

A genome-wide analysis in *Anopheles gambiae* mosquitoes reveals 46 male accessory gland genes, possible modulators of female behavior

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The male accessory glands (MAGs) of many insect species produce and secrete a number of reproductive proteins collectively named Acps. These proteins, many of which are rapidly evolving, are essential for male fertility and represent formidable modulators of female postmating behavior. Upon copulation, the transfer of Acps has been shown in *Drosophila* and other insects to trigger profound physiological and behavioral changes in females, including enhanced ovulation/oviposition and reduced mating receptivity. In *Anopheles gambiae* mosquitoes, the principal vectors of human malaria, experimental evidence clearly demonstrates a key role of MAG products in inducing female responses. However, no Acp has been experimentally identified to date in this or in any other mosquito species. In this study we report on the identification of 46 MAG genes from *An. gambiae*, 25 of which are male reproductive tract-specific. This was achieved through a combination of bioinformatics searches and manual annotation confirmed by transcriptional profiling. Among these genes are the homologues of 40% of the *Drosophila* Acps analyzed, including Acp70A, or sex peptide, which in the fruit fly is the principal modulator of female postmating behavior. Although many *Anopheles* Acps belong to the same functional classes reported for *Drosophila*, suggesting a conserved role for these proteins in mosquitoes, some represent novel lineage-specific Acps that may have evolved to perform functions relevant to *Anopheles* reproductive behavior. Our findings imply that the molecular basis of *Anopheles* female postmating responses can now be studied, opening novel avenues for the field control of these important vectors of human disease.

reproduction | malaria | mating | Acp | seminal fluid

A*nopheles gambiae* mosquitoes, the major vectors of human malaria, transmit *Plasmodium* parasites with a remarkable efficiency. The ability of this mosquito species to function as a malaria vector depends on multiple factors, including a strong preference for humans as hosts for blood feeding, a high susceptibility to parasite infections, a long lifespan, and a high reproductive rate. The identification of *Anopheles* genes regulating vectorial capacity is therefore a research priority, because their manipulation would provide important clues for the development of novel vector control measures aimed at fighting this devastating disease.

An. gambiae females, similar to females from a number of other insect species, undergo a series of changes upon insemination that dramatically modify their physiology and behavior: the phase of the flight activity rhythm shifts from crepuscular to nocturnal, ovulation and oviposition are triggered, and lifetime refractoriness to further copulation is induced, with multiple matings occurring in only a small percentage of individuals (1). In the fruit fly *Drosophila melanogaster* postmating responses are modulated by components of the seminal fluid, produced primarily by the male accessory glands (MAGs) and transferred to females during mating. Besides stimulating egg laying and triggering reduced sexual receptivity, MAG secretions have been shown to induce expression of immune peptides and reduction of female lifespan (reviewed in refs. 2–4). MAG products therefore are essential modulators of female be-

havior and represent important targets for biological and genetic control of insect pests (4).

The major components of MAG secretions are proteins, collectively named Acps. Thus far 59 of the 112 Acps postulated to be present in the *D. melanogaster* genome have been identified, and a small number has been shown to play a role in reproduction (5–11). The majority of these genes encode proteins with secretory signal peptides and exhibit male-specific expression that is highly enriched in MAGs, two criteria used to define bona fide Acps (6, 11). They include some of the most rapidly evolving genes in the *Drosophila* genome (10), and accordingly numerous attempts to identify their orthologues in other insects have failed (12–14).

In *An. gambiae* mosquitoes a crucial role for MAG secretions in modulating female postmating responses is well established. Experiments based on normal and forced copulation demonstrated that substances from the MAGs are necessary and sufficient to induce refractoriness to subsequent inseminations in females and to stimulate oviposition (15, 16). Females mated to males with degenerate testes and accessory glands failed to oviposit and readily remated, whereas females mated to males with degenerate testes but fully developed accessory glands laid (unfertilized) eggs and did not remate (15, 16). Unlike other insect species in which Acps are introduced into females as fluid secretions, MAG products in *Anopheles* are transferred during mating mainly as a solid mass (called the mating plug) (17) that slowly dissolves in the female reproductive tract. The means of delivery of MAG peptides in *An. gambiae* appears to be essential for their function because crude MAG homogenates do not produce measurable effects on mating refractoriness when injected directly into females (18). Despite the unquestionable importance of *Anopheles* mosquitoes for human health and the potential for their control presented by the manipulation of female postmating responses, no *Anopheles* MAG gene has been identified.

Here we report on the identification of 46 genes from the MAGs of *An. gambiae* mosquitoes, including the putative orthologues of 25 *D. melanogaster* Acps. The majority of these genes are specifically expressed in the male reproductive tract and show a low level of homology with their *Drosophila* orthologues. Comparative structural analysis suggests that MAG proteins share similar functions in the two insect species. However, a number of *Anopheles*-specific Acps are found, mostly organized in a densely populated cluster on chromosome arm 3R. These findings increase our knowledge of the

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Abbreviation: MAG, male accessory gland.

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molecular components of reproductive behavior in mosquitoes and expand the field of research on the role of MAG proteins, so far principally focused on studies in the *Drosophila* genus, to other medically and economically important insect species.

Results

Identification of Acp Genes in *An. gambiae*. With the aim to identify male genes modulating female postmating responses, the *An. gambiae* genome was searched for orthologues of 59 known *D. melanogaster* Acps (5–10). Initial standard sequence similarity (BLAST) and sequence colinearity (synteny) searches identified in *Anopheles* 20 putative Acp orthologues, mainly encoding large proteins. RT-PCR showed that 15 of these genes, similar to their *Drosophila* orthologues (www.flyatlas.org), were expressed in most tissues analyzed, which included the MAGs, testes, male carcasses (i.e., males deprived of their internal reproductive organs), and whole females (Table 1). The remaining five genes identified by the BLAST analysis were not detected in MAGs [[supporting information \(SI\) Table 2](#)].

This initial analysis did not allow the identification of the orthologues of any of the male-specific Acps that in *Drosophila* trigger female postmating behavioral changes. To significantly improve the depth of our bioinformatics analysis, homology searches were performed by using the Smith–Waterman and Needleman–Wunsch approaches, as implemented in the Bioinformatics toolbox in the Matlab programming environment (for a detailed explanation of the bioinformatics analysis procedure see *Materials and Methods* and [SI Appendices 1 and 2](#)). Remarkably, this analysis identified 16 *Anopheles* genes abundantly expressed in MAGs, 12 of which are specifically expressed in the male reproductive tract, encoding the homologues of 11 additional *Drosophila* Acps (Table 1). These genes, encoding proteins <100 aa (with one exception), show very low identity (19–29%) to their *Drosophila* homologues, demonstrating the power of this bioinformatics analysis (Table 1). An additional seven putative orthologues instead could not be detected in MAGs but in some cases were detected in the testes or other male tissues ([SI Table 2](#)).

Several of the *An. gambiae* Acps identified were organized in a cluster on chromosome arm 3R (Fig. 1, blue-shaded area). A manual annotation of the region encompassing this cluster uncovered the presence of 15 additional genes, all encoding proteins with predicted signal peptides. RT-PCR analysis revealed all 15 genes to be highly expressed in MAGs, nine being MAG-specific and four being restricted to the male reproductive tract (MAGs and testes) (Table 1 and Fig. 1). The *Drosophila* orthologues of these genes were identified by using the Matlab programming environment as above and did not encode additional known Acps.

In total, these analyses identified 46 genes showing strong expression in the MAGs of *Anopheles*, 25 being male reproductive tract-specific (17 specifically expressed in MAGs and eight restricted to MAGs and testes) (Table 1 and Fig. 1). Among these genes are the homologues of 25 *Drosophila* Acps, corresponding to >40% of the fruit fly genes analyzed here.

Male Reproductive Tract-Specific Acps. The 25 genes specifically expressed in the male reproductive tract identified by bioinformatics analysis and manual annotation meet the stringent criteria that define bona fide Acps in *Drosophila* (6). All 25 genes encode proteins with predicted signal peptides, exhibit expression profiles restricted to the male reproductive tract and highly enriched in the MAGs, and lack any previously assigned non-Acp function. Importantly, 14 of these *An. gambiae* Acps are homologues of *Drosophila* Acps, showing low levels of identity (19–29%), and none could be identified by standard BLAST or synteny analysis (Table 1 and [SI Appendices 1 and 2](#)).

Included among these 25 genes are the homologues of *Drosophila* Acps implicated in the induction of female postmating responses. We have identified a putative orthologue of Acp70A, commonly

referred to as sex peptide, which is by far the best-characterized Acp in *Drosophila*. Sex peptide has been shown to induce mating refractoriness, trigger ovulation, elicit feeding behavior, cause induction of the immune response, and reduce female lifespan (2, 4, 19). Like its identified *An. gambiae* orthologue, 09352 ([SI Appendix 1](#)), sex peptide is primarily expressed in MAGs but is also found in the testes at low levels (6). [Note that the *An. gambiae* genes are referred to with the last five digits of the VectorBase identifiers (i.e., 06418 refers to AGAP006418).]

An additional *An. gambiae* male-specific Acp is a homologue of the *Drosophila* Acp62F, a cysteine-rich trypsin-like protease inhibitor that has been demonstrated to be toxic to females when ectopically expressed (20). Allelic variation in Acp62F in males is associated with their success in sperm competition and the fecundity of their mating partners (21). The *Anopheles* homologue of Acp62F (06587) is clustered with the homologue of another *Drosophila* Acp, Acp63F, on chromosomal arm 2L (06585). This cluster contains three additional closely related genes expressed in MAGs and also in females (Table 1 and Fig. 1, green-shaded area).

Allelic variation in two other *Drosophila* Acps, Acp26Ab and Acp53Ea, for which putative orthologues were identified in our screen, is associated with sperm defense, the ability of an ejaculate present in a female to resist displacement by a second ejaculate (22). Among the *Drosophila* Acps for which we found male-specific orthologues in *Anopheles*, Acp70A, Acp62F, Acp26Ab, Msopa, and CG17575 are transferred to the female during copulation and enter the female circulatory system shortly after transfer (23).

A total of 12 male-specific Acps show similarity to *Drosophila* proteins with either unknown function or not known as MAG proteins. Two highly similar genes (09358-RB and 09358-RA) are related to the neuropeptide hormone Neuropeptide-like precursor 4 (Nplp4) (Table 1). A further two genes, 09355 and 09357, which are 100% identical to each other, are related to the antimicrobial peptide Drosocin (Dro-PA) (Table 1). In *Drosophila*, Drosocin is a proline-rich peptide, highly induced in the fat body by bacterial challenge, which upon mating is also found in the oviduct of egg-laying females (24). Another *Anopheles* gene, 09368, shows similarity to CG14770, which has not been described as an Acp in *Drosophila* but is up-regulated in MAGs (www.flyatlas.org) and has an orthologue in the medfly *Ceratitis capitata*, which has also been shown to be MAG-specific (12) (Table 1).

The striking majority (22 of 25) of the male-specific genes are organized in the 3R cluster. This region, spanning 48 kb, contains exclusively MAG-expressed genes (Fig. 1, blue-shaded area) and appears to have evolved as a male “fertilization island” in the *An. gambiae* genome. The presence of this island represents a major difference with the organization of Acps in *Drosophila*, where, although some clustering exists in the form of tandem duplications, genes are predominantly dispersed throughout the genome. Several groups of paralogues are found in the cluster, indicating that some of these genes are likely to be the result of recent gene duplication. Large clusters of coordinately expressed genes have been documented in *Drosophila* in the case of genes coexpressed in spermatogenesis and coregulated during circadian cycles (25). The presence of this highly populated cluster suggests that coregulation of these MAG genes may be important for male reproductive biology.

Comparative Structural Modeling of *An. gambiae* Acps. Because sequence similarity does not necessarily imply functional conservation, we used comparative structural analysis to help elucidate the function of 22 *Anopheles* Acps ([SI Table 3](#)). As expected, most of these genes fall in the same protein classes as their *Drosophila* orthologues, as described below.

