Review

Function and composition of male accessory gland secretions in *Anopheles gambiae*: a comparison with other insect vectors of infectious diseases

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Human malaria, a major public health burden in tropical and subtropical countries, is transmitted exclusively by the bite of a female *Anopheles* mosquito. Malaria control strategies aimed at inducing sexual sterility in natural vector populations are an attractive alternative to the use of insecticides. However, despite their importance as disease vectors, limited information is available on the molecular mechanisms regulating fertility in *Anopheles* mosquitoes. In the major malaria vector, *An. gambiae*, the full complement of sperm and seminal fluid required for a female's lifelong egg production is obtained from a single mating event. This single mating has important consequences for the physiology and behavior of *An. gambiae* females: in particular, they become refractory to further insemination, and they start laying eggs. In other insects including *Drosophila*, similar post-copulatory changes are induced by seminal proteins secreted by the male accessory glands and transferred to the female during mating. In this review, we analyze the current state of knowledge on the function and characterization of male seminal proteins in *An. gambiae*, and provide a comparative assessment of the role of these male reproductive factors in other mosquito vectors of human disease in which female post-copulatory behavior has been studied. Knowledge of the factors and mechanisms regulating fertility in *An. gambiae* and other vectors can help the design of novel control strategies to fight the spread of disease.

Keywords: Anopheles, Fertility, Seminal fluid, Sperm, Post-mating response, Vector control, Malaria, Protease, Redox, Acps, Copulation, Reproduction, Sex, Sterile

Introduction

Mosquitoes transmit a variety of infectious diseases that severely affect human health. Malaria alone, transmitted by the bite of female *Anopheles* mosquitoes, annually infects more than 200 million people and causes nearly one million deaths. Infections by dengue and yellow fever virus, transmitted by *Aedes* mosquitoes, are a leading cause of illness and death in many tropical and subtropical countries. Current strategies aimed at targeting vector populations are mainly based on the use of insecticides; however, such efforts are hampered by the emergence of insecticide resistance in mosquitoes combined with the lack of novel chemicals. There is an urgent need for novel strategies to control mosquito disease-transmitting populations.

Among the hundreds of extant anopheline species, *An. gambiae* is the most important vector of human malaria. *Plasmodium* parasites, the causative agents of malaria, are transmitted when a female mosquito feeds on the blood of a host, releasing infective sporozoites into the blood stream.[1] As blood feeding is necessary for egg production, the parasite exploits the mosquito's reproductive needs to achieve its own transmission between vertebrate hosts. The high reproductive rate of *An. gambiae* mosquitoes is a major component of their capacity as malaria vectors. A female of this species can generate more than a hundred eggs from each blood meal, and can fertilize her lifetime egg production using sperm acquired from a single mating and stored in her sperm storage organ.

The acquisition of sperm by a female is a potential target for intervention aimed at vector control: *An. gambiae* females generally mate only once[2] as mating with one male permanently switches off their receptivity to further insemination with other males and stimulates oviposition.[3] This dependence of lifetime reproductive success on a single mating event offers an excellent target for intervention; interfering with insemination or oviposition would have a large impact

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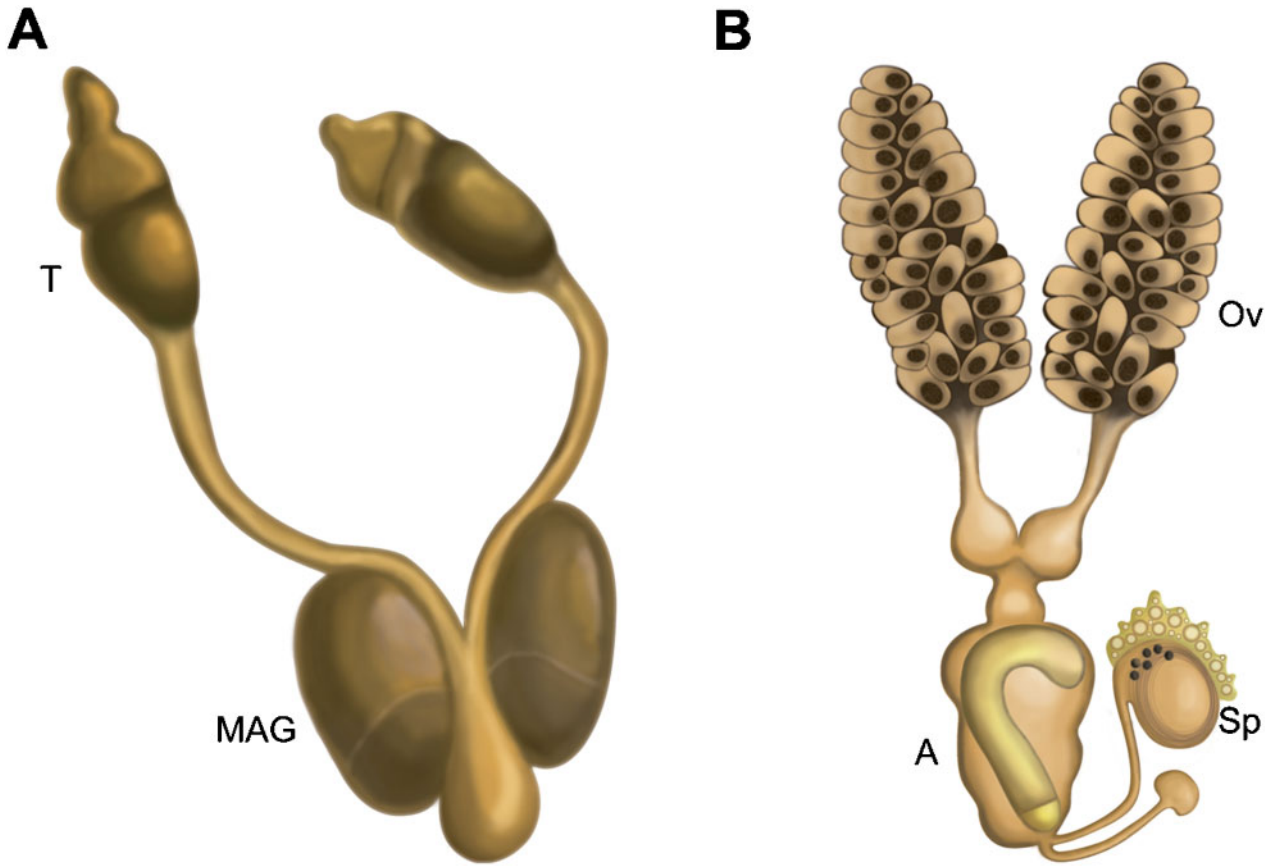

