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

W. Robert Shaw[a,1], Eleonora Teodori[b,1], Sara N. Mitchell[c], Francesco Baldini[b,2], Paolo Gabrieli[b], David W. Rogers[a,3], and Flaminia Catteruccia[a,b,c,4]

[a]Department of Life Sciences, Imperial College London, London SW7 2AZ, United Kingdom; [b]Dipartimento di Medicina Sperimentale e Scienze Biochimiche, Università di Perugia, Terni 05100, Italy; and [c]Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115

Edited by Anthony A. James, University of California, Irvine, CA, and approved March 12, 2014 (received for review January 28, 2014)

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.

Author contributions: W.R.S., E.T., F.B., P.G., D.W.R., and F.C. designed research; W.R.S., E.T., F.B., P.G., and D.W.R. performed research; W.R.S., E.T., S.N.M., F.B., P.G., and F.C. analyzed data; and W.R.S., E.T., and F.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

The microarray data reported in this paper have been deposited in the ArrayExpress database, www.ebi.ac.uk/arrayexpress (accession no. E-MTAB-2429).

[1]W.R.S. and E.T. contributed equally to this work.

[2]Present address: Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow G12 8QQ, United Kingdom.

[3]Present address: Max Planck Research Group for Experimental Evolution, Max Planck Institute for Evolutionary Biology, 24306 Plön, Germany.

[4]To whom correspondence should be addressed. E-mail: fcatter@hsph.harvard.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1401715111/-/DCSupplemental.

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 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

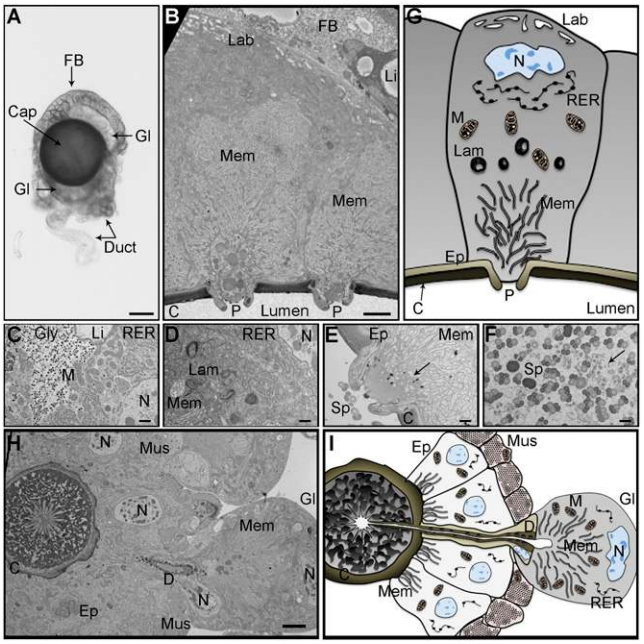

Fig. 1. Ultrastructural features of spermathecal cell types. Transmission EM images of spermathecae from virgin or mated (24 hpm) with or without blood meal females. (*A*) Spermatheca at low magnification, showing the arrangement of secretory glandular cells and fat body cells around a capsule (mated). (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.

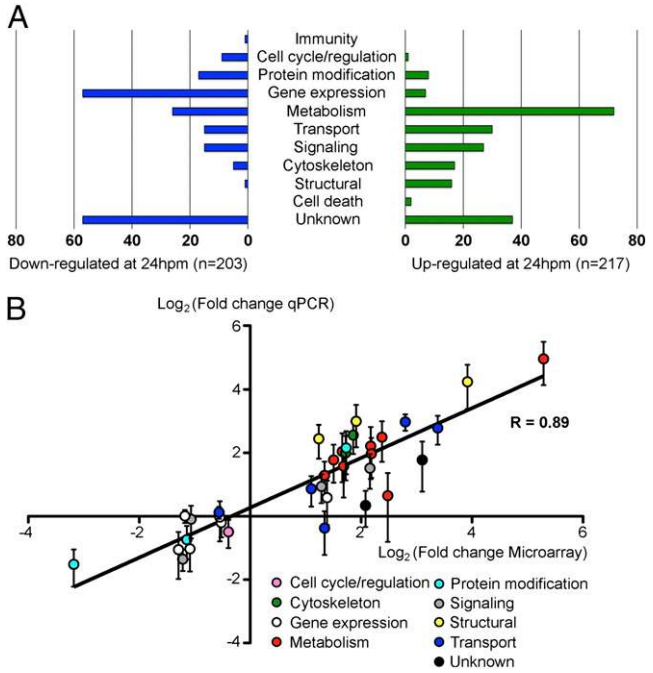

Fig. 2. Mating causes large gene expression changes in the spermatheca. (*A*) Functional classes of genes up-regulated (green) or down-regulated (blue) at 24 hpm with adjusted $P < 0.005$. The number of genes in each group is on the *x* axis. (*B*) Validation of the microarray data by correlation to 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 ± 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, phosphofructokinase, 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. 2*B* 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

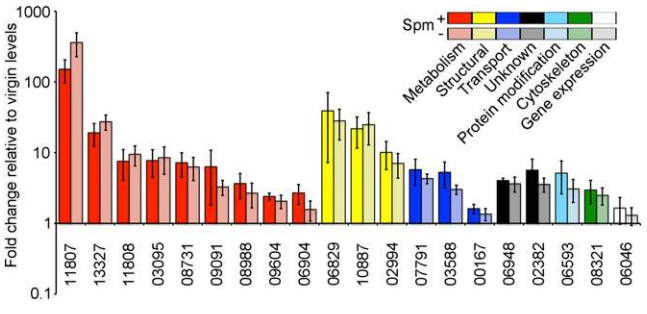

Fig. 3. Sperm transcripts do not contribute to gene expression changes observed in the spermatheca. Expression levels of 20 mating-induced genes were determined by qRT-PCR in the lower reproductive tracts of females mated to sperm-carrying (Spm+) or spermless (Spm–) males and compared with age-matched virgin females. Different functional categories are color-coded. Mean fold changes ± 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: $t_{0.05(2),1} = 12.706$).

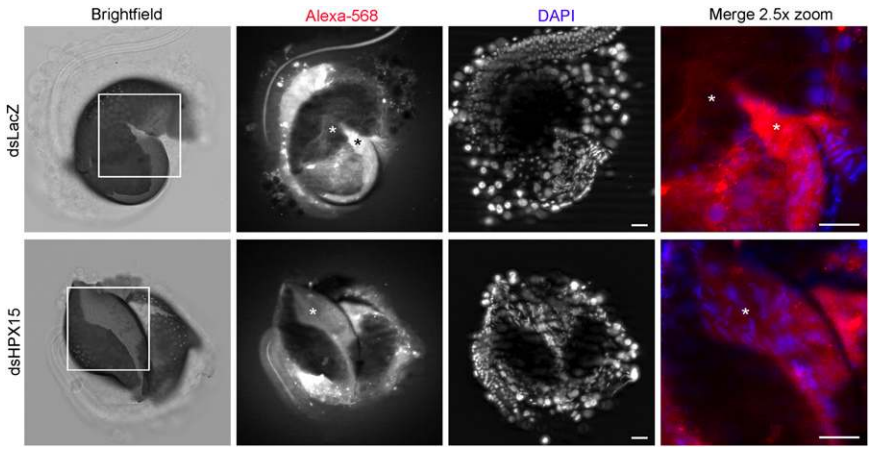

Fig. 4. HPX15 activity is associated with sperm in the lumen of the spermatheca at 24 hpm. Peroxidase activity in the spermatheca of mated (24 hpm) females injected with ds*LacZ* or ds*HPX15* was detected by the deposition of fluorescently labeled tyramide (Alexa-568 in red). A clear fluorescent signal on sperm bundles (asterisk) is visible only in control ds*LacZ* females, whereas fluorescence disappears in ds*HPX15* samples. Signal from the glandular cells surrounding the capsule (overexposed to visualize the weaker signal localized to sperm tails) is instead present in control and experimental spermathecae. Nuclei are labeled in DAPI and are visible for sperm and spermathecal cells (blue). (*Right*) Merged images of the boxed region in the bright-field panels at 2.5× zoom. Fluorescent images are flattened stacks of six *z*-slices, covering 6 μm. (Scale bar: 20 μm.)

IMPer (25); Dataset S1). Peroxidases prevent cellular damage caused by free hydroxyl radicals by catalyzing the reduction of hydrogen peroxide to water. We therefore reasoned that the function of this enzyme after copulation might provide a protective mechanism to prevent damage to sperm or to spermathecal cells. The peroxidase-based fluorescent tyramide signal amplification assay identified peroxidase activity in glandular cells of mated spermathecae and also in close association with sperm tails (Fig. 4). Activity on sperm tails was specific for HPX15 as the fluorescent signal was abolished after silencing this peroxidase (injecting females with dsRNA fragments targeting *HPX15*, i.e., ds*HPX15*; Fig. 5*A* and Fig. S1*A*). The signal in the glandular cells instead remained visible in ds*HPX15* females, possibly because of the activity of additional peroxidase enzymes in those cells (Fig. 4). Consistent with the detection of peroxidase activity on sperm cells, western blotting analysis of mated spermathecae in which the sperm bundle and capsule had been separated detected HPX15 in both fractions (Fig. 5*B*), suggesting that the protein is secreted into the spermatheca lumen [in agreement with observations by others of HPX15 secretion in the midgut (25)]. All together, these findings suggest HPX15 is required to protect sperm rather than spermathecal cells, possibly from oxidative damage.

HPX15 Is Important for Mosquito Fertility During Multiple Gonotrophic Cycles. *HPX15* remained up-regulated over the course of multiple days after mating and in the presence of blood feeding, suggesting a long-term role in sperm protection from possible oxidative damage (Fig. 5*A* and Fig. S2). We tested this hypothesis by performing functional RNAi analysis of this enzyme across repeated blood feeding and egg laying (gonotrophic) cycles. Females injected with ds*HPX15* or with control ds*RNA* (targeting the bacterial gene *LacZ*) were mated and blood-fed, and the number of fertile eggs laid by each female was determined. After a first blood feeding, ∼5% of eggs were infertile in control and ds*HPX15*-injected females ($P = 0.0672$, Mann–Whitney test; Fig. 5*C*). However, infertility of ds*HPX15* females increased to 10% after the second blood feeding, and a third blood meal induced a further increase to 19%, whereas no increase was seen in control females in either condition ($P < 0.0001$ after second and third blood meal, Mann–Whitney test; Fig. 5*C*).

Given the time elapsed between mating and the fertilization of the second or third egg batch (more than 5 d), the observed increase in infertility in *HPX15*-silenced females could represent damage accumulated over time rather than a specific effect of multiple blood feedings. To separate these possibilities, we performed injections and matings as before, but waited 5 d before giving females a blood meal, so that this first blood feeding would occur at the same time as the second blood feeding had occurred in the previous experiment. We observed no difference in the fertility of control and ds*HPX15*-injected females ($P = 0.3201$, Mann–Whitney test), demonstrating that the increase in the number of infertile eggs detected in ds*HPX15*-injected females was a result of repeated blood feedings (Fig. 5*C*).

HPX15 is also induced 24 h after blood feeding in the midgut (Fig. S1*B*), at which time work from others has shown it forms a cross-linked barrier that protects *Plasmodium* parasites and gut microbiota against the mosquito immune response (25). Our RNAi injections abolished the midgut-specific up-regulation of *HPX15* caused by blood feeding (Fig. S1*B*), but no difference in the number of eggs developed and laid was observed between control and ds*HPX15*-injected females at any of the blood meals tested (Fig. S3). Based on these results, we rule out any major reproductive effect of *HPX15* silencing other than in the spermatheca, as silencing of this gene in the midgut would most likely affect egg development rather than sperm functionality. No *HPX15* expression was detectable in the ovaries after mating (Fig. S2) or blood feeding (26).

***HPX15* Expression Is Regulated by the Steroid Hormone 20E.** During mating, *An. gambiae* males transfer large amounts of the steroid hormone 20E to the female reproductive tract (3, 4). After each blood feeding, 20E is also produced by the female (27), but at much lower levels than those transferred by males (3, 4). An analysis of the promoter region of *HPX15* using Matinspector software (www.genomatix.de) detected five putative binding sites for transcription factors responsive to 20E. Moreover, we noted that the expression pattern of this enzyme in the spermatheca and midgut coincides with 20E peaks after mating and blood feeding (4, 27). We therefore tested whether *HPX15* transcription may be under hormonal regulation. Virgin females injected with 20E showed induced expression of *HPX15* specifically in the lower reproductive tract at 24 h after injection ($P < 0.05$, one-way ANOVA; Fig. 6*A*). This effect was concentration-dependent, as lower concentrations of 20E were not able to induce expression, similar to what had been previously observed in the 20E-regulated induction of the mating-induced gene *Mating Induced Stimulator of Oogenesis* (*MISO*) in the female atrium (4). Signaling via 20E is generally mediated by a nuclear receptor complex formed by a heterodimer of EcR and Ultraspiracle (28, 29). When we examined transcript levels after mating in females injected with ds*RNA* targeting *EcR*, *HPX15* induction in the spermatheca was significantly reduced in ds*EcR* females compared with controls ($P < 0.001$, one-way ANOVA; Fig. 6*B*). All together, these results strongly indicate that the interaction of male-transferred 20E with its nuclear receptor in the female is required for the normal mating-induced expression of this gene.

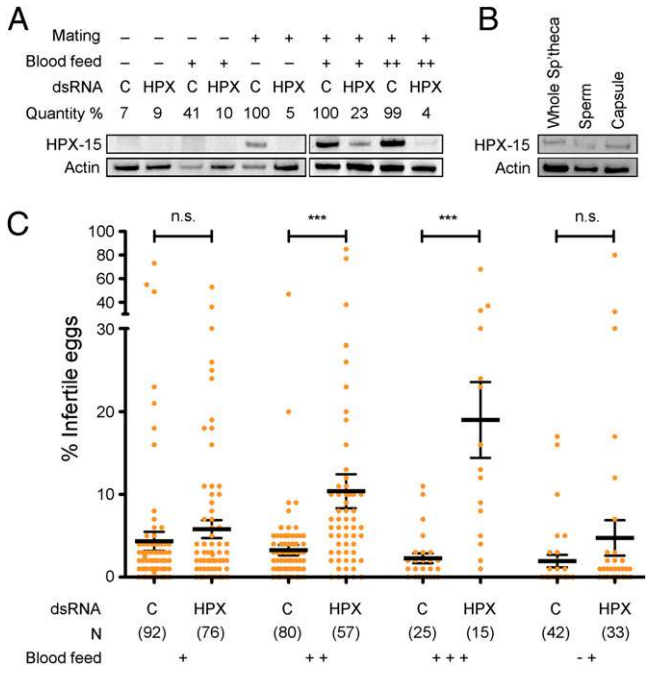

Fig. 5. HPX15 is expressed in the spermatheca after mating and is required to prevent infertility. (*A*) Western blot analysis of HPX15 in virgin (–), mated (+), and mated blood-fed spermathecae at 24 hpm and one (+) or two (++) blood feedings. C, ds*LacZ* control injection; HPX, ds*HPX15* injection; "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 ds*LacZ* (*C*) or ds*HPX15* (HPX). "–+" signifies the first blood feed was at the time of the second blood feed in "++" experiments. Bars represent mean ± 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 ds*HPX15*-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 ds*HPX15* 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