CAP (CRISP/Antigen5/PR-1) Superfamily. One *Anopheles* male-specific Acp, 06418, contains 10 conserved cysteine residues in the N-terminal CAP and hinge domains characteristic of mem-

Table 1. *An. gambiae* genes expressed in the MAGs

<i>Anopheles</i> gene	<i>Drosophila</i> homologue	Identity, %	Length, aa	Functional class	Method	Chr
MAG-only						
06418	CG17575	27	266	CAP	M	2L
06585	Acp63F	29	83	Protease inhibitor	M	2L
06587	Acp62F	27	91	Protease inhibitor	M	2L
(12682)	CG15353*	30	48		C	3R
09353 (12680)	Msopa	21	81		M	3R
09358-RB	Nplp4-PA*	24	80		C	3R
09358-RA (12830)	Nplp4-PA*	25	80		C	3R
09359	Mst57Da	25	71		M	3R
12718 [†]	CG15065*	26	52		C	3R
09362	CG6409*	19	225		C	3R
09365 [†]	CG5793*	42	309	Transport protein	C	3R
09367	Acp26Ab	22	91		M	3R
09368	CG14770*	21	129		C	3R
09369	Acp53Ea	19	100		M	3R
09370 (12706)	Acp53Ea	22	98		M	3R
09371	CG14302*	25	72		C	3R
09373	CG16707 [‡]	22	210		C	3R
MAG, T						
09352 (12681)	Acp70A	27	56		M	3R
09354	Mst57Da	25	71		M	3R
09355	Dro-PA*	26	55		C	3R
09356	Mst57Da	24	71		M	3R
09357	Dro-PA*	26	55		C	3R
09360 (12807)	CG13230*	17	68		C	3R
09361	Acp95EF	25	54		M	3R
09372	CG32726*	21	69		C	3R
MAG, T, F						
COEBE4D	EST-6	31	557	Carboxylesterase	B	2L
COEBE1D	EST-6	32	570	Carboxylesterase	B	2L
06583	Acp63F	22	81	Protease inhibitor	M	2L
01424 [†]	CG5520	62	959	Chaperone	B	2R
04428 [†]	CG3359	39	822	Cell adhesion	B	2R
09429	Anp	26	75		M	3R
MAG, RB, F						
06581	Acp62F	23	94	Protease inhibitor	M	2L
06586	Acp62F	24	94	Protease inhibitor	M	2L
03083	CG17097	39	428	Lipase	B	2R
09364	CG5793*	52	310	Transport protein	C	3R
Ubiquitous						
05246	CG9334	38	395	Protease inhibitor	B	2L
07049	CG10433	37	136	β -Defensin	B	2L
07088	CG2852	72	202	Isomerase	B	2L
07491	CG4670	27	661	Redox	B	2L
SRPN9	CG10956	18	416	Protease inhibitor	B	2R
Calreticulin	Crc	71	406	Chaperone	B	2R
TEP15	CG10363	34	1422	α 2-Macroglobulin	B	3R
08822 [†]	CG9847	62	175	Isomerase	B	3R
08968	CG31704	42	63	Protease inhibitor	B	3R
09363 [†]	CG11466*	36	508	Cytochrome P450	C	3R
09842	CG8194	35	312	Ribonuclease	B	3R

The table contains 46 *An. gambiae* genes that are expressed exclusively or abundantly in the MAGs and their putative *D. melanogaster* homologues. Genes are grouped according to their expression profile, as determined by RT-PCR. T, testes; RB, rest of the body (whole males deprived of reproductive apparatus); F, whole females. The percentage of identity between putative homologues and the length of the *Anopheles* proteins are provided, as are their putative function when determined (see SI Table 3). The chromosomal arm on which genes are localized is indicated. The methods used for the initial identification of the *Anopheles* homologues are indicated (M, MatLab; B, BLAST searches; C, cluster on chromosome arm 3R). The *Anopheles* genes in parentheses represent putative alleles from an alternative haplotype. Gene 12682 is in parentheses because in Ensembl it is located on an unmapped scaffold, whereas our analysis predicts its localization on chromosome arm 3R. *Anopheles* gene 12718 is described with the first five digits of the old AgamP3 Ensembl identifier (ENSANGG00000012718) because it has not been assigned a new identifier in the AgamP3.4 gene build. 09358-RA and 09358-RB are labeled in AgamP3.4 as alternative transcripts of the same gene; however, they encode two separate genes and therefore are identified here by their transcript numbers. Chr, chromosome arm.

