arthropods (“peptides” in Table 1). Six of these proteins had no homologs in any of the other three Diptera species (*Cx. pipiens*, *An. gambiae*, and *D. melanogaster*) investigated; one of the eight proteins had a homolog only in *Cx. pipiens* (the most closely related species); and one had homologs in all of the other three Diptera. The latter protein (Supp_Aedes002310) is similar in sequence to a *D. melanogaster* protein involved in olfactory learning (Dubnau et al., 2003). We have no functional information on the remaining seven proteins in this group, but these seven proteins are of particular interest because all four of the proteins in this group that we tested had either AG/ED-specific or AG/ED-enriched expression.

3.5.H Similarities to salivary gland and digestive tract proteins of blood-feeding arthropods—Surprisingly, the suite of putative *Ae. aegypti* mRGPs identified in this study resembles the suite of proteins found in the salivary glands and digestive tract of blood-feeding arthropods. For example, in *Ae. aegypti*, calreticulins, fibrinogen/fibronectins, serpins, phosphatidylethanolamine-binding proteins, odorant-binding proteins, alkaline phosphatases, lectins, and hydrolases are expressed in both the male AG/ED and the female salivary glands (Valenzuela et al., 2002; Ribeiro et al., 2007). Genes encoding proteins in these classes are also expressed in the salivary glands and digestive tracts of other blood-feeding arthropods (Jaworski et al., 1995; Ribeiro et al., 2004; Arca et al., 2005; Andersen et al., 2007).

Four proteins of particular interest in this group are AAEL008620, AAEL009239, Supp_Aedes004111, and Supp_Aedes002636. AAEL008620, a predicted odorant binding protein, shares highest sequence similarity with the *Ae. albopictus* gene, D7L2 (Arca et al., 2007), a member of the D7 family of proteins (long form). Prior to our study, members of this protein family have previously only been reported from the salivary glands of blood-feeding Diptera (Calvo et al., 2006), to our knowledge. In the salivary glands, D7 proteins are proposed to act as scavengers of biogenic amines to fight the defenses of their vertebrate hosts. Consistent with this hypothesis, an *Ae. aegypti* long form D7 protein and several short form *An. gambiae* D7 proteins bind serotonin, histamine, and norepinephrine *in vitro* (Calvo et al., 2006).

AAEL009239, a predicted CRISP (discussed in section 3.5.D), shares highest sequence similarity with a gene expressed in the salivary glands of female *Ae. albopictus* that is part of a family of proteins (Antigen-5) found in the salivary glands of several blood-feeding

arthropods and in the venom of vespid wasps (Arca et al., 2007). Supp_Aedes004111, a predicted Kazal-type serpin discussed above, shares high sequence similarity with proteins with anticoagulant properties found in the digestive tracts of blood-feeding insects (*Dipetalogaster maximus*: Mende et al., 1999; *Triatoma infestans*: Lovato et al., 2006). A predicted ACE (the same protein class as Supp_Aedes002636) has also been found in the salivary glands of the sand fly, *Lutzomyia longipalpis* (Valenzuela et al., 2004), although the function of this protein in the salivary glands has not been elucidated.

While the relationship between expression of these proteins in male reproductive tissues and those expressed in the salivary glands and digestive tracts of blood feeding arthropods is not entirely clear, these similarities suggest that the proteins identified in this study may play a role in blood meal digestion and, indirectly, in egg production. This topic and the potential for indirect male mosquito investment in their progeny warrant further investigation.

4. Conclusions

We have identified several male-derived reproductive proteins that are likely to be transferred to females during mating in *Ae. aegypti*. Most of these proteins fall into similar biochemical protein classes as male-derived reproductive proteins in other insects and salivary gland and digestive tract proteins in blood-feeding arthropods. However, two lines of evidence suggest that several *Ae. aegypti* mRGPs are rapidly evolving. First, some mRGPs within the aforementioned conserved classes lack homologs identifiable by BLAST in all three of the other Dipteran species that we tested (*Cx. pipiens*, *An. gambiae*, and *D. melanogaster*). Second, we identified several proteins that did not fall into any predicted protein class found in seminal fluid or tissues of other arthropods. Interestingly, the genes we identified as having AG/ED-specific or male- or AG/ED-enriched expression show a pattern consistent with more rapid evolution than those we identified as more ubiquitously expressed, a pattern also found in *Drosophila* (Haerty et al., In press).

