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A comparative analysis of reproductive biology of insect vectors of human disease

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Abstract

Studying the reproductive strategies of insect species that transmit diseases to humans can identify new exploitable targets for the development of vector control methods. Here we describe shared characteristics and individual features of the reproductive biology of three major disease vectors: *Anopheles gambiae*, *Aedes aegypti* and *Glossina morsitans*. Current studies are identifying i) species-specific molecular cascades that determine female monandrous behavior, ii) core aspects of egg development that could be disrupted for controlling natural populations, and iii) the increasingly apparent role of resident microbiota in shaping reproductive success and disease transmission potential. The recent completion of multiple genome sequencing projects is allowing comparative genomics studies that not only increase our knowledge of reproductive processes but also facilitate the identification of novel targets for vector control.

Introduction

The global burden of diseases spread by the biting of insect vectors is a heavy one: more than 17% of all infectious diseases are transmitted by vectors, and it is estimated over half the world's population is at risk, with more than 1 million deaths every year (World Health Organization; URL: www.who.int). Blood feeding is necessary for insect vectors to obtain nutrients required for energy and reproduction, and various viruses and parasites have evolved to exploit this requirement as a way to move between hosts. Current vector control strategies heavily rely on insecticides, which are nevertheless thwarted by the spread of resistance alleles in insect populations. Studying the basic reproductive biology of insect vectors of human disease can identify broad-ranging or species-specific reproductive targets

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that can be exploited for the development of novel control methods as alternatives or to complement the use of insecticides.

In this review, we will focus on recent findings concerning the reproductive biology of disease vectors from the genera *Anopheles* (malaria), *Aedes* (yellow fever, dengue fever, chikungunya), and *Glossina* (human African trypanosomiasis), which account for the vast majority of global vector-borne mortality. Crucial aspects of mating, egg development and symbiotic relationships will be discussed with the end goal to highlight possible weak links in these life cycles that can be exploited for disease control.

Mating strategies and post-mating behavior of insect vectors

Insect vectors show various mating strategies depending on species-specific behaviors and ecologies. Mosquitoes (sub-Order Nematocera) are distant relatives of the tsetse flies (sub-Order Brachycera). Nevertheless, their mating behaviors bear some similarities (Table 1). Most *Anopheles* species mate in crepuscular swarms formed over particular markers on the ground [1-3], where males gather at dusk and attract females by as yet unknown mechanisms, likely based on visual and chemical cues. *Aedes* mosquitoes, although they may show swarming behavior, prefer instead to mate in proximity to the hosts on which they feed [3]. Similarly, tsetse flies mate in close proximity to their vertebrate hosts and utilize visual cues to identify mating partners. Mating begins once a contact-based pheromone on the female is detected by the male [4] and pairs must remain coupled for 1.5-2 hours for the pairing to be successful [5]. In contrast, mosquito matings are short (10-20 seconds).

Regardless of the mating strategy, in all three genera sperm transferred during mating are stored in a dedicated sperm storage organ: a single spermatheca in *Anopheles*, two in tsetse, and two spermathecae and a bursa in seminalis in *Aedes*. Males of most *Anopheles* species are exceptional in the coagulation of their seminal fluid to form a mating plug, a gelatinous rod rich in proteins, lipids and steroid hormones produced in the male accessory glands (MAGs) which upon sexual transfer is processed in the female reproductive tract [6-11]. In *An. gambiae*, the major malaria vector, transfer of the mating plug is linked to sperm storage, as females that do not receive a plug do not store sperm in their spermatheca [7]. *Aedes* seminal fluid is not coagulated but nevertheless contains a complex mix of bioactive peptides [12, 13].