**Drosophila* genes that are not known to be expressed in the male accessory glands in the fruit fly.

[†]*Anopheles* genes whose full-length sequence has been reconstructed (using Fgenesh, Fgenesh+, Wise2, and Genescan gene-finding software).

[‡]This result was not confirmed by reverse BLAST analysis.

bers of the cysteine-rich secretory protein family (CRISPs), but like its *Drosophila* orthologue CG17575 lacks the ICR/CRD domain of true CRISPs. The 3D structure reveals two conserved histidine residues (H119 and H184) located in a pocket that likely

form the Ca²⁺-binding site characteristic of CRISPs (SI Fig. 3A). CAP proteins lacking the ICR/CRD domain are known to function as sperm chemoattractants and inhibitors of sperm-egg fusion (26).

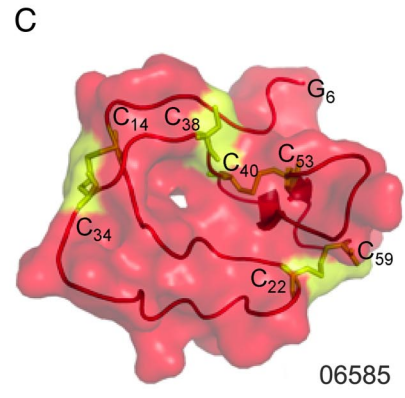
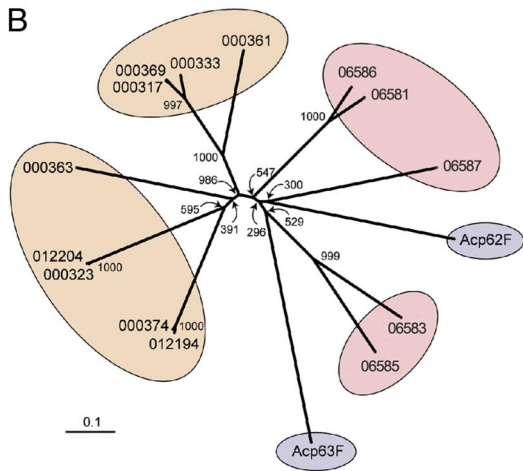
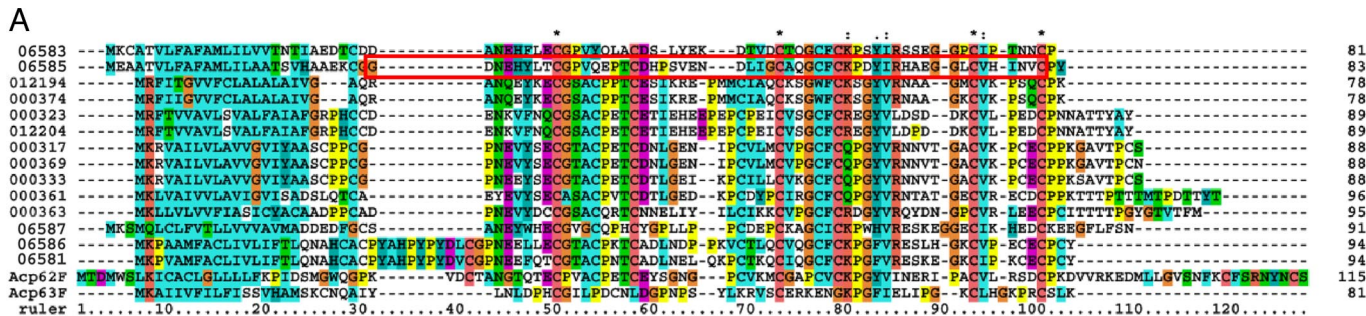


Fig. 2. Phylogenetic analysis of serine protease inhibitors in three insect species. (A) Multiple protein sequence alignment for the *D. melanogaster* (Acp62F and Acp63F), *An. gambiae* (06587, 06586, 06585, 06583, and 06581), and *Ae. aegypti* (identified by the last six digits of their Ensembl entry codes omitting the AEEL code: 012194, 000374, 000323, 012204, 000317, 000369, 000333, 000361, and 000363) homologues generated by using Clustal X and Clustal W. Conserved cysteine residues are indicated by an asterisk. The red box in the sequence of 06585 specifies the region used for the 3D model shown in C. (B) Unrooted phylogenetic tree was constructed by the neighbor-joining method based on the sequence alignment as above. *Anopheles* genes are shaded in pink, *Drosophila* genes are shaded in blue, and *Aedes* genes are shaded in orange. The bootstrap values of 1,000 replicates are indicated. The scale bar represents the amino acid divergence. (C) Three-dimensional model of the *Anopheles* putative serine protease inhibitor 06585. The cysteine residues engaged in disulfide bridges and the free Cys-59 are shown in ball-and-stick form in yellow.

Redox Proteins. Two redox proteins are expressed in MAGs. One, 09363, is a previously characterized microsomal cytochrome P450 (CYP9M1), and the other, 07491, is a thioredoxin-like protein belonging to an antioxidant class of proteins involved in protection from oxidative stress.

Discussion

We have achieved the identification of the first MAG genes from a mosquito vector of human malaria. The 46 *An. gambiae* Acps found here, 25 of which are male reproductive tract-specific, encode the homologues of 25 *Drosophila* known Acps, bridging the gap in knowledge between *Anopheles* and *Drosophila*. This result is remarkable considering that many Acps are rapidly evolving and diverge extensively among related insect species. Indeed, with the exception of the large proteins, most *Anopheles* Acps identified here show very low homology to their *Drosophila* orthologues, ranging from 19% to 29%.

We estimate that the genes identified here correspond to the majority of male reproductive tract-specific Acps present in *Anopheles*. The *An. gambiae* Affymetrix microarray database, available at www.angened.bio.uci.edu, contains a total of 32 nonredundant *Anopheles* genes that potentially meet the stringent criteria for genuine Acps described above (6, 11), because they show a predicted signal peptide, their transcripts are enriched at least 5-fold in males than in female at any stage, and they do not have an assigned non-Acp function. Twenty of these 32 genes (therefore 62.5%) correspond to the male-specific Acps identified in our bioinformat-

ics and molecular analyses (Table 1 and Fig. 1). The remaining five male-specific Acps do not have corresponding probe sets. Considering that the additional male-specific genes found in the above database will not necessarily be all expressed in the MAGs, we can conservatively estimate that we have identified two-thirds of bona fide Acps in *An. gambiae*. Future functional analyses are needed to clarify the role of single Acps in modulating female postmating behavior. However, given the number of *Anopheles* Acps identified here, it is reasonable to believe that indeed at least some of these proteins are transferred to females and perform reproductive functions.

In our analysis we found proteins belonging to a series of functional classes described for *Drosophila* Acps, including lipases, chaperones, serpins, and antimicrobial peptides (Table 1 and SI Table 3). However, some differences were found, and a number of *Anopheles* MAG genes have homologues in *Drosophila* that are not known as Acps. The microarray database of *Drosophila* available at www.flyatlas.org shows that transcripts of these fruit fly genes are not enriched in MAGs, suggesting that the *An. gambiae* homologues may represent lineage-specific Acps that have evolved to perform functions relevant to mosquito fertility. It is tempting to speculate that such proteins could account for the differences in postmating behavior observed between the two insect species, for instance the lifelong refractoriness of most *An. gambiae* females compared with the resumption of mating behavior observed in *D. melanogaster* 7 days after mating. The collection of *Anopheles* Acp genes identified here constitutes therefore an important reference set of mosquito

male fertility genes that will undoubtedly benefit future analyses in other insect species and may provide novel leads for vector control strategies based on the manipulation of important female postmating responses such as oviposition and remating behavior.