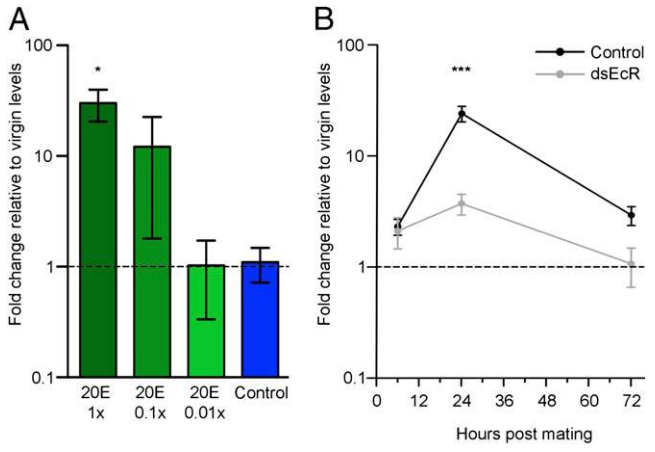

Fig. 6. *HPX15* is regulated by the hormone 20E. (*A*) *HPX15* expression in virgin females injected with different concentrations of 20E in a 10% (vol/vol) ethanol solution (control). Fold changes are expressed relative to untreated virgins. *HPX15* is induced by 20E injections to levels similar to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

ACKNOWLEDGMENTS. The authors thank all members of the laboratory of F.C. for helpful discussions, Janis Thailayil for the spermless matings samples, and the Harvard Medical School Electron Microscopy facility for help with performing electron microscopy. This study was sponsored by European Research Council FP7 Starting Grant project "Anorep" Grant 260897, Wellcome Trust Grant 093553, Biotechnology and Biological Sciences Research Council Grant BB/I002898/1, and National Institutes of Health Grant 1R01AI104956-01A1 (to F.C.).

1. World Health Organization (2011) *World Malaria Report* (World Health Organization, Geneva).
2. Rogers DW, et al. (2009) Transglutaminase-mediated semen coagulation controls sperm storage in the malaria mosquito. *PLoS Biol* 7(12):e1000272.
3. Pondeville E, Maria A, Jacques JC, Bourgouin C, Dauphin-Villemant C (2008) Anopheles gambiae males produce and transfer the vitellogenic steroid hormone 20-hydroxyecdysone to females during mating. *Proc Natl Acad Sci USA* 105(50):19631–19636.
4. Baldini F, et al. (2013) The interaction between a sexually transferred steroid hormone and a female protein regulates oogenesis in the malaria mosquito Anopheles gambiae. *PLoS Biol* 11(10):e1001695.
5. Takken W, Klowden MJ, Chambers GM (1998) Effect of body size on host seeking and blood meal utilization in Anopheles gambiae sensu stricto (Diptera: Culicidae): The disadvantage of being small. *J Med Entomol* 35(5):639–645.
6. Scott TW, Takken W (2012) Feeding strategies of anthropophilic mosquitoes result in increased risk of pathogen transmission. *Trends Parasitol* 28(3):114–121.
7. Clements AN (1999) *The Biology of Mosquitoes* (CABI, Wallingford, UK), 2nd Ed.
8. Schnakenberg SL, Matias WR, Siegal ML (2011) Sperm-storage defects and live birth in Drosophila females lacking spermathecal secretory cells. *PLoS Biol* 9(11):e1001192.
9. Allen AK, Spradling AC (2008) The Sf1-related nuclear hormone receptor Hr39 regulates Drosophila female reproductive tract development and function. *Development* 135(2):311–321.
10. Anderson RC (1945) A study of the factors affecting fertility of lozenge females of Drosophila melanogaster. *Genetics* 30(3):280–296.
11. Sun J, Spradling AC (2013) Ovulation in Drosophila is controlled by secretory cells of the female reproductive tract. *Elife* 2:e00415.
12. Prokupek AM, Kachman SD, Ladunga I, Harshman LG (2009) Transcriptional profiling of the sperm storage organs of Drosophila melanogaster. *Insect Mol Biol* 18(4): 465–475.
13. Prokupek AM, Eyun SI, Ko L, Moriyama EN, Harshman LG (2010) Molecular evolutionary analysis of seminal receptacle sperm storage organ genes of Drosophila melanogaster. *J Evol Biol* 23(7):1386–1398.
14. Baer B, Eubel H, Taylor NL, O'Toole N, Millar AH (2009) Insights into female sperm storage from the spermathecal fluid proteome of the honeybee Apis mellifera. *Genome Biol* 10(6):R67.
15. Collins AM, Williams V, Evans JD (2004) Sperm storage and antioxidative enzyme expression in the honey bee, Apis mellifera. *Insect Mol Biol* 13(2):141–146.
16. Rogers DW, et al. (2008) Molecular and cellular components of the mating machinery in Anopheles gambiae females. *Proc Natl Acad Sci USA* 105(49):19390–19395.
17. Kumar S, et al. (2003) The role of reactive oxygen species on Plasmodium melanotic encapsulation in Anopheles gambiae. *Proc Natl Acad Sci USA* 100(24):14139–14144.
18. Reinhardt K, Ribou A-C (2013) Females become infertile as the stored sperm's oxygen radicals increase. *Sci Rep* 3:2888.
19. Schirmer T, Evans PR (1990) Structural basis of the allosteric behaviour of phosphofructokinase. *Nature* 343(6254):140–145.
20. Jurica MS, et al. (1998) The allosteric regulation of pyruvate kinase by fructose-1,6-bisphosphate. *Structure* 6(2):195–210.
21. Xu X, et al. (2012) Insulin signaling regulates fatty acid catabolism at the level of CoA activation. *PLoS Genet* 8(1):e1002478.
22. Zhang Y, Chen D, Wang Z (2009) Analyses of mental dysfunction-related ACSl4 in Drosophila reveal its requirement for Dpp/BMP production and visual wiring in the brain. *Hum Mol Genet* 18(20):3894–3905.
23. Sumner JP, et al. (1995) Regulation of plasma membrane V-ATPase activity by dissociation of peripheral subunits. *J Biol Chem* 270(10):5649–5653.
24. Thailayil J, Magnusson K, Godfray HC, Crisanti A, Catteruccia F (2011) Spermless males elicit large-scale female responses to mating in the malaria mosquito Anopheles gambiae. *Proc Natl Acad Sci USA* 108(33):13677–13681.
25. Kumar S, Molina-Cruz A, Gupta L, Rodrigues J, Barillas-Mury C (2010) A peroxidase/dual oxidase system modulates midgut epithelial immunity in Anopheles gambiae. *Science* 327(5973):1644–1648.
26. Marinotti O, Nguyen QK, Calvo E, James AA, Ribeiro JM (2005) Microarray analysis of genes showing variable expression following a blood meal in Anopheles gambiae. *Insect Mol Biol* 14(4):365–373.
27. Bai H, Gelman DB, Palli SR (2010) Mode of action of methoprene in affecting female reproduction in the African malaria mosquito, Anopheles gambiae. *Pest Manag Sci* 66(9):936–943.
28. Thomas HE, Stunnenberg HG, Stewart AF (1993) Heterodimerization of the Drosophila ecdysone receptor with retinoid X receptor and ultraspiracle. *Nature* 362(6419):471–475.
29. Yao TP, et al. (1993) Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. *Nature* 366(6454):476–479.
30. Vernet P, Faure J, Dufaure JP, Drevet JR (1997) Tissue and developmental distribution, dependence upon testicular factors and attachment to spermatozoa of GPX5, a murine epididymis-specific glutathione peroxidase. *Mol Reprod Dev* 47(1):87–98.
31. Chabory E, et al. (2009) Epididymis seleno-independent glutathione peroxidase 5 maintains sperm DNA integrity in mice. *J Clin Invest* 119(7):2074–2085.
32. Hasson C, Sugumaran M (1987) Protein cross-linking by peroxidase: Possible mechanism for sclerotization of insect cuticle. *Arch Insect Biochem Physiol* 5:13–28.
33. Chapman T, et al. (2003) The sex peptide of Drosophila melanogaster: Female postmating responses analyzed by using RNA interference. *Proc Natl Acad Sci USA* 100(17):9923–9928.
34. Liu H, Kubli E (2003) Sex-peptide is the molecular basis of the sperm effect in Drosophila melanogaster. *Proc Natl Acad Sci USA* 100(17):9929–9933.
35. McGraw LA, Gibson G, Clark AG, Wolfner MF (2004) Genes regulated by mating, sperm, or seminal proteins in mated female Drosophila melanogaster. *Curr Biol* 14(16):1509–1514.