Figure 1 Cartoon representing male and female reproductive tracts in *An. gambiae*. (A) The male reproductive tract, showing the testes (T) and male accessory glands (MAG). (B) The reproductive tract of a freshly mated female, showing the ovaries (Ov), the atrium (A) containing a mating plug, and the spermatheca (Sp) filled with sperm.

on the size of natural mosquito populations. Fertility is a target in control strategies, such as the sterile insect technique (SIT),[4] aimed at natural insect pests. SIT relies on the massive release of sterilized males into field populations. Females mated to sterile males lay infertile eggs, with a consequent decrease in population size. Despite the use of this technique for the control of many insect pests, to date SIT against *Anopheles* species has not been very successful.[5] A deeper knowledge of mating and other processes underlying *Anopheles* fertility would definitely benefit the chances of SIT success, and would identify targets for the development of novel vector control strategies.

In this review, we describe the current understanding of reproductive biology in *An. gambiae*, with a particular focus on the mechanisms known to regulate female receptivity to mating and ability to lay eggs. In doing so, we provide a comparison of the events that influence female post-copulatory biology in other disease-transmitting mosquitoes, such as *Aedes* and *Culex*, and relate these factors to the primary model of insect reproductive biology, *Drosophila melanogaster*.

Mating Behavior and Physiology in *Anopheles* and Other Mosquitoes

Mosquitoes are members of a family of the nematocerid flies: the Culicidae. This family consists of three subgroubs: the toxorhynchitinae, the anophelinae, and the culicinae. Blood feeding mosquitoes, including vectors of human diseases, belong to the two latter groups. Anophelines mate predominantly in crepuscular station-keeping swarms formed by large aggregations of males above inanimate markers.[6–12] Virgin females enter the swarm, are captured by a male, and leave the swarm while in copula. Most male culicines also aggregate in the proximity of visual markers, although members of the *Aedes* subgenus are known to swarm and mate in the vicinity of the host.[13–18] There is strong evidence that males and females recognize each other by the wing beat frequency specific to each species[19,20] and interact acoustically by shifting their harmonic overtones to match.[21,22] Furthermore, spatial segregation of the swarms may contribute to reproductive isolation of different species, as observed for the incipient M and S forms of *An. gambiae* species.[23,24]

Anopheline and culicine females are generally monandrous as after mating they become refractory to further insemination.[2,25–29] Field studies show that remating does not occur in anophelines,[29] or is observed at very low rates.[2,27,28] During mating, male mosquitoes transfer sperm, and seminal secretions from the male accessory glands (MAGs) (see Fig. 1 for a representation of the male and female reproductive tracts). Sperm are stored by the female in

a dedicated sperm storage organ named the spermatheca. While *Anopheles* mosquitoes have a single spermatheca, *Aedes* and *Culex* have three, like *Drosophila*.[30] Seminal secretions from the MAGs are transferred to the female reproductive tract, and in some anophelines including the major malaria vectors (*An. gambiae*, *An. arabiensis*, and *An. funestus*), these secretions are coagulated into a gelatinous mating plug that is deposited in the atrium (sometimes called the uterus).[31]

The Role of MAG Secretions in Modulating Female Behavior after Mating

In many insect species, the MAGs exert powerful control over female reproduction and behavior. In *D. melanogaster*, a wealth of studies has demonstrated that MAG secretions, composed of proteins, carbohydrates, and lipids,[32] play a major role in inducing behavioral and physiological changes in the female. These changes include loss of mating receptivity, increased oogenesis and oviposition, increased feeding and sleeping activity, induction of immune responses, and decreased longevity (reviewed in Refs. 33 and 34). Seminal fluid proteins are also associated with the behavioral and physiological changes (namely, the loss of mating receptivity and increased oogenesis and oviposition) observed in females of some mosquito species after mating (reviewed in Refs. 32 and 35). The physical act of copulation is not always required to induce these post-mating responses; in *Aedes* and *Culex* species, simply transplanting the MAGs or injecting MAG extracts into the hemolymph of virgin females is sufficient to induce life-long mating refractoriness and to trigger oviposition.[26,36–40] Early studies showed some promise in the identification of MAG factors modulating oviposition and female remating.[26,38–40,41] However, in spite of recent advances in characterizing the components of the seminal fluid in *Aedes* mosquitoes,[42,43] the nature of the molecule(s) responsible for inducing post-mating behavior remains elusive.

The mode of action of key seminal fluid proteins appears to be conserved across several species; heterologous transplant of MAGs between *Ae. aegypti*, *C. pipiens*, and *D. melanogaster* stimulates oviposition in virgins of all species.[44] Similarly, oviposition can be triggered in virgin *Ae. aegypti* by the transplantation of MAGs from *Ae. triseriatus*.[45] However, some functions of the seminal fluid may be species specific. Yeh and Klowden[46] found that implanting MAGs or injecting MAG homogenates from a conspecific male stimulated pre-oviposition behavior (i.e. the attraction of gravid mosquitoes to mating sites) in *Ae. aegypti*. However, injection of MAG extracts from other mosquito species failed to induce pre-oviposition behavior.

The role of MAG secretions in shaping two of the major female post-mating responses in *Anopheles*, such as the acquisition of sexual refractoriness and the induction of oviposition, is controversial (see Table 1 for a summary of the experiments). Early studies, based on hybrid mating, suggested a role for the MAGs in triggering oviposition. Virgin *An. gambiae*, which rarely lay eggs,[47] can be induced to oviposit unfertilized eggs if mated to hybrid *An. gambiae*/*An. melas* males with degenerate testes but normal accessory glands.[48] However, attempts to replicate the MAG transplant and injection experiments that provide such clear results in other Diptera have produced mixed results in anophelines. Intraspecific MAG implant induced loss of mating receptivity in virgin *An. quadrimaculatus*[26] but not in

Table 1 Induction of post-mating response experiments in *Anopheles*

Reference	Species	Mating status	Operation	Behavioral change
26	*An. quadrimaculatus*	Virgin	MAG implant	Refractoriness
48	*An. gambiae*	Mated	Mated males with abnormal testes and normal MAGs	Refractoriness,* oviposition
64	*An. gambiae*	Mated	Mated males with abnormal testes and abnormal MAGs	No refractoriness, no oviposition†
49	*An. gambiae*/*An. albimanus*	Virgin	MAG implant	No refractoriness, no oviposition
49	*An. gambiae*	Virgin	Injection of MAG homogenate (intra-abdominal)	No refractoriness, no oviposition
49	*An. gambiae*	Virgin	Injection of MAG homogenate (into the genital tract)	No refractoriness
49,51	*An. gambiae*	Virgin	Implant of spermatheca from a mated female	No refractoriness, no oviposition
49,51	*An. gambiae*	Mated	Removal of spermatheca	No oviposition
50	*An. gambiae*/*An. stephensi*	Virgin	Injection of MAG homogenate (intra-thoracic)	Refractoriness
53	*An. gambiae*	Mated	Mated males with no sperm cells and functional MAGs	Refractoriness, oviposition

Notes: MAG, male accessory gland.
Behavioral changes include the ability to lay eggs (oviposition) or the inhibition of remating (refractoriness) after treatment.
*Further mating was performed by forced copulation. No insemination was detected.
†Oviposition occurred at very low levels similar to those observed in virgins.

An. gambiae and *An. albimanus*.[49] Furthermore, Klowden found that intra-abdominal injections of MAG extracts had no effect on mating receptivity or oviposition behavior of virgin *An. gambiae*.[49] In contrast, recent experiments by Shutt *et al.*[50] showed that intra-thoracic injections of MAG extracts into virgin *An. gambiae* females reduced their likelihood of subsequently becoming inseminated. To explain this discrepancy, Shutt *et al.* suggested that putative MAG protein receptors in *An. gambiae* might be located in the thorax, and that this would reduce the efficacy of abdominal injections.[50] There might be several explanations for these conflicting results. In the field, *Anopheles* remating occurs at very low rates[2,27–29] while it is much higher under laboratory conditions.[51–53] A number of parameters can influence the frequency of mating (and remating) — as well as oviposition — in laboratory cages. These parameters include cage size, female age, female fitness after the injection (MAG homogenates contain many proteolytic enzymes that when injected may interfere with normal female physiology), and length of time that females are exposed to males.[51] Further studies are needed to clarify the role of MAG secretions in modulating female post-mating changes. MAG products may need to be processed by atrial proteases in order to stimulate ovulation and oviposition, explaining why MAG implants or extract injections are not effective. In support of this hypothesis, proteases associated with processing of peptide hormones[54] are highly expressed and synthesized in virgin atria.[55]

Sperm Effect in Female Post-mating Responses

Injection of MAG secretions or purified components into female *Drosophila* induces post-mating changes for 1–2 days.[56] The maintenance of the response for up to a week, as seen in natural matings, requires transfer of sperm,[57–59] a phenomenon known as the 'sperm effect'. The sperm effect is mediated by the binding of MAG peptides to the sperm cell flagella; seminal proteins bound to sperm tails are carried into the female sperm storage organs, where their gradual release maintains the post-mating response.[60–62]

Early studies suggested that sperm transfer could play an important role in influencing female behavior in mosquitoes. Gwadz found that refractoriness to mating was induced within 15 seconds of mating in female *Ae. aegypti*,[63] in contrast to the 4 hour latency arising from MAG transplants described by Craig.[26] This difference could be due to the time required by the injected females to recover from the operation or can be interpreted as an evidence for a sperm effect with the immediate post-mating response triggered by the filling of a female's bursa by sperm.[63] If a sperm effect is present in *Ae. aegypti*, it would only be effective over the short-term, unlike the long-term maintenance observed in *D. melanogaster*. Removal of the aedine spermathecae, where sperm migrate after their initial deposition in the bursa, prior to copulation did not interfere with the induction of oviposition upon mating.[51]

Due to the ambiguous results of MAG transplantation and injection experiments, sperm has often been viewed as a key trigger of post-mating changes in anophelines (Table 1). As with studies of the effects of MAG secretions, early studies of the sperm effect examined hybrid matings. Hybrid *An. gambiae*/*An. melas* males with degenerate testes but with well-developed MAGs were still capable of inducing post-mating behavior,[48] whereas hybrid males lacking both MAGs and testes were not,[64] suggesting that sperm are not required to trigger female behavioral changes. In a recent study, the role of sperm in modulating female behavior in *An. gambiae* has been unambiguously established by RNA interference-mediated silencing of zero population growth, a germ cell differentiation gene whose knockdown results in males lacking sperm cells but with functional accessory glands. These spermless males were capable of inducing oviposition and inhibiting remating in females,[53] confirming the original findings by Bryan that in this species sperm have no role in triggering post-mating behavior. Furthermore, spermless males induced female transcriptional responses similar to those triggered by normal males.[53]

Although sperm do not play a major role in triggering behavioral changes in female *An. gambiae*, an intact (and possibly innervated) spermatheca may be needed for such responses to take place. Surgical removal of the spermatheca from mated *An. gambiae* results in inhibition of oviposition, while implantation of a mated spermatheca into virgin females does not stimulate egg laying or loss of sexual receptivity.[49,51] In the grasshopper *Gomphocerus rufus*, microinjection of MAG secretion into the spermatheca induces female mating refractoriness and sperm have no role in the process.[65] In this insect spermathecal gland cells digest and resorb seminal secretions, and the neural pathway between the spermatheca and the ventral nerve cord is required to maintain sexual refractoriness.[66] Recently, it has been shown in *Drosophila* that spermathecal secretory cells attract sperm into the female sperm storage organ and participate in modulating sperm motility and stimulating oviposition.[67] A possible role of spermathecal cells in *Anopheles* post-mating behavior needs to be fully established.

MAG Proteins in *An. gambiae* and Other Insects

Despite the crucial role of MAG components in regulating many aspects of mosquito reproduction, elucidation of the seminal proteomes of mosquitoes has only been established in the last few years.[42,43,68,69]

The molecular composition of mosquito MAG secretions remained entirely unknown until Dottorini *et al.*'s[69] bioinformatic comparison of *D. melanogaster* and *An. gambiae* identified 46 MAG-expressed anopheline genes. Since then, further components have been identified in *An. gambiae*[68] and the *Ae. aegypti* seminal proteome has been well characterized.[42,43] These analyses have identified 71 genes expressed in the MAGs of *An. gambiae*.[68–70] Moreover, for the purpose of this review, we have identified 50 additional *An. gambiae* MAG-specific genes by analyzing the data produced in a recent whole genome microarray study where gene expression was determined in multiple male and female tissues[71] (Table 2).

These studies allow comparative analysis of the MAG proteomes in *An. gambiae*, *Ae. aegypti*, and *D. melanogaster*. Many of the functional classes of MAG proteins are shared between the two mosquito species and *D. melanogaster* (Fig. 2 and Table 2). In all three species, accessory gland proteins (Acps) form the most abundant category. Acps are defined as MAG-specific proteins that are secreted and do not contain known functional domains. In the fruitfly, many of these proteins are known to be transferred to females and control a number of responses to mating. For instance, sex peptide (Acp70A) has been implicated in the inhibition of remating,[56,61,62,72] increased egg production,[73,74] decreased longevity,[75] alteration of locomotion and feeding behaviors,[76,77] and stimulation of the immune system.[78,79] Sex peptide is detected by sensory neurons in the female reproductive tract[80,81] where it binds to a G protein coupled receptor[82] leading to the alteration of female physiology and behavior. Another category abundant in *An. gambiae* and *D. melanogaster* comprises peptides that have putative hormonal function. For instance, the hormone ovulin (Acp26Aa) is an important regulator of ovulation in *D. melanogaster*.[83] Although functions have yet to be ascribed to MAG peptide hormones in *An. gambiae*, putative orthologues of Acp53Ea, another peptide hormone that in *Drosophila* is involved in sperm competition,[84,85] were localized in close proximity to the spermatheca, suggesting a role in sperm function.[86] In *Ae. aegypti*, a head peptide expressed primarily in the MAGs[87] has been implicated in the short-term inhibition of host-seeking behavior in females.[88]

Proteases and peptidases are represented at high levels in all three species. These enzymes can be involved in the activating cleavage of many seminal fluid protein.[83,89] In *Drosophila*, ovulin is transferred to females as a preprohormone where it is processed by a seminal astacin-like protease.[89] Other proteases can play roles in controling the activity of these processing proteases.[90,91] Similarly abundant are serpins and other protease inhibitors, which have been shown to have a role in male mammalian fertility,[92] and chaperones, which can facilitate protein folding and sperm-egg interactions.[93] Cysteine-rich secretory proteins (CRISPs) that in ascidians are involved in gamete interactions[94] are instead more frequently observed in *D. melanogaster* than in mosquito species. Lipases are also more abundant in *D. melanogaster* than in mosquitoes; in *Drosophila* these enzymes are transferred to females during mating,[95] influence egg-laying behavior and possibly receptivity to remating,[96] and provide energy to sperm.[97] Contrary to *Drosophila* and *Aedes*, in *Anopheles*, no lectins have been identified to date in the MAGs. Lectins are postulated to play a role in sperm-oocyte recognition.[98]

Finally, it is notable that proteins that participate in oxidation/reduction (redox) processes are more abundant in mosquitoes compared to *D. melanogaster*. In the fruitfly, many MAG-expressed redox proteins are prolyl 4-hydrohylases that are involved in the hydroxylation of collagen, whose function may be needed to ensure the integrity and functionality of the extracellular matrix, possibly necessary for the activity of the MAGs.[99] In *An. gambiae* MAGs, the majority of identified redox proteins are oxidases. Among these, a number are involved in the synthesis of ecdysteroid hormones, which are transferred to females during mating[70] and that control egg production after a blood meal.[100] By contrast, in *Ae. aegypti*, MAG-specific redox proteins are mostly dehydrogenases involved in energetic metabolism, and subunits of the ATP synthase protein complex, which might supply the energetic requirement for protein synthesis in this secretory glands.[42,43] A Rab3-like protein appears instead to be specific for *An. gambiae* MAGs. Rabs are proteins that regulate membrane trafficking and in particular Rab3 is associated with secretory vesicles.[101]

Although functional classes of seminal proteins are conserved across the species, the MAG-expression of individual genes rarely is. As shown in Table 2, among the 121 genes expressed in the *An. gambiae* MAGs, 109 and 71 have putative annotated *D. melanogaster* and *Ae. aegypti* orthologues, respectively. However, by comparing the tissue expression of orthologues in the three species, it is clear that only a small number of these are expressed in the MAGs of more than one species. Only 17% of the *An. gambiae* genes have orthologues in either *D. melanogaster* and/or *Ae. aegypti* that are expressed in the MAGs. Only two genes are expressed in the MAGs of all three species: they encode for a protein disulphide isomerase, which may promote protein folding, and a CRISP protein. A total of 16 genes are expressed in the MAGs of both *An. gambiae* and *D. melanogaster* but not *Ae. aegypti*, comprising predicted pro-hormonal peptides, antimicrobial peptides, protease inhibitors, and proteases.

Table 2 Genes identified in the MAGs of *An. gambiae*

Functional class	*Anopheles*	*Drosophila*	*Aedes*	Expression data (%)
Acps				
	AGAP001510	nd	AAEL017145	100
	AGAP006362	CG13699	AAEL014767	100
	AGAP006581	Acp62F*	AAEL000356	13
	AGAP006583	Acp63F*	nd	100
	AGAP006585	Acp63F*	nd	**100**
	AGAP006586	Acp62F*	AAEL000356	17
	AGAP006587	Acp62F*	nd	**100**
	AGAP006589	nd	nd	100
	AGAP008116	nd	AAEL010264	100
	AGAP008968	CG31704	nd	nd
	AGAP009352 (AGAP012681)	Acp70A*	nd	100
	AGAP009353 (AGAP012680)	Msopa*	nd	**100**
	AGAP009354	Mst57Da*	nd	**100**
	AGAP009355	Dro-PA	nd	100
	AGAP009356	Mst57Da*	nd	**100**
	AGAP009357	Dro-PA	nd	100
	AGAP009358 (AGAP012682/ AGAP012830)	Nplp4	nd	**100**
	AGAP009359	Mst57Da*	nd	**100**
	AGAP009360 (AGAP012807)	CG13230	nd	98
	AGAP009361	Acp95EF*	nd	nd
	AGAP009362	CG6409	nd	**100**
	AGAP009367	Acp26Ab*	nd	**100**
	AGAP009368	CG14770	nd	**100**
	AGAP009369	Acp53Ea*	nd	**100**
	AGAP009370 (AGAP012706)	Acp53Ea*	nd	**100**
	AGAP009371	CG14302	nd	**100**
	AGAP009372	CG32726	nd	nd
	AGAP009373	vsg	Supp02310*	**100**
	AGAP013714	nd	nd	100
	AGAP013731	CG13230	nd	98
	AGAP013734	CG15065	nd	100
	AGAP013776	Nplp4	nd	**100**
Redox				
	AGAP001039	spo	AAEL009762	100
	AGAP003067	Cyp304a1	AAEL014413	100
	AGAP005784	PAM	AAEL007732¤	100
	AGAP007420	PHM	AAEL001394	0
	AGAP007491	CG4670	AAEL012054	35
	AGAP008019	Cyp12b2	AAEL002031¤	99
	AGAP008203	Cyp6a2	AAEL009120	100
	AGAP009363	Cyp9f2	AAEL001312¤	0
	AGAP009584	Trx-2	AAEL010777	24
	AGAP012855	Cyp6a2	AAEL009120	100
	CYP302A1	dib	AAEL015655	85
	CYP306A1	phm	AAEL004888	88
	CYP314A1	shd	AAEL011850	98
	CYP315A1	sad	AAEL010946	100
Proteases				
	AGAP005791	CG12951	nd	**100**
	AGAP005792	CG12951	nd	**100**
	AGAP008276	Try29F	nd	**100**
	AGAP008277	Try29F	nd	**100**
	AGAP008997	CG8172	AAEL000238	100
	AGAP012315	CG34290	AAEL012447¤	100
	AGAP013150	CG9806*	AAEL009108	**100**
	ENSP017764	nd	nd	100
	ZCP1	nd	nd	100
	ZCP3	nd	nd	100
	ZCP4	nd	nd	100
	ZCP6	nd	nd	nd
	ZCP7	nd	nd	**100**
	ZCP9	nd	nd	100
Nucleic acid binding				
	AGAP000355	Mkrn1	AAEL007476	100
	AGAP000754	CG15439	AAEL003032	100

Table 2 Continued

Functional class	*Anopheles*	*Drosophila*	*Aedes*	Expression data (%)
	AGAP000916	pAbp	nd	100
	AGAP000918	pAbp	nd	100
	AGAP000920	pAbp	nd	100
	AGAP003844	cwo	AAEL010513	100
	AGAP009339	CG6654	AAEL005029	100
	AGAP009699	sens-2	AAEL001243	100
	AGAP010358	gsb	nd	100
	AGAP010359	prd	nd	100
Hydrolases				
	AGAP001649	CG31414	AAEL014321‡	**100**
	AGAP005255	Rab3	AAEL006267	100
	AGAP006425	CG9701	nd	100
	AGAP010720	pom1	AAEL013237	100
	COEBE1D	EST-6	nd	nd
	COEBE4D	EST-7	nd	nd
Protein folding				
	AGAP000831	CG7872	AAEL013114	100
	AGAP001424	CG5520	AAEL012827	nd
	AGAP001502	CG11267	AAEL001052	10
	AGAP007088	CG2852	AAEL013279	3
	AGAP008822	CG9847	AAEL004313	0
	AGAP012407	PDI*	AAEL000641*	4
Transferases				
	AGAP000843	CG4774	AAEL012719‡	100
	AGAP009099	CG7356	nd	**100**
	AGAP009190	CG4688	AAEL007955	100
	AGAP009191	CG16936	AAEL007954	100
	AGAP009365	CG5973	AAEL001297	**100**
	AGAP009377	CG5973	AAEL001297	93
Protein binding				
	AGAP000927	sqh	AAEL008921	100
	AGAP004761	mwh	AAEL008269	100
	AGAP005684	Syx17	AAEL000282‡	100
	AGAP007041	fibrinogen	AAEL001713*	**100**
	AGAP012986	spi	AAEL011205	100
Transmembrane proteins				
	AGAP000107	pyx	AAEL004179	98
	AGAP002824	Takr86C	AAEL017414	100
	AGAP010637	Toll6	nd	100
	AGAP010861	CG1698	AAEL003626	100
Immune peptides				
	AGAP007049	CG10433	AAEL009861	2
	AGAP009429	Anp*	nd	98
	TEP15	CG10363	AAEL014755	nd
Serpins				
	AGAP005246	CG9334	AAEL007765	17
	SRPN9	CG10956	AAEL008364	nd
Nucleic acid metabolism				
	AGAP000139	tam	AAEL015671	100
	AGAP009842	CG8194	AAEL001159	36
Protein metabolism				
	AGAP000926	l(1)G0196	AAEL008950‡	100
	AGAP009673	QPCT	AAEL010727	**100**
Lipases				
	AGAP003083	CG17097	AAEL012343‡	100
	AGAP003749	CG6296	AAEL012790	100
CRISPs				
	AGAP006418	CG17575*	AAEL009239*‡	**100**
Kinases				
	AGAP002181	CG5644	AAEL010062	100
Others				
	AGAP004428	CG3359	AAEL014917	3
	AGAP005239	CG8323	AAEL006262	93
	AGAP005504	scramb1	AAEL010661	100
	AGAP007339	TpnC47D	AAEL000744	96
	AGAP009001	Hdc	AAEL014632	100
	AGAP009189	Eps-15	AAEL007950	100
	AGAP009364	CG5793	AAEL001308	0
	CALRETICULIN	Crc	AAEL001005‡	nd
Unknown				
	AGAP003736	CG30053	nd	100

Table 2 Continued

Functional class	*Anopheles*	*Drosophila*	*Aedes*	Expression data (%)
	AGAP005859	nd	nd	100
	AGAP008439	CG31705	AAEL005017	100

Notes: *Acps, accessory gland proteins; CRISPs, cysteine-rich secretory proteins.*
The table contains all 121 genes identified to date in the accessory glands of *An. gambiae* (*Anopheles*) males, identified by a number of strategies (as described in the text).[68–71] Genes are organized based on their functional class. When known, the putative *D. melanogaster* (*Drosophila*) and *Ae. aegypti* (*Aedes*) orthologues are indicated. In the *Anopheles* column, identifiers in bold represent genes whose products have been detected in the male accessory glands (MAGs) by mass spectrometry[68] and/or reverse transcription PCR (RT-PCR) data.[68–70] The '*' symbol indicates *D. melanogaster* and *Ae. aegypti* genes that are expressed in the MAGs of these species. The 'ב' symbol in the *Aedes* column indicates the presence of multiple putative orthologues (only the most similar orthologue is reported). The expression data column refers to the degree of MAG specificity of the *An. gambiae* genes, as obtained from MozAtlas website (http://www.tissue-atlas.org/); the degree of specificity is reported as the percentage of expression observed in the MAGs relative to all male and female tissues where expression was present in a minimum of 3/4 calls.[71] Numbers are rounded to the nearest integer. Numbers in bold represent expression data obtained by transcriptional and/or immuno blotting analyses.[68–70] Please note that *An. gambiae* genes in the following groups share a common probeset in mozatlas: (1) AGAP009354, AGAP009356, AGAP009359; (2) AGAP009355, AGAP009357; (3) AGAP009358, AGAP0013776; (4) AGAP009360, AGAP0013731; (5) AGAP009365, AGAP009377; and (6) AGAP009369, AGAP009370.

Putative orthologues of many *Drosophila* accessory gland peptides were identified in *An. gambiae*: among these is the putative *An. gambiae* orthologue of sex peptide. A further two genes are expressed in the MAGs of both *An. gambiae* and *Ae. aegypti*, but not *D. melanogaster*: a fibrinogen and a visgun-like peptide, whose functions in reproduction are unknown. The diversity of the factors synthetized in the MAGs may highlight different reproductive roles for these male reproductive tissues among insects, stressing the need for a detailed analysis of the reproductive molecular machinery in each species.

An. gambiae MAG Secretions are Coagulated to Form a Mating Plug

Unlike most mosquito species, *An. gambiae* (and its close relatives) transfers its seminal secretions as a gelatinous mating plug, which becomes coagulated during copulation. Many insects produce mating plugs with different functions. In the lepidopteran *Cressida cressida*, males transfer an external plug termed a sphragis that blocks the female copulatory opening, physically preventing remating by other males.[102] In the hymenopteran *Bombus terrestris*, a linoleic fatty acid present in the mating plug renders females refractory to further insemination for life.[103] In Diptera, there are examples of mating plugs that prevent remating, such as in the dung fly *Coprocica vagans*,[104] while in *D. hibisci*, the mating plug is associated with female loss of sexual receptivity and correct sperm storage.[105,106] In *D. melanogaster*, mating plug function and composition have been well characterized. The plug is divided into anterior

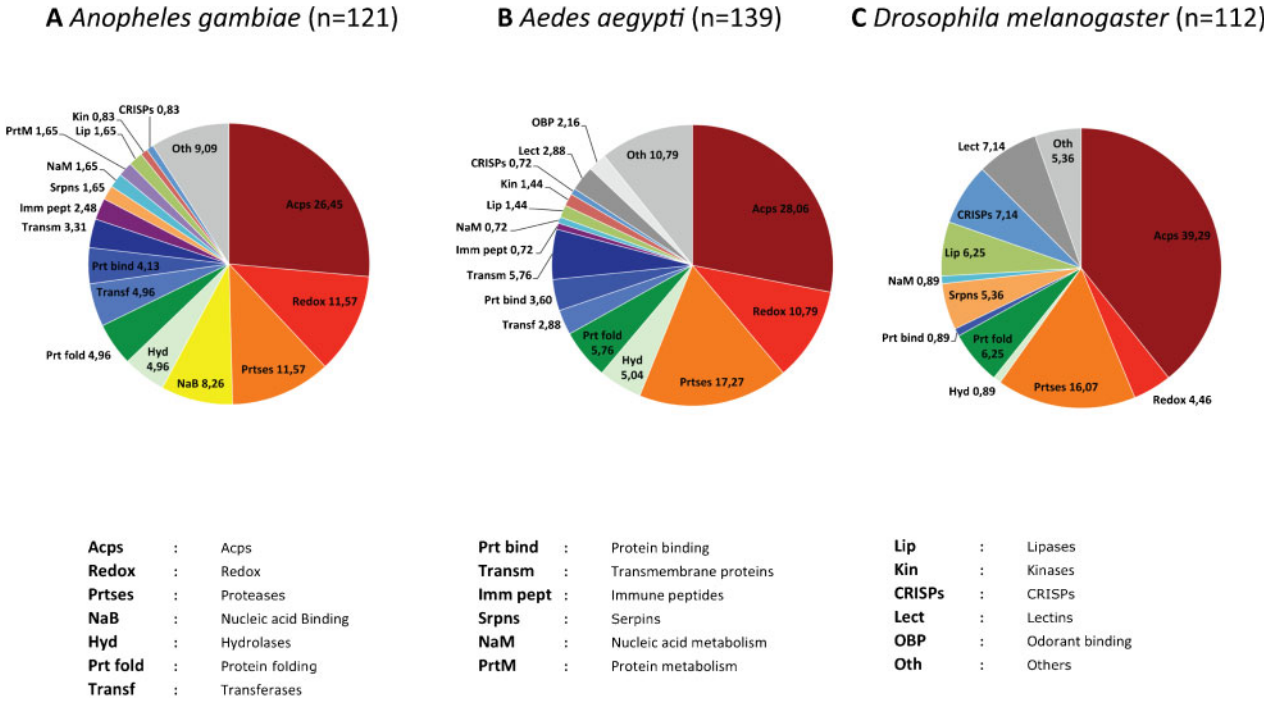

Figure 2 Functional classes of MAG-specific genes in *An. gambiae*, *Ae. aegypti*, and *D. melanogaster*. The pie charts represent the functional classes of genes that are expressed in the male accessory glands of *An. gambiae* (A), *Ae. aegypti* (B), and *D. melanogaster* (C). Values indicate the percentage of genes that belongs to each class in the three species. These charts are based on published data derived from bioinformatics,[34,42,69] transcriptional[34,42,68,69,71] and proteomic[34,43,68] analyses.

and posterior regions: the posterior part is composed of male ejaculatory bulb proteins (PEB-me, PEBII, and PEBIII) and is formed in the female reproductive tract 3 minutes after the start of mating but before sperm transfer.[107] Acquisition of short-term refractoriness is associated with PEBII as shown in knockdown studies.[108] The anterior region of the plug is formed after the transfer of sperm, and it is needed to prevent sperm backflow from the storage organs and is composed of MAG proteins such as Acp36DE.[109] Acp36DE binds to sperm and enters the sperm storage organs,[110,111] where it enhances the rate of sperm accumulation.[112]

Among mosquitoes mating plugs are only found in anophelines.[113] The role of the plug in reproduction has been controversial for decades and various hypotheses ranged from a physical barrier against re-insemination or sperm loss to a vestigial trait with no function.[31,114–116] Recently, it has been shown that in *An. gambiae*, mating plug transfer is crucial for correct sperm storage by the female after mating.[68] Through RNAi-mediated knockdown of a MAG-specific plug-forming transglutaminase (TGase), Rogers *et al.*[74] were able to show that females mated to males who failed to form and transfer the plug could not store sperm in their spermatheca, uncovering a crucial role of this feature in the reproduction of *An. gambiae*. This MAG-specific TGase coagulates seminal secretions by cross-linking other MAG secreted proteins, primarily Plugin, a glutamine-rich protein that is highly abundant in the mating plug.[68] This mechanism is remarkably similar to semen coagulation in mammals.[117] While *An. gambiae* has three TGases, of which only one is active in the MAGs, the genomes of the culicines *Ae. aegypti* and *C. quinquefasciatus* contain only two TGase and none shows activity in the male glands, consistent with the inability of *Aedes* and *Culex* mosquitoes to produce a mating plug.

The *An. gambiae* plug is digested in the female atrium during the first 24–36 hours post-mating, possibly by female proteases,[68,118] and this processing may produce factors that affect female post-copulatory behavior. This might explain the frequently observed inability of MAG transplantation or extract injections to induce oviposition and sexual refractoriness in *An. gambiae*, in contrast to what is observed in culicines.

Conclusions and Outlook

To date, the most effective strategies for the control of *An. gambiae* mosquitoes rely on the use of insecticides through indoor residual sprays and long-lasting insecticide treated bednets.[119] In many regions where these tools are used, the size of vector populations is decreasing significantly, contributing to reducing malaria transmission and therefore placing these regions in the Malaria Elimination Group.[120] However, in much of sub-Saharan Africa, the use of insecticides is not sufficient to stop the spread of disease. Furthermore, the origin and spread of insecticide resistance in vector populations is reducing the effectiveness of insecticide-based strategies.[121] In this scenario, the study of the processes shaping the biology and physiology of *An. gambiae* mosquitoes and other disease vectors brings new promise to the generation of novel ideas and to the identification of targets for the manipulation of the mosquito vectorial capacity. Recent studies reviewed here have identified the factors produced and secreted by the MAGs in several species. However, the functions of the majority of these factors remain unknown.

Characterizing the effects of MAG proteins and of the female genes that they target can provide a gateway to understanding the genetic modulation of mosquito reproduction. This knowledge would benefit the development of novel control programs based on the genetic modification of the vector at two different but complementary levels. On the one hand, it may help generate males with increased mating competitiveness, crucial for a successful deployment of SIT and related strategies. Laboratory reared mosquitoes generally show extremely low mating success in competition with their wild counterparts (reviewed in Ref. 122). There is growing evidence that the MAGs are associated with male reproductive success across insects. In some species, including mosquitoes,[123] depletion of the MAGs may result in infertility long before males exhaust their supplies of sperm. Moreover, seminal fluid availability can contribute to male motivation to mate in the first place,[124] and a lack of accessory gland material as a result of sexual immaturity or exhaustion is often associated with low mating rates.[125] In laboratory-reared anophelines, male mating rate reaches a maximum approximately 3–7 days after eclosion[12,126–128] which corresponds to the period of time required to synthesize MAG secretions (72–100 hours).[129] Improving the diets of adult male mosquitoes may be a simple way to improve mating competitiveness by promoting the rapid maturation and final size of the MAGs. For instance, supplementing the pre-release diets of males with protein or juvenile hormone analogues has been shown to dramatically increase the mating competitiveness of sterile males in several fruit fly species (reviewed in Ref. 130).

On the other hand, the study of reproductive biology will identify male genes important for fertility that could be targeted to induce genetic sterility in males for release, while genes responsible for female fertility could be disrupted in homing endonuclease mediated population depletion.[131] Understanding the

mode of action of MAG proteins may also allow the development of novel chemosterilants. Synthetic compounds could be developed that mimic the behavior-modulating effects of MAG proteins, or prevent the function of factors essential for fertility. Inducing the post-mating response, particularly the inhibition of remating, in virgin females would provide an excellent addition to the vector control arsenal. Novel chemosterilants would provide a second line of defense when used in combination with traditional insecticides used as indoor residual sprays or insecticide treated bednets, as resistant mosquitoes that escape insecticide action would be rendered sterile, preventing the spread of resistance genes. Moreover, as many MAG genes evolve rapidly[132] and are therefore highly divergent between closely related species, it may be possible to develop chemosterilants that would precisely target only the desired vector. One example might be the MAG-specific plug forming transglutaminase identified by Rogers *et al.*[68] whose function is important for ensuring correct storage of sperm by the female and which has no direct orthologue in aedine or culicine mosquitoes. A molecule that specifically inhibits this TGase, but not other related enzymes, could provide a specific mechanism for reducing the fertility of anopheline mosquitoes. Although speculative at present, these strategies are potentially highly rewarding. The full feasibility of such measures will only become clear once we have an improved understanding of the multiple functions of the MAGs.

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