Ammonium transporter expression in sperm of the disease vector *Aedes aegypti* mosquito influences male fertility

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The ammonium transporter (AMT)/methylammonium permease (MEP)/Rhesus glycoprotein (Rh) family of ammonia (NH_3/NH_4^+) transporters has been identified in organisms from all domains of life. In animals, fundamental roles for AMT and Rh proteins in the specific transport of ammonia across biological membranes to mitigate ammonia toxicity and aid in osmoregulation, acid–base balance, and excretion have been well documented. Here, we observed enriched *Amt* (*AeAmt1*) mRNA levels within reproductive organs of the arboviral vector mosquito, *Aedes aegypti*, prompting us to explore the role of AMTs in reproduction. We show that AeAmt1 is localized to sperm flagella during all stages of spermiogenesis and spermatogenesis in male testes. AeAmt1 expression in sperm flagella persists in spermatozoa that navigate the female reproductive tract following insemination and are stored within the spermathecae, as well as throughout sperm migration along the spermathecal ducts during ovulation to fertilize the descending egg. We demonstrate that RNA interference (RNAi)-mediated AeAmt1 protein knockdown leads to significant reductions (∼40%) of spermatozoa stored in seminal vesicles of males, resulting in decreased egg viability when these males inseminate nonmated females. We suggest that AeAmt1 function in spermatozoa is to protect against ammonia toxicity based on our observations of high NH_4^+ levels in the densely packed spermathecae of mated females. The presence of AMT proteins, in addition to Rh proteins, across insect taxa may indicate a conserved function for AMTs in sperm viability and reproduction in general.

spermatogenesis | Culicidae | insect physiology | insect reproduction | dengue

Ammonium transporters (AMTs), methylammonium permeases (MEPs), and Rhesus glycoproteins (Rh proteins) comprise a protein family with three clades, and homologs from each have been identified in virtually all domains of life (1). AMT proteins were first identified in plants (2) with the simultaneous discovery of MEP proteins in fungi (3), followed by Rh proteins in humans (4). Ammonia (NH_3/NH_4^+) is vital for growth in plants and microorganisms and is retained in some animals for use as an osmolyte (5, 6), for buoyancy (7, 8), and for those lacking sufficient dietary nitrogen (9). In the majority of animals, however, ammonia is the toxic by-product of amino acid and nucleic acid metabolism and, accordingly, requires efficient mechanisms for its regulation, transport, and excretion (10–13). AMT, MEP, and Rh proteins are responsible for the selective movement of ammonia (NH_3) or ammonium (NH_4^+) across biological membranes, a process that all organisms require. Unlike their vertebrate, bacterial, and fungal counterparts which function as putative NH_3 gas channels (14–18), a myriad of evidence suggests that plant AMT proteins and closely related members in some animals are functionally distinct and facilitate electrogenic ammonium (NH_4^+) transport (17, 19–22). In contrast to vertebrates which only possess Rh proteins (23), many invertebrates are unique in that they express both AMT and Rh proteins, sometimes in the same cell (24–28). Among insects, the presence of both AMT and Rh proteins has been described in *Drosophila melanogaster* (29, 30) and mosquitoes that vector disease-causing pathogens, *Anopheles gambiae* (22, 31) and *Aedes aegypti* (32, 33). It is unclear whether, in these instances, AMT and Rh proteins can functionally substitute for one another, but in the anal papillae of *A. aegypti* larvae, knockdown of either Amt or Rh proteins causes decreases in ammonia transport, suggesting that they do not (32–34). To date, studies on ammonia transporter (AMT and Rh) function in insects have focused on ammonia sensing and tasting in sensory structures (22, 30, 31, 35), ammonia detoxification and acid–base balance in muscle, digestive, and excretory organs (15, 36), and ammonia excretion in a variety of organs involved in ion and water homeostasis (9, 24, 32–34).

A. aegypti is the primary vector for the transmission of the human arboviral diseases Zika, yellow fever, chikungunya, and dengue virus, which are of global health concern due to rapid increases in the geographical distribution of this species, presently at its highest ever (37, 38). In light of the well-documented evolution of insecticide resistance in mosquitoes (39–42), more recent methods to control disease transmission such as the sterile insect technique (43), transinfection and sterilization of mosquitoes with the bacterium *Wolbachia* (44), and targeted genome editing rendering adult males sterile (45) have proven effective. These methods take advantage of various aspects of mosquito reproductive biology; however, an understanding of male reproductive

Significance

Ammonium transporters (AMT) have been implicated in facilitating nitrogen assimilation in plants and bacteria and ammonia (NH_3/NH_4^+) transport for nitrogen regulation and excretion in animals. However, the involvement of AMT proteins in mediating reproductive processes remains unexplored. We report that an insect AMT, AeAmt1, is expressed in the flagellum of spermatozoa from the dengue vector mosquito *Aedes aegypti* during sperm development through to egg fertilization. We provide evidence that AeAmt1 expression in spermatozoa is critical for sperm survival and overall male fertility. This description of ammonia transporter (AMT and Rh families) expression in the sperm of an animal provides a striking role for these evolutionarily conserved protein families and contributes to our understanding of mosquito reproductive biology.

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The authors declare no competing interest.

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biