

Mating activates the heme peroxidase HPX15 in the sperm storage organ to ensure fertility in *Anopheles gambiae*

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Anopheles gambiae mosquitoes are major African vectors of malaria, a disease that kills more than 600,000 people every year. Given the spread of insecticide resistance in natural mosquito populations, alternative vector control strategies aimed at reducing the reproductive success of mosquitoes are being promoted. Unlike many other insects, *An. gambiae* females mate a single time in their lives and must use sperm stored in the sperm storage organ, the spermatheca, to fertilize a lifetime's supply of eggs. Maintenance of sperm viability during storage is therefore crucial to the reproductive capacity of these mosquitoes. However, to date, no information is available on the factors and mechanisms ensuring sperm functionality in the spermatheca. Here we identify cellular components and molecular mechanisms used by *An. gambiae* females to maximize their fertility. Pathways of energy metabolism, cellular transport, and oxidative stress are strongly regulated by mating in the spermatheca. We identify the mating-induced heme peroxidase (HPX) 15 as an important factor in long-term fertility, and demonstrate that its function is required during multiple gonotrophic cycles. We find that *HPX15* induction is regulated by sexually transferred 20-hydroxy-ecdysone (20E), a steroid hormone that is produced by the male accessory glands and transferred during copulation, and that expression of this peroxidase is mediated via the 20E nuclear receptor. To our knowledge, our findings provide the first evidence of the mechanisms regulating fertility in *Anopheles*, and identify *HPX15* as a target for vector control.

The *Anopheles gambiae* mosquito is the major vector for malaria, an infectious disease that accounts for more than 600,000 deaths per year (1), mostly in sub-Saharan Africa. The high reproductive capacity of these mosquitoes contributes to their role as disease vectors, with a single female able to produce several hundred progeny during her lifetime. To reproduce, the male transfers to the female sperm, produced by the testes, and seminal secretions from the male accessory glands (MAGs), delivered as a coagulated gelatinous mating plug. The mating plug is composed of proteins and lipids, and delivers the male-produced steroid hormone 20-hydroxy-ecdysone (20E) (2–4). Unlike other insects, *An. gambiae* females mate a single time in their lives and must therefore preserve the viability of stored sperm for their lifetime in a specialized sperm storage organ, the spermatheca. As replenishment of sperm stores does not occur, multiple pathways must be in place to nourish and protect sperm in storage in the event of physiological stresses, such as repeated blood feedings needed for egg development during multiple gonotrophic cycles (5, 6). The maintenance of sperm viability therefore is a crucial stage in the mosquito life cycle that could be targeted to reduce the fertility of field malaria-transmitting populations.

Unlike *Drosophila melanogaster* and *Aedes aegypti*, which possess three sperm storage organs, *An. gambiae* females have a single spermatheca (7), enclosing sperm within a cuticular capsule produced by a thin covering of epithelial cells. Behind this layer are secretory glandular cells, connected by pores to the capsule

lumen, and fat body cells, containing energy stores. Sperm enter and exit the spermatheca via a single duct that is connected to the posterior of the lower reproductive tract (7).

Studies in *Drosophila* have shown that the sperm storage organs act to nourish and protect sperm by creating an appropriate environment through secretion: genetic or targeted disruption of the secretory glandular cells or of the secretory pathway results in nonfunctional spermathecae and reduced fertility phenotypes (8–11). Transcriptional and proteomic profiling of sperm storage organs and their secretions in *Drosophila* and *Apis* have identified pathways involved in carbohydrate and lipid metabolism, oxidative stress, and antimicrobial responses that are likely to play roles in nourishing and/or protecting sperm from damage caused by infection and reactive oxygen species (ROS) (9, 12–15). Additionally, these studies identified an array of proteases and detoxification enzymes that may process small molecules (peptides and steroid hormones) transferred from males during mating, and may act to regulate postmating reproductive processes, including sperm storage and activation (9, 12, 13).

Significance

Successful fertilization requires viable sperm and eggs to meet. Some insects, such as the *Anopheles gambiae* female mosquito, the principal vector of malaria, mate only once and keep sperm received from a male in a specialized sperm storage organ while eggs are developed after taking a blood meal. Sperm are kept functional for several weeks, but the factors and mechanisms that achieve this preservation are unknown in this mosquito. Here we identify a heme peroxidase *HPX15* and other mechanisms activated by sex that are important to preserve the functionality of stored sperm and long-term fertility. Disrupting the reproductive cycle in field *Anopheles* would reduce numbers of mosquitoes transmitting malaria, aiding in the fight against one of the world's deadliest diseases.

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In contrast to *Drosophila* and *Apis*, very little is known in *An. gambiae* about the factors and mechanisms that preserve sperm functionality in the spermatheca, and, to date, few spermathecal genes have been identified in these mosquitoes (16). Exposure to repeated blood feedings, each generating large amounts of ROS (17), may pose particular challenges for sperm function, as ROS can reduce the viability of stored sperm in insects (18).

Here we identify factors and pathways triggered by mating in the spermatheca that are important for ensuring fertility of *An. gambiae* females. By using a combination of morphological, transcriptional and functional analyses, we demonstrate that the mating-induced heme peroxidase *HPX15* is required for preserving long-term fertility during multiple cycles of blood feeding and egg development. Moreover, we show that *HPX15* expression after mating is regulated by the sexually transferred steroid hormone 20E via its nuclear receptor Ecdysone receptor (EcR). Our results demonstrate that sperm function can be impaired in these important malaria vectors, affording opportunities for vector control.

Results

EM Reveals Important Features of Spermathecal Cells. The spermatheca must act to preserve sperm quality and function for several weeks by controlling the physiological environment in which sperm are stored. To identify cellular components important for sperm functionality, we initially performed an ultrastructural analysis of virgin and mated spermathecae at 24 h post mating (hpm), and also included samples derived from 24-hpm females that were blood-fed immediately after copulation. EM analyses detected features that may be indicative of processes needed for sperm preservation, although they did not reveal major differences between treatments. Three major cell types (fat body, secretory, and epithelial) were identified (Fig. 1) by comparison with similar work done in other mosquitoes (7). Fat body cells have large nuclei, numerous mitochondria, rough endoplasmic reticulum (RER), and are filled with glycogen granules and lipid globules, providing potential energy stores for sperm (Fig. 1 *B* and *C*). Secretory cells contain an extensive RER, a basal labyrinth, and membranous vesicles or lamellar bodies (Fig. 1*D*), suggesting high transport and secretory capabilities into the spermatheca lumen. In addition, a complex array of apical membranes (Fig. 1*B*) appears to secrete very electron-dense particles (Fig. 1*E*). We detected a very thin layer of epithelial cells immediately behind the cuticular capsule, likely producing it. The cuticle contains pores 2–3 μm in diameter, through which secretory and epithelial cells project into the lumen (Fig. 1 *B* and *E*). Sperm contained in the lumen are in contact with glandular cell secretions and are aligned to form bundles. Damage to sperm cells was visible as disrupted axoneme microtubules in two of three blood-fed females (Fig. 1*F*).

During mating sperm enter the spermatheca via a long duct originating from the atrial lumen. Transverse sections of the spermathecal duct showed a concentric layered structure (Fig. 1 *H* and *I*) around a star-shaped lumen. Outside the exterior muscle layer, possibly regulating duct contractions, large secretory glandular cells filled with many mitochondria and membranes are connected to the duct lumen by radial ductules crossing all underlying layers (Fig. 1*H*). These ductules are produced within specialized epithelial cells, and also have a cuticle layer in their lumen. Diagrams representing the organelles found within a typical secretory cell and a section through the duct are shown in Fig. 1*G* and Fig. 1*I*.

The Spermatheca Undergoes Large Transcriptional Changes After Mating. Based on previous data showing that mating induces a large transcriptional response in whole females (16), we decided to determine which mating-induced changes occur specifically within the spermatheca to achieve its function. We