Even though male mosquitoes do not ingest blood and thus are not likely to play a direct role in the transmission of pathogens, studies of *Ae. aegypti* and other Diptera suggest that their mRGPs are likely to have an important influence on female behavior, physiology, survival and reproduction thus playing a crucial role in disease vector biology. Further investigation of mosquito mRGPs identified in this study, and in a recent study of *An. gambiae* (Dottorini et al., 2007), may provide targets for controlling the reproductive output of these important disease vectors. Furthermore, promoters of genes expressed exclusively in the male reproductive accessory glands can be used to drive expression of a number of useful constructs. For example, one could use the promoter and protein sequence of such a gene to engineer a secretable GFP-fusion protein in the male reproductive accessory glands (as has been done in *D. melanogaster*; Lung and Wolfner, 1999) and use it as a marker to track mating patterns in field cages. More broad comparative studies of male mRGPs across organisms may also reveal the evolutionary patterns that shape and influence post-copulatory effects in mating biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Proteins from male accessory glands and ejaculatory ducts and virgin and mated female bursae inseminalis and spermathecae. Boxes indicate protein bands that are present in the male and mated female samples, but absent or lighter in the virgin female sample. Samples were acquired by centrifuging tissues of 20 individuals of each class for 30 minutes and then subjecting the entire supernatant to electrophoresis on a 5-15% SDS-polyacrylamide gel.

Figure 2.

Examples of reverse transcription PCR of *Aedes aegypti* genes with accessory gland/ejaculatory duct (AG/ED) specific, male enriched, and multi-tissue expression. PCR was conducted on two independent samples for each tissue type (labeled “1” and “2”). RNA levels of the ribosomal protein gene S17 were used to equalize amounts of template cDNA used. Tissue of expression is categorized as “AG/ED” (expressed exclusively in the male accessory gland/ejaculatory duct (AG/ED)); “Multiple; Male enriched (expressed in all tested tissues, but at a higher level in the male tissues); “Multiple” (expressed approximately equally in all tested tissues).

Table 1

Putative *Ae. aegypti* male reproductive gland proteins.

Predicted protein class	Gene ^a	ID method ^b	Protein in female RT ^c	Tissue of expression ^d	<i>Cx. Pipiens</i> homolog ^e	<i>An. gambiae</i> homolog/exp ^f	<i>D. melanogaster</i> homolog/exp ^g	Predicted size (kD)	Size on gel (kD)
Alkaline Phosphatase	00931	B	N	Multiple	N	10596/Y	CG1462/Y	61.6	NA
Aminotransferase	09872	P	N	NT	B13237	00901/Y	CG1640/Y	59.5	50-55
ATPase	07777	P	Y	Multiple	J2523.m000285	03879/Y	CG8029/Y	43.4	16-36
Calcium-binding	14271/Supp011593	B	N	NT	B09053	04631/Y	CG17271/Y	27	NA
Chaperone	01005/11773	B/P	Y	Multiple	B06878	04212 /Y	CG9429/Y	46.7	50-55
	12827	B/P	N	Multiple	B02228	01424 /Y	CG5520/Y	91.1	
	Supp05941/Supp05917	B/P	Y	Multiple	B03317	04192/Y	CG4147/Y	72.3	64-90
CRISP	09239	P	Y	Multiple; AG/ED enriched	N	N	N	29.2	16-36
Defensin	09861	B/P	N	NT	V10860	07049 /Y	CG10433/Y	14.3	6-22
Fibrinogen/Fibronectin	01713	P	N	NT	B05964	11307/Y	N	26.2	16-36
Gelsolin precursor	06096/06102	P	Y	Multiple	B04312	N	N	43	40-50
Hydrolase	01965	P	N	NT	B02428	08061/Y	N	48.7	50-55
	02023	P	Y*	Multiple	B02429	08060/Y	CG1780/Y	48	40-50
Kinase	12359	P	Y	Multiple	V11116	07120/Y	CG2210 /Y	18.5	6-22
Lectin	01121	B	N	Multiple	V10242	12256/Y	CG30463/Y	73.3	NA
	10596	B	N	Multiple	B01557	04375/Y	CG10000/Y	65.1	NA
Lipase	07063	P	N	NT	B03950	11683/N	N	35.3	16-36
	12311/05815	P	N	NT	B14868	05103/Y	CG5162 /Y	41.2	40-50

Predicted protein class	Gene ^a	ID method ^b	Protein in female RT ^c	Tissue of expression ^d	Cx. <i>Pipiens</i> homolog ^e	<i>An. gambiae</i> homolog/exp ^f	<i>D. melanogaster</i> homolog/exp ^g	Predicted size (kD)	Size on gel (kD)
Odorant binding	05772	P	N	NT	B16102	00278/Y	CG18111/N	15.9	6-25
	08620	P	N	NT	N	06278/Y	N	35.2	24-40
Peptidase	04240/11823	B		NT	B16380	N	CG6461/Y	62.4	NA
Peptide	10824	P	Y	AG/ED	B15151	N	N	24.4	6-25
	Supp00077/Supp10999	P	N	NT	N	N	N	30.1	40-50
	Supp02310	B	N	NT	VSNAP18197	10727/N	CG16707/Y	18.8	NA
	Supp03543	P	Y	Multiple; AG/ED enriched	N	N	N	20.8	16-36
	Supp04095	P	N	NT	N	N	N	34.7	40-50
	Supp04104	P	Y	AG/ED	N	N	N	33	100-148
	Supp04124	P	Y	Multiple; AG/ED enriched	N	N	N	15.9	6-25
Phosphatidylethanolamine-binding protein	Supp09007/Supp12267/Supp07647/Supp10064/Supp08422/Supp05282/Supp07551/Supp14289/Supp07636	P	N	NT	N	N	N	11.9	50-55
	11263	P	Y	Multiple; Male enriched	B08056	02049/Y	N	23.4	16-30
Protease	02000	P	Y	AG/ED	B02422	N	N	47.9	40-50
	02610	P	N	NT	N	N	N	48.3	55-64
	02833	P	N	NT	B06044	N	CG6692 /Y	38	25-40
	05269	P	N	NT	B11067	06099/Y	CG4169/Y	45.6	40-50
	05521	P	Y	Multiple	B12340	00994/N	CG2493/Mixed (2 of 4)	53.2	48-55
	06169	P	N	NT	B15894	03277/Y	CG1548/Y	41.8	25-40
	06323	P	N	NT	N	N	N	97	90-100
	06403	P	Y	Multiple; Male enriched	N	N	N	27.3	16-36
	06414	P	Y	Multiple; AG/ED enriched	N	N	N	27.9	16-36
	07201	P	N	NT	B10309	03077/N	CG8773/N	113.8	100-140
	09291	P	N	NT	B08009	07505/Y	CG4572/Y	54.5	50-55
	11558/13449	P	Y	AG/ED	N	N	CG15253/N	29.4	16-30
	12217	P	Y*	Multiple	B13591	00885/N	CG11956/N	109	100-140
	14005/06576	P	N	NT	N	N	N	33.5	40-50

Predicted protein class	Gene ^a	ID method ^b	Protein in female RT ^c	Tissue of expression ^d	Cx. <i>Pipiens</i> homolog ^e	<i>An. gambiae</i> homolog/exp ^f	<i>D. melanogaster</i> homolog/exp ^g	Predicted size (kD)	Size on gel (kD)
Protease Inhibitor	15110/15386	P	Y	Multiple	B05153	08176/N	CG11034/Y	84.3	64-98
	Supp02636/Supp02632	P	Y*	Multiple; AG/ED enriched	B08491	09754/N	CG8827/Y	74.5	64-90
	00333	B	N	NT	N	06582/Y	CG33259/Y	9.2	NA
	00369/00317	B	N	NT	V2504	N	CG1262/Y	9.1	NA
	00551	P	N	NT	B10197	11319/Y	N	37.5	16-36
	02715	P	Y	AG/ED	B06549	N	N	42.7	40-50
	02720	P	Y	Multiple	B06547	N	N	43.8	40-50
	08364	P	Y	Multiple	B10931	03139/Y	CG18525/Y	48	40-50
	Supp04111	P	N	NT	B09777	N	N	26.1	25-40
Protein folding	00641	B/P	Y*	Multiple; AG/ED enriched	B04885	12407/Y	CG6988/Y	56	50-55
	01432	P	Y	Multiple	B14716	07393/Y	CG8983/Y	55.2	50-55
	04313	B		Multiple	V7794	08822/Y	CG9847/Y	23.2	
	11758	P	Y*	Multiple	N	00462/Y	CG9916/Y	22.3	6-25
	13279	B/P	Y*	Multiple	B04666	07088/Y	CG2852/Y	21.4	6-25
Reductase	04873	P	N	NT	B08103	04551/Y	CG9796/Y	26.3	25-40
	10065	P	N	NT	B06823	01919/Y	CG5809/Y	47.7	50-55
Rnase	01159	B	N	Multiple	V6938	09842/Y	CG8194/Y	37.1	NA
Transferrin	15458/15639/Supp05593	P	Y*	Multiple	B14626	00376/Y	CG6186/N	70.6	55-64
Von Willebrand factor	08274	P	N	NT	J4820.m000018	N	N	31.5	40-50

^aGene identification numbers are from Vectorbase and are presented without the proceeding “AAEL0”. Multiple entries separated by a “/” indicate genes with high sequence similarity.

^bB: Identified through bioinformatics comparison with *D. melanogaster* accessory gland proteins; P: Identified through proteomics.

^cRT: Reproductive tract; Y: Found in RT of mated females; Y*: Found in RT of both virgin and mated females.

^dMultiple: Expressed in female whole body, male whole body without accessory glands/ejaculatory duct, and male accessory glands/ejaculatory duct. NT: Not tested

^eGene identification numbers are from Vectorbase. B: JHB Strain BROAD CDSs; J: JHB Strain JCVI CDSs; V: JHB Strain VectorBase CDSs.

^fGene identification numbers are from Vectorbase and are presented without the preceding “AGAP0”. Y: Gene is expressed in males; N: Gene is not expressed in males; Genes in bold were also found in a recent study of *An. gambiae* mRCPs (Dottorini et al.2007).

^gY: Gene is expressed in male reproductive accessory glands; N: Gene is not expressed in male reproductive accessory glands. Genes in bold have upregulated expression in the AG relative to other tissue, but have not been previously identified as *Drosophila melanogaster* Acps; the proteins encoded by these genes all had predicted secretion signal sequences except CG99J6.