Materials and Methods

Identification of the *Anopheles* Homologues of *Drosophila* Acps. Selected *D. melanogaster* Acps were initially subjected to BLAST searches at NCBI (www.ncbi.nlm.nih.gov/BLAST) and at Ensembl (www.ensembl.org), at both the protein level and the DNA level. For all annotated *Drosophila* Acp genes we also analyzed the genome colinearity (synteny) data between *Drosophila* and *Anopheles* (36) present in AnEST (<http://web.bioinformatics.ic.ac.uk/AnoEST/anoest.php>), and the automatic clustering of orthologues and in-paralogues from Inparanoid (<http://inparanoid.sbc.su.se>). For more stringent and sensitive bioinformatics analyses, homology searches were performed by using the Smith–Waterman and Needleman–Wunsch approaches, as implemented in the Bioinformatics toolbox in the Matlab programming environment (Matlab version 7.0.4), to identify homology between a selected set of Acp proteins from different *Drosophila* species (*D. melanogaster*, *Drosophila mauritiana*, *Drosophila simulans*, *Drosophila sechellia*, *Drosophila subobscura*, *Drosophila madeirensis*, and *Drosophila pseudoobscura*, downloaded from Swissprot, <http://expasy.org/sprot>) and the *An. gambiae* superset of translated sequences from known or novel gene predictions, as downloaded from Ensembl (database release version 42.3e, February 2006). Alignment tests were done by using different scoring matrices: generally the best results were obtained by using BLOSUM as scoring matrix, with a percent identity level of 62. Various penalties were assigned for opening or extending a gap during sequence alignment. Each run of the application resulted in a list of candidate amino acid sequences in the *Anopheles* genome that were subjected to reciprocal best-hit analysis against the *D. melanogaster* genome using the same parameters used in the initial similarity searches. For examples and references of the bioinformatics searches, see *SI Appendices 1 and 2*. The Matlab script that was used to obtain the results and the input data are readily available upon request.

Mosquito Dissections and RT-PCR Analysis. Tissues from MAGs, testes, and male carcasses lacking the genitalia were dissected in 1× PBS solution, and total RNA was extracted from these tissues as well as from whole adult *An. gambiae* female mosquitoes (G3 strain) using TRI Reagent (Helena Biosciences, Gateshead, U.K.), according to the manufacturer's instructions. The RNA was reverse transcribed by using oligo d(T)s and M-MLV Reverse Transcriptase (Invitrogen, Paisley, U.K.) following standard protocols. RT-PCRs were carried out by using the HotStarTaq DNA Polymerase (Qiagen, Valencia, CA) as described by the manufacturer, using primer sets specific for each gene. A total of 35 cycles per reaction were run. At least two replicates were performed for each gene analyzed in each tissue. As a control for the specificity of our amplifications, we used primers for the testis-specific β 2-tubulin gene (37) on MAGs and testes cDNA samples. The ribosomal gene S7 was used as a positive control for all cDNA samples. In five cases, the amplified products were cloned and sequenced to confirm their identity. In all cases the sequences obtained corresponded to the original Ensembl gene prediction.

Phylogenetic Analysis of Serpins. The selected *Drosophila*, *Anopheles*, and *Aedes* amino acid sequences were subjected to multiple alignments by means of the Clustal W (www.ebi.ac.uk/Tools/clustalw) and Clustal X (Version 1.83) algorithms. Phylogenetic analysis was performed by using TreeView software (Version 1.6.6) (38). A phylogenetic tree was constructed by the neighbor joining method by using *p*-distance estimates and tested by using the interior-branch test. Reliability of each node was assessed with 1,000 bootstrap replications.

Generation of 3D Models. Three-dimensional templates were generated for 22 of the 46 *Anopheles* Acp proteins. See the legend of *SI Table 3* for details.

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