Seminal fluids in *Glossina* are transferred to the female reproductive tract, where they coagulate into a structure called a spermatophore, which also contains the sperm bundle [14]. After mating, the spermatophore is broken down over 24 hours and sperm migrate to the spermathecae. The constituent proteins and chemical moieties associated with this structure remain however undefined. Despite wide evolutionary distance, *Anopheles*, *Aedes* and *Glossina* share a female monandrous behavior (i.e. the occurrence of a single mating event during the female's lifespan). This mating strategy could potentially be targeted using chemical analogs that mimic monandry-inducing factors, preventing virgin females from mating, thereby decreasing vector populations. The male triggers of monandry have been recently identified in *An. gambiae*: high titers of the steroid hormone 20-hydroxyecdysone (20E) transferred to the female atrium (uterus) within the mating plug contribute to

switching the female to a mated status, rendering her refractory to further copulation (among other physiological changes – see below)[10]. As discussed later, both 20E and its precursor ecdysone (E) are also produced by the female after a blood meal, where they are essential for egg development.

Conversely, the molecular basis of monandry is unknown in *Aedes*, although a number of early studies suggest a role for peptides synthesized in the MAGs [15]. MAG protein extracts were able to induce mating refractoriness and oviposition when injected into virgin female mosquitoes [16, 17]. Recent work aimed at molecularly characterizing components of *Aedes* seminal fluid has identified a number of male proteins that are transferred to the female, so that the specific factors required to induce monandry in these mosquitoes may be pinned down in the near future [12, 13].

Female tsetse flies also become refractory to further copulation after sex [18], a behavior that starts 24 hours after mating [19, 20]. Injection of MAG extracts can induce mating refractoriness [21], suggesting that factors produced by the male glands are the trigger of this behavior. Spermatophore digestion over 24 hours correlates with the initiation of refractoriness behavior in females, but the nature of the molecular triggers is not known.

Egg development is a conserved process in different vectors

Much of what we know of the molecular mechanisms of oogenesis comes from studies in *Ae. aegypti*. Here, egg development is triggered by both the nutritional status of the female and the taking of a blood meal (reviewed in [22]). After emergence, the sesquiterpene juvenile hormone (JH) is secreted by the corpora allata in the brain and coordinates the maturation of multiple tissues. As JH levels increase and peak over the first 2 days of adulthood, the multifunctional fat body, with roles in nutrient storage, detoxification and protein synthesis, undergoes structural remodeling and large JH-dependent changes in gene expression that render this tissue competent to respond to ecdysone produced by the ovary after blood feeding [23]. JH also causes the pre-vitellogenic development and maintenance of ovarian follicles, which accumulate lipids and transcripts for key proteins involved in uptake of yolk protein precursors (YPPs) [24, 25]. Moreover, JH delivered by *Ae. aegypti* males during mating [26] also increases female fecundity [17, 27] by directing available nutrient resources towards reproduction, enlarging ovarian follicles and preventing follicle resorption [28].

After taking a blood meal *Aedes* mosquitoes develop eggs over 2-3 days. The mosquito brain stops JH synthesis and releases the ovarian ecdysiotropic hormone (OEH)[29], triggering the ovaries to produce the steroid hormone ecdysone [30]. Ecdysone is hydroxylated in turn to 20E in the fat body. Through the 20E receptor EcR/USP and early-acting genes E74, E75 and Broad [31-33], 20E stimulates the transcription of YPPs, such as vitellogenin and lipophorin, which are released into the hemolymph and taken up by the ovaries by receptor-mediated endocytosis [34-36]. Additionally, levels of extracellular amino acids released by blood meal digestion trigger YPP production via the TOR-signaling pathway [37].

These oogenic processes are considered largely conserved in *Anopheles* as many of the molecular components are found in the genome [38], however published data confirming a role for JH and OEH is limited [39, 40]. In *An. gambiae*, fecundity is also augmented by mating, and the mating-induced trigger of egg development occurs via the interaction between sexually transferred 20E received in the mating plug and the atrial protein Mating-Induced Stimulator of Oogenesis (MISO) [9]. The MISO-dependent increase in egg numbers is characterized by an enhanced expression of the YPP lipophorin after a blood meal, possibly suggesting that male 20E primes the fat body for the production of YPPs.