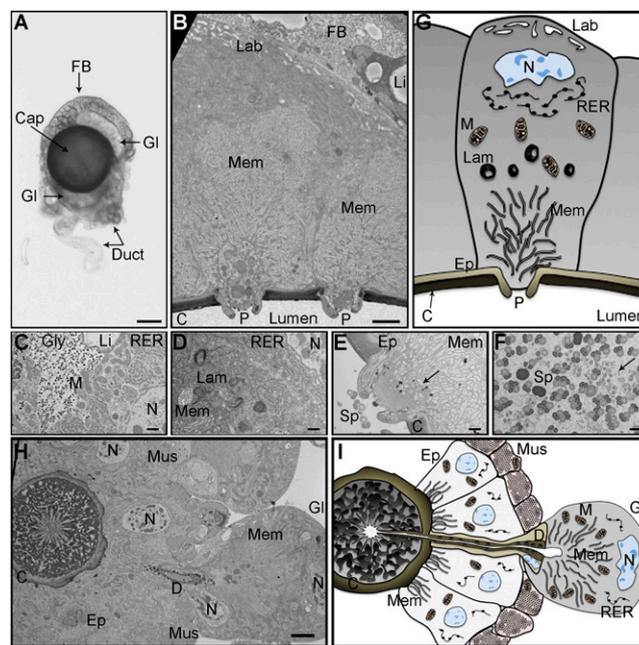


Fig. 1. Ultrastructural features of spermathecal cell types. Transmission EM images of spermathecae from virgin or mated (24 hpm) with or without blood meal females. (A) Spermatheca at low magnification, showing the arrangement of secretory glandular cells and fat body cells around a capsule (mated). (Scale bar: 50 μm .) (B) Secretory cells contain a membrane network connected to the lumen via apical pores in the cuticle. Fat body cells containing lipids and glycogen are associated with the basal side of secretory cells (virgin). (Scale bar: 2 μm .) (C) Fat body cells also contain RER and mitochondria (virgin). (D) Secretory cells contain lamellar bodies, mitochondria, and RER immediately basal to the membrane network (virgin). (E) Secretory cell pore. Electron-dense particles (arrow) are observed within the membrane network (mated). (F) Sperm are aligned and associate into bundles in the lumen. Damaged sperm (free microtubules marked by arrow) were observed in two of three blood-fed females (mated and blood-fed). (Scale bars: C–F, 500 nm.) (G) Diagram of a secretory cell, showing locations of the organelles. (H) Concentric layers of muscle and epithelial cells, cuticle, and electron-dense material, surround the star-shaped lumen of the spermathecal duct. Glandular cells and part of a duct cell can be seen traversing the concentric layers. Glandular cells contain a large membrane network and extensive mitochondria and RER (mated and blood-fed). (Scale bar: 1 μm .) (I) Diagram of the spermathecal duct. C, cuticle; Cap, capsule; D, duct cell; Ep, epithelial cell; FB, fat body cell; Gl, glandular cell; Gly, glycogen; L, lumen; Lab, basal labyrinth; Lam, lamellar body; Li, lipid globule; Mem, membrane network; Mus, muscle; N, nucleus; P, pore; Sp, sperm.

compared transcript levels in spermathecae dissected from virgin and mated females at 24 hpm by using whole-genome microarrays. We anticipated that this time point would identify candidate genes up- or down-regulated to ensure sperm viability over the longer term. Hits (adjusted $P < 0.005$) were compared to the *An. gambiae* genome (PEST, gene set version 3.6) using basic local alignment search tool and mapped to 217 up-regulated and 203 down-regulated genes (Dataset S1), which were assigned to one of several functional groups by using Gene Ontology terms, Vectorbase annotations (www.vectorbase.org), and functional information from orthologous genes (Fig. 2*A*).

The largest class of up-regulated genes included factors involved in metabolism (70 genes; Dataset S1). This group comprised a number of genes regulating oxidative stress, including the strongly induced heme peroxidase *HPX15*, and detoxifying cytochrome p450 enzymes. Such a strong response against oxidative stress may be essential to prevent sperm damage by ROS, which, even at mildly elevated levels, can reduce sperm function and increase female infertility in insects (18). A high demand for

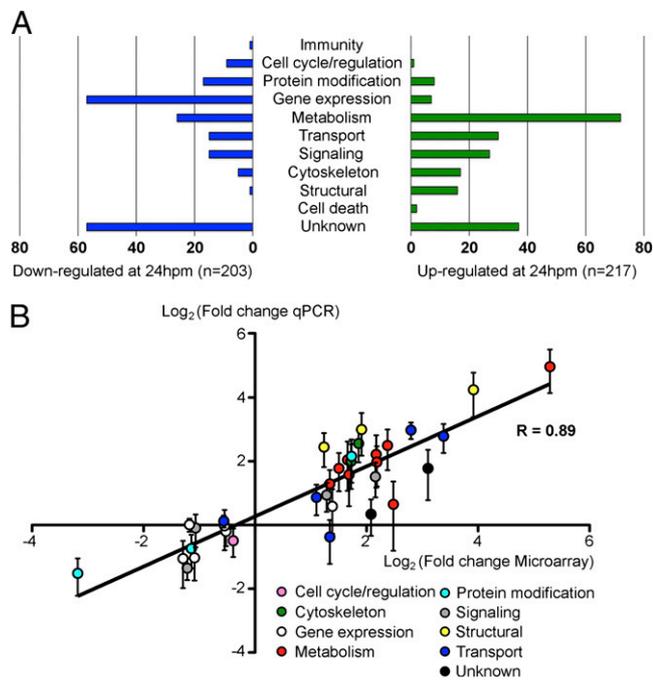


Fig. 2. Mating causes large gene expression changes in the spermatheca. (A) Functional classes of genes up-regulated (green) or down-regulated (blue) at 24 hpm with adjusted $P < 0.005$. The number of genes in each group is on the x axis. (B) Validation of the microarray data by correlation to data from qRT-PCR on 35 genes (24 up-regulated, 11 down-regulated) on three independent biological samples of 10 spermathecae. Genes from each functional group were tested. Filled circles represent mean fold change \pm SEM (Pearson correlation coefficient $R = 0.89$; 95% CI, 0.79–0.94).

energy production in the spermatheca after copulation was suggested by the up-regulation of pathways associated with energy metabolism. These included enzymes for the production of hexoses from oligosaccharides, such as trehalases and α -amylases, and the glycolytic enzymes triose phosphate isomerase, phosphoglycerate mutase, phosphofruktokinase, and pyruvate kinase, the latter two being energetically irreversible and major points of glycolysis regulation (19, 20). This increase in glycolysis may be linked to a breakdown of lipids. Indeed, we detected the up-regulation of a predicted lipase that converts triglycerides to fatty acids and glycerol, and of three acyl-CoA ligases, whose orthologs in *Drosophila* are involved in the activation of long-chain fatty acids (21, 22). A glycerol-3-phosphate dehydrogenase was also identified, which may convert glycerol produced by triglyceride breakdown to dihydroxyacetone phosphate and feed it into glycolysis as a triose. Two enzymes in the tricarboxylic acid cycle were also significantly up-regulated: malate dehydrogenase and an α -ketoglutarate dehydrogenase. Mating also induced many P-type and V-type ATPases involved in cell active transport systems, including the activity-regulating C-subunit, critical in assembly of inactive V0 and V1 subcomplexes into functional H^+ -pumping ATPases (23). Proton gradients generated can be used to drive antiporters of other cations, and an ortholog of the *Drosophila* antiporter *Nha1* (AGAP002093), which exchanges H^+ ions for K^+ ions, was also found among the up-regulated genes. Finally, a possible hardening of the spermathecal capsule after mating was suggested by the induction of multiple genes associated with cuticular sclerotization, such as eight genes belonging to three families of cuticular proteins (TWDL, CPR, and CPAP3), as well as dopa decarboxylase and laccase enzymes.

A similar number of genes were down-regulated in the spermatheca after mating. Factors regulating gene expression represented the largest group (57 genes), and included subunits of

RNA polymerase II and III, RNA splicing factors, ribosome subunits, tRNA-loading enzymes, and protein isomerase chaperones. Genes involved in cell-cycle regulation were also down-regulated, implying that some cell types may become quiescent in response to mating. These genes included a DNA polymerase subunit, DNA helicase, replication factor C, tumor suppressor p53, and mitotic spindle assembly checkpoint protein MAD1. A decrease in the cell cycle rate would also reduce requirements for cell-cycle gene expression. Functional enrichment analysis of the data with the Database for Annotation, Visualization and Integrated Discovery online tool (<http://david.abcc.ncifcrf.gov/>) identified four significant clusters of terms in the up-regulated genes, including energy metabolism from carbohydrates, amine biosynthesis, and ATP-linked ion transport (Table S1). Among the down-regulated genes, the “RNA recognition motif” and “RNA polymerase activity” clusters were significantly enriched.

We went on to validate the microarray data by testing the expression of 35 genes for mating-induced changes by quantitative RT-PCR (qRT-PCR). By using independent virgin and mated spermathecal samples, we observed a good correlation between microarray and qRT-PCR data [$R = 0.89$ on \log_2 (fold change); 95% CI, 0.79–0.94; Fig. 2B and Table S2]. To rule out the possibility that some of the transcriptional changes identified in our microarray analysis were derived from transcripts from sperm cells present in the mated samples, we tested 10% of mating-induced genes in available cDNA samples derived from reproductive tissues dissected from females mated to spermless males (24) (*SI Materials and Methods*). As these females had not received sperm, we reasoned that mating-induced changes in gene expression would be abolished had they derived from sperm transcripts. All 20 genes analyzed were instead induced similarly in the presence or absence of sperm (Fig. 3), demonstrating that the mating-induced transcriptional response occurs in spermathecal cells. Moreover, these data suggest that transfer of sperm is not a trigger of the spermathecal transcriptional response, confirming previous findings that female postmating physiology is not modulated by sperm (24) and pointing at a prominent role for MAG secretions as triggers of transcriptional changes.

HPX15 Is an Active Peroxidase in the Spermatheca. Our microarray analysis revealed the up-regulation of genes involved in limiting oxidative stress, and one of the genes highly induced by mating was the heme peroxidase *HPX15* [AGAP013327, also known as

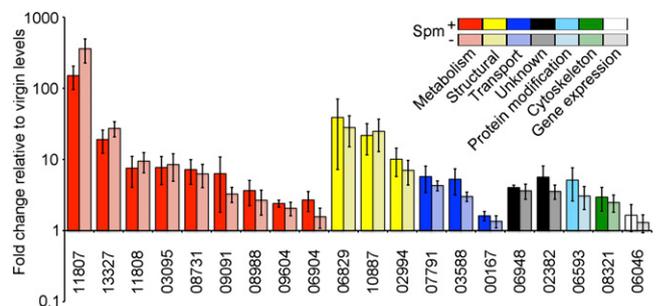


Fig. 3. Sperm transcripts do not contribute to gene expression changes observed in the spermatheca. Expression levels of 20 mating-induced genes were determined by qRT-PCR in the lower reproductive tracts of females mated to sperm-carrying (Spm+) or spermless (Spm-) males and compared with age-matched virgin females. Different functional categories are color-coded. Mean fold changes \pm SEM are plotted. Genes are identified by the last five digits of their Vectorbase identifiers (e.g., 13327 corresponds to AGAP013327). No significant differences were observed in fold changes between spermless and sperm-carrying males (Student t test, critical value: $t_{0.05(2),1} = 12.706$).

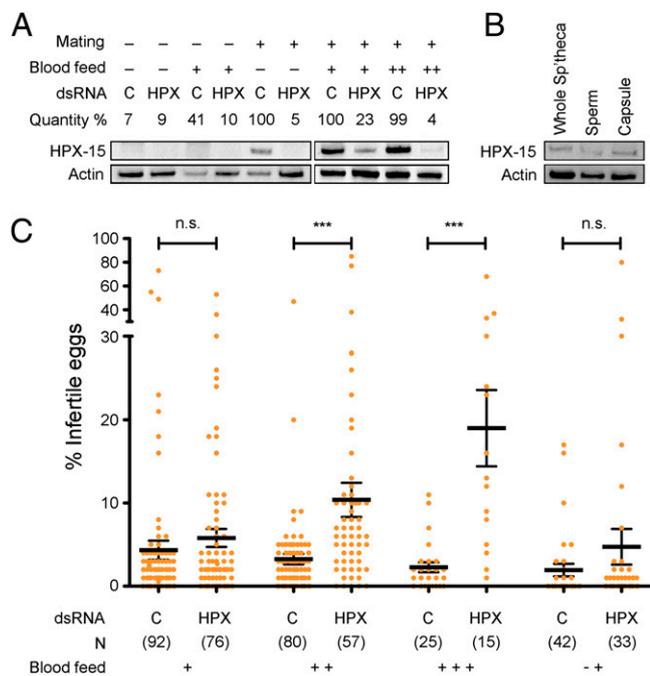


Fig. 5. HPX15 is expressed in the spermatheca after mating and is required to prevent infertility. (A) Western blot analysis of HPX15 in virgin (–), mated (+), and mated blood-fed spermathecae at 24 hpm and one (+) or two (++) blood feedings. C, dsLacZ control injection; HPX, dsHPX15 injection; “Quantity %” represents quantities calculated from mean band intensities on the western blots (SI Materials and Methods). HPX15 silencing exhibited a >70% knockdown compared with control levels (Fig. S1). (B) Western blot analysis of HPX15 in whole mated spermathecae (Sp'theca), sperm bundles isolated from mated spermathecae (Sperm), and the remaining spermathecal cells after sperm bundle removal (Capsule). (C) Percentage of eggs that are infertile laid by females injected with dsLacZ (C) or dsHPX15 (HPX). “–+” signifies the first blood feed was at the time of the second blood feed in “++” experiments. Bars represent mean \pm SEM (*** P < 0.0001, Mann–Whitney U test); n.s., not significant. A minimum of three biological replicates was performed.

Discussion

To our knowledge, this study provides the first characterization of the spermatheca of *An. gambiae* mosquitoes at the morphological, transcriptional, and functional levels. This characterization highlights pathways involved in the regulation of oxidative stress, energy metabolism, and membrane transport as key to maintaining the metabolism and viability of stored sperm, and identifies possible targets to reduce fertility of field populations. We found evidence of a mating-induced response to combat oxidative stress in the spermatheca (Dataset S1), and functional analysis by RNAi showed that expression of HPX15 is required to prevent infertility after multiple blood meals (Fig. 5). To our knowledge, HPX15 is the first factor with a demonstrated role in maintaining fertility in *Anopheles* mosquitoes.

An. gambiae females feed and lay eggs multiple times during their lifetime, and may take multiple blood meals even during the same gonotrophic cycle (5, 6). Therefore, the reduction in fertility seen in our experimental settings might be highly relevant to wild mosquito populations. Although HPX15 levels did not respond significantly to blood feeding (Fig. S2), expression was detected for at least 7 d after mating and blood feeding, suggesting that the induction of this gene has a crucial role in long-term protection of sperm. Despite a good level of silencing, the infertility phenotype was detectable in just a proportion of eggs and after repeated blood meals, suggesting a possible genetic redundancy with other mating-induced antioxidant factors found in our analysis (laccase, vitellogenin, cytochrome p450). Indeed, we observed peroxidase

activity in spermathecal secretory cells even after HPX15 knockdown. Although our data do not prove that HPX15 is induced to neutralize ROS, increased levels of ROS in stored sperm can cause infertility in insects (18), and mechanisms to prevent this damage, such as the expression of antioxidants, should evolve, and have evolved, in other insects with sperm storage strategies (14, 15, 18). HPX15 could be protecting sperm cells directly, as indicated by its secretion into the spermathecal lumen and by the presence of peroxidase activity on sperm tails, or could play a role in maintaining the correct physiology of spermathecal cells. Close association of peroxidase with sperm cells is reminiscent of the mammalian system, in which glutathione peroxidase GPX5 is secreted within the epididymis lumen, the site of sperm storage and maturation in males, and is found associated with sperm cell membranes, in which it prevents DNA damage, lipid peroxidation, and premature activation of the sperm by ROS (30, 31). Downstream effects on embryogenesis caused by fertilization by partially damaged sperm may also cause or contribute to the lower fertility of dsHPX15-injected females.

HPX15 was previously shown to regulate *Plasmodium* infections. This enzyme is required in the midgut for the formation of cross-linked dityrosine bonds within the peritrophic ECM surrounding the blood meal; in dsHPX15 females, the increased permeability of the matrix allows bacterial elicitors released from the gut microbiota to stimulate the mosquito immune system, leading to decreased *Plasmodium* loads (25). Besides a possible role against damaging ROS, it is possible that HPX15 may function to protect sperm from the female immune system by a cross-linked matrix. We did not, however, detect any additional matrix barrier surrounding sperm by EM. Alternatively, peroxidase activity may be required within the cuticle for cross-linking via catechols (32) to form a more protective hardened capsule. This hypothesis is supported by the coordinated up-regulation of cuticular proteins and enzymes important in sclerotization detected by microarrays. Further work is required to determine the exact mechanisms of HPX15 function.

In *Drosophila*, sperm transfer plays an important role in the female postmating behavior, as it is necessary to extend beyond

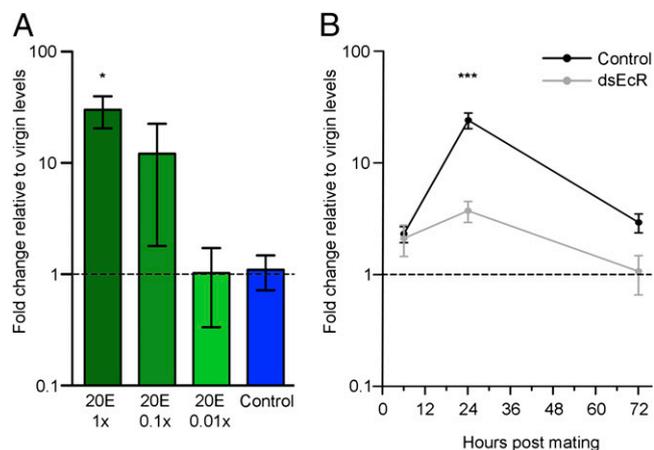


Fig. 6. HPX15 is regulated by the hormone 20E. (A) HPX15 expression in virgin females injected with different concentrations of 20E in a 10% (vol/vol) ethanol solution (control). Fold changes are expressed relative to untreated virgins. HPX15 is induced by 20E injections to levels similar to those induced by mating (one-way ANOVA followed by Dunnett multiple comparison test, * P < 0.05). (B) HPX15 expression in females injected with dsRNA targeting the control gene *gfp* or *EcR* and then mated normally. Fold changes are expressed relative to dsRNA-injected virgins (one-way ANOVA followed by Tukey multiple comparison test, *** P < 0.001).

the first day after copulation the response triggered by the MAG-produced Sex Peptide (33, 34). Consistently, almost one third of all transcriptional changes induced by mating in whole females are caused by sperm, although at low levels (35). In our experiments, we tested 10% of spermathecal genes up-regulated by mating and observed no differences between gene expression in females mated to spermless and sperm-carrying males (Fig. 3). This observation strongly suggests that the regulation of gene expression within the mated spermatheca is largely independent of the presence of sperm, in agreement with our previous findings demonstrating that sperm transfer does not modulate the *An. gambiae* female postmating responses (24). We cannot rule out that sperm transfer might affect untested genes, but our results suggest a prominent role for MAG secretions in regulating postmating transcriptional activity. Indeed, we provide evidence that *HPX15* expression within the female reproductive tract is under the regulation of sexually transferred 20E, which is synthesized in the MAGs and transferred as part of the mating plug (3, 4). Interestingly, a number of other up-regulated genes in our dataset have orthologs in *Drosophila* that may also be regulated by 20E (Table S3), and sexually transferred 20E has previously been shown to increase oogenesis in *An. gambiae* via the up-regulation of *MISO* expression in the female reproductive tract (4). Further studies are needed to determine the role of 20E and other MAG secretions in regulation of gene expression in the spermatheca.

This study underlines the potential for *HPX15* to be targeted in natural populations, now as a two-pronged approach against malaria transmission: reduced *HPX15* activity leads to decreased

mosquito fertility, and simultaneously lowers *Plasmodium* infection rates, impacting the total number of mosquitoes and disease transmission. Additional work to characterize the roles of other genes relevant to sperm function will offer new targets for vector control strategies.

Materials and Methods

An. gambiae mosquitoes (G3 strain) were separated by sex as pupae to ensure the virgin status of females. Mating couples were collected *in copula* as previously described (16). Age-matched virgin females were raised identically and dissected at the same time as mated females. Spermathecae not containing visible sperm were discarded. Samples of the lower reproductive tract from females mated to spermless males used in this study were already available in the laboratory (24). For reproductive assays, females were mated and blood-fed 1 d after *dsRNA* injection, and put into individual cups to lay eggs 2 d later. The numbers of hatched and unhatched eggs were counted after 3 d, and the proportion of infertile eggs calculated. Each egg was probed with a needle to determine the presence of a larva inside. *SI Materials and Methods* contains details of RNA extraction and cDNA synthesis, microarray experiments and analysis, qRT-PCR experiments and analysis, *dsRNA* and 20E injection experiments, reproductive assays, antibody generation and western blotting, electron microscopy, and peroxidase assays.

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