The fundamental biology behind oocyte development in tsetse is similar to that observed in other Diptera, yet is markedly reduced in scale; tsetse ovaries are significantly smaller and contain only two ovarioles per ovary for a total of four ovarioles. During each gonotrophic cycle a single oocyte develops at any given time, independently of mating. The remaining ovarioles are held in a state of arrest that is broken when oocyte development is complete and the oocyte is ovulated into the uterus. Flies utilize lipase-derived YPPs (similar to other Brachyceran flies) that are synthesized and secreted exclusively by the ovarian follicle cells, as opposed to mosquitoes that utilize vitellogenins secreted by the fat body [41]. Egg development processes in mosquitoes and tsetse are summarized in Figure 1.

After development in the ovary, oocytes are ovulated and fertilized as they move through the female's reproductive tract. In mosquitoes, fertilized embryos are laid seconds later on (*Anopheles*) or close to (*Aedes*) water, where they hatch into an aquatic larval life stage (Figure 2). As virgins can develop eggs but do not lay them, mating triggers ovulation and egg-laying behavior. In *An. gambiae* once again the male-transferred 20E has recently been identified as both a necessary and sufficient trigger of oviposition [10]: 38% of females that were mated to males with experimentally reduced 20E levels were not able to lay their eggs compared to 14% of females mated to control males; consistently, oviposition was stimulated in virgin blood-fed females by the injection of 20E in a dose-dependent manner [10]. 20E also regulates fertility over multiple blood feedings preserving sperm function by up-regulating a spermathecal detoxifying enzyme, the heme peroxidase HPX15, and possible other mechanisms [42]. No information is yet available on the identity of the triggers of oviposition in *Aedes*, although, again similar to *Drosophila*, small proteins and peptides produced by the MAGs are likely candidates.

In tsetse, the oocyte is ovulated and fertilized as it enters into the uterus. Ovulation in tsetse is dependent upon the mating and pregnancy status of the fly [18]. Like mosquitoes, unmated females develop oocytes but are unable to ovulate until after mating. MAG proteins do not appear to regulate ovulation as mechanical stimulation by intrauterine implantation of glass beads has the same effect as mating [21]. However, ovulation can be induced in virgin females by the injection of hemolymph from mated pregnant females, which indicates an ovulation factor is systemically released in response to mating and oogenic stimuli [5].

At this point tsetse's reproductive cycle veers dramatically from that of oviparous insects (Figure 2). The embryo is retained within the uterus and hatches into a larva, which grows within the mother for its entire 3 instar developmental cycle and is fed by nutrients generated by an adapted female accessory gland termed the milk gland. This is the defining

characteristic of obligate adenotrophic viviparity. The milk secretions generated by the mother consist of roughly 50% lipids and 50% protein. During lactation stored lipids are mobilized from the fat body to the milk gland, and this transfer is regulated by JH and insulin signaling [43]. Milk contains at least 12 proteins, 10 of which are specific to tsetse [44] and are partly regulated in a milk gland- and pregnancy-specific manner by a transcription factor called Ladybird late [45]. This factor/regulatory system appears conserved among the Brachyceran dipterans and may also regulate female accessory function in other vector species within the Nematocera.

Microbes influence the reproductive success of insect vector species

It has long been known that tsetse fly reproduction is strongly dependent upon its relationship with an obligate endosymbiont, *Wigglesworthia* [46]. *Wigglesworthia* live intracellularly in a specialized organ in the gut named the bacteriome, and extracellularly within the lumen of the milk gland, allowing bacteria to be vertically transmitted to the developing larva [47, 48]. These symbionts supplement tsetse's rich but nutritionally limited blood diet with essential nutrients and cofactors required for energy metabolism. The *Wigglesworthia* genome encodes the synthesis pathways for multiple B vitamins, one of which (vitamin B₆) functions as a co-factor for proline biosynthesis in the fly [49]. Proline functions as tsetse's primary energy source and is required to maintain the energetic process of milk production during pregnancy. In addition to *Wigglesworthia*, laboratory strains of tsetse have endosymbiotic relationships with *Sodalis* and *Wolbachia* bacteria. *Sodalis* do not affect tsetse's reproductive physiology but are required for longevity and can reduce trypanosome infection intensity [50], while *Wolbachia* induce strong cytoplasmic incompatibility [51], a phenomenon discussed below. All three symbionts may be exploited in paratransgenic anti-pathogen strategies to create trypanosome-resistant tsetse populations [52].

In mosquitoes, antibiotic treatment does affect reproductive output, indirectly implying roles for microbiota in reproductive fitness [53, 54]. Well studied are the reproductive effects of *Wolbachia*, an endosymbiont which resides within the germlines of many arthropod species (including tsetse and some mosquitoes), and is vertically transmitted from the female parent to progeny. Initially characterized in *Culex* mosquitoes [55], *Wolbachia* can cause cytoplasmic incompatibility (CI - reviewed within [56]) whereby matings between uninfected females and infected males result in embryonic lethality in the progeny, while matings of infected females produce fertile progeny regardless of the infection status of the male. *Wolbachia* infection can cause additional reproductive phenotypes, including increased fecundity and hatching rates in *Aedes albopictus* [57, 58]. Because of these reproductive phenotypes, *Wolbachia* infections can rapidly spread through natural insect populations, and have been detected in *Aedes* [59], *Glossina* [51, 60] and more recently in *Anopheles* species from West Africa [61]. Additionally, *Wolbachia* infections have also been shown to block human pathogen transmission in *Ae. aegypti* [62, 63] and in *An. stephensi* [64, 65], prompting their current and proposed use in disease control programs [61, 66, 67] (Eliminate Dengue Program; URL: www.eliminatedengue.com). A specific effect of *Wolbachia* on trypanosome infection intensity or on tsetse fecundity has not yet been fully established.

Comparative genomics to identify shared and species-specific reproductive pathways

Recent publication of the genomes of the major tsetse fly vector species *G. morsitans morsitans* [68] and 16 *Anopheles* species [69] has provided exceptional opportunity to study key biological questions of vector species. How do tsetse achieve their unusual reproductive biology? What are the individual and common determinants of reproductive success in anophelines? What species are likely to be targeted by particular reproductive control strategies? The anticipated genome release of the invasive mosquito species *Ae. albopictus* [70] and improvements to the assembly of the *Ae. aegypti* genome [71] will enable similar comparative analysis for *Aedes* mosquitoes.

The analysis of the tsetse genome has allowed a first insight into how reproduction diverged so significantly in these vectors [68]. The reduced capacity for oogenesis in tsetse may have resulted in the reduction in YPP genes; while close relatives of tsetse carry three or more YPPs, tsetse has only one, YP1. Conversely, the evolution of lactation has resulted in the expansions of families of milk protein genes, likely via a series of gene duplication events. Nine of these genes are clustered within a single 40 kb region of the genome and have no known orthologs in other Diptera. They are only expressed in the secretory cells of the milk gland and in coordination with the pregnancy status of the mother. Novel insights into the reproductive biology of *Anopheles* mosquitoes have been provided by the 16 genome sequencing project [69]. Previous work in the African vector *An. gambiae* had shown that formation of the mating plug depends on the crosslinking activity of a MAG-specific transglutaminase enzyme, AgTG3 [7, 72]. Phylogenetic analysis across 16 anophelines showed that AgTG3 is highly divergent and more rapidly evolving than the other two TGs present in the genome [69], possibly reflecting divergence in mating plug phenotypes. This hypothesis was confirmed by a phenotypic study where semen coagulation and 20E synthesis by the MAGs were determined in eight *Anopheles* species besides *An. gambiae*. While three anophelines (*An. arabiensis*, *An. funestus* and *An. stephensi*) had a fully coagulated plug and high 20E levels in the MAGs similar to *An. gambiae*, others (*An. atroparvus*, *An. dirus*, *An. farauti*, and *An. sinensis*) showed intermediate coagulation and hormone synthesis, while the New World species *An. albimanus* was the only species that completely lacked both plug formation and male 20E synthesis [11]. These findings indicate the occurrence of different reproductive strategies across the *Anopheles* genus. Ancestral state reconstruction analyses determined that plug coagulation and 20E synthesis in the MAGs are derived characters that have co-evolved in anophelines from a plugless and 20E-less ancestor [11]. Given the role of 20E transfer in switching off the female receptivity to further mating and in inducing oviposition [10], these data suggest that strategies targeting this steroid hormone may be successful in preventing successful mating and reproduction in a number of malaria vectors, demonstrating the power of comparative genomics.

Conclusions

The 200 million years of divergence separating mosquitoes and tsetse flies is reflected in the remarkable differences in their reproductive biology. Control methods based on reducing the

reproductive output of these insect vectors of disease are likely to be highly specific compared to wide-spectrum insecticides, and less harmful to the native ecology. On the other hand, these species share a male-triggered monandrous behavior that suggests these species could all be vulnerable to control strategies that either mimic or disrupt key factors transferred at mating, such as the steroid hormone 20E in *Anopheles*. The slow, reduced rate and unusual method of reproduction in tsetse makes this species an especially attractive target for control strategies based on disrupting the reproductive cycle, perhaps by interfering with milk protein production in lactation or exploiting its obligate symbiotic relationships. The next steps are to translate these laboratory findings into effective strategies to control insect populations in disease-endemic areas, producing a significant impact on the global burden of vector-borne disease.

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Highlights

1. Targeting reproduction may lead to new ways to control vector-borne disease
2. Monandry is a weak link in the life cycles of disease vectors
3. Tsetse fly reproduction is unique and includes pregnancy and lactation
4. The microbiome can influence the reproductive success of disease vectors
5. Comparative genomics powerfully reveals shared and species-specific mechanisms

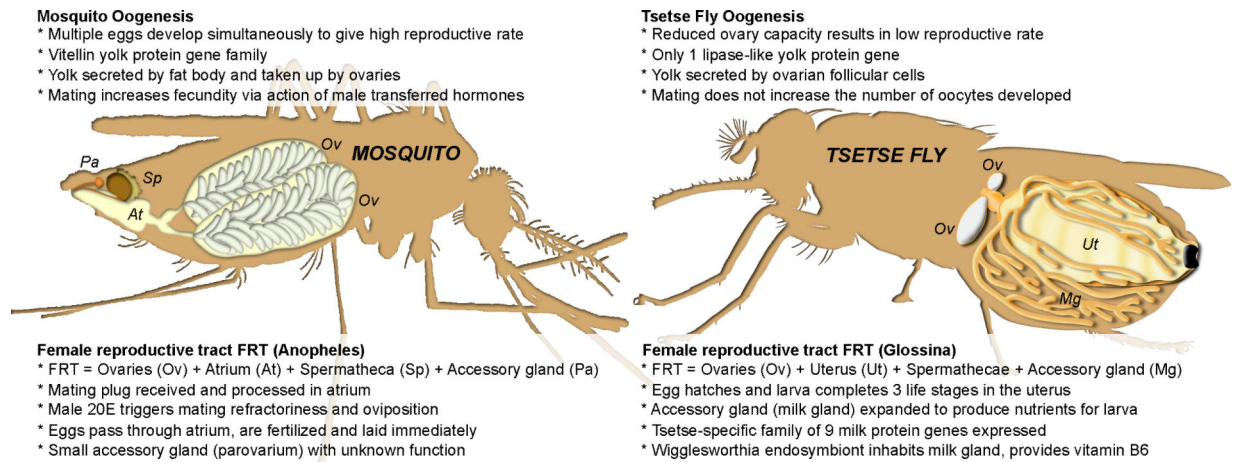


Figure 1. Oogenesis in mosquitoes and tsetse flies

An *Anopheles* mosquito is shown on the left and a tsetse fly on the right. Eggs are shown within the ovaries. The spermathecae of the tsetse fly are not shown for clarity and a 3rd instar larva is shown within the uterus. *Aedes* mosquitoes have a similar reproductive tract structure to *Anopheles*, but have three sperm storage organs (two spermathecae and a bursa in seminalis).

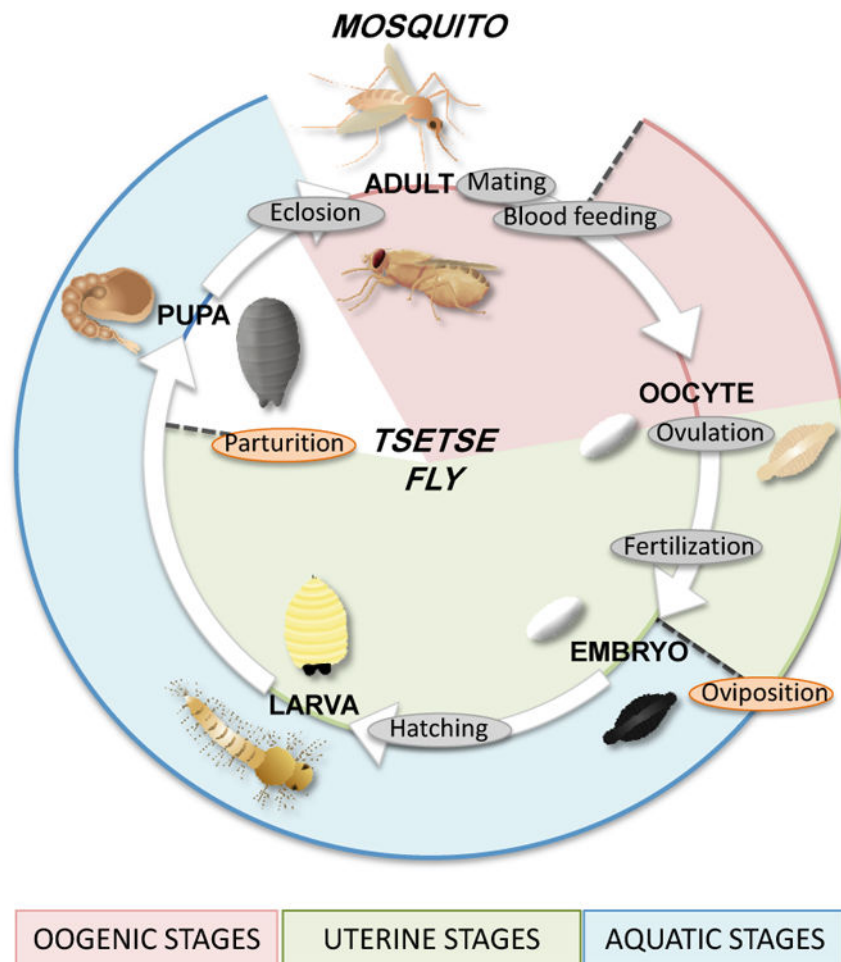


Figure 2. Life cycles of mosquitoes and tsetse flies

Life cycles are shown on the same circle, with mosquitoes on the outside and tsetse flies on the inside of the circle. Common events are labeled in gray, and species-specific events in orange. After blood feeding, mosquitoes develop multiple oocytes within the ovaries, which are ovulated and fertilized as they pass through the reproductive tract. The embryos are laid immediately on (*Anopheles*) or close to (*Aedes*) water and hatch into larvae. The larvae molt through 4 instars and become pupae, before eclosing into adult mosquitoes. Tsetse flies begin egg development during the pupal stage and continue to develop oocytes after blood meals. A single oocyte is developed at one time and is ovulated into the uterus. The oocyte is fertilized and the embryo remains in the uterus, where it hatches into a larva. The larva is fed by nutrients from the milk gland and completes 3 further molts within the uterus. The female gives birth to a 3rd instar larva, which rapidly pupates, before eclosing into an adult.

Table 1
Comparison of the mating biology of major disease vectors

Mating biology	<i>Anopheles</i>	<i>Aedes</i>	<i>Glossina</i>
<i>Mating in swarms</i>	Yes	Possible	No
<i>Mating near host</i>	No	Yes	Yes
<i>Mating duration</i>	15-20 sec	10-15 sec	90-120 min
<i>Coagulated seminal fluids (SF)</i>	Yes	No	Yes
<i>Identified SF components</i>	20E, TG3	JH	None
Post-mating changes			
<i>Female monandry</i>	Yes	Yes	Yes
<i>Fecundity increased</i>	Yes	Yes	No
<i>Ovulation induced</i>	Yes	Yes	Yes
<i>Sperm storage</i>	Yes	Yes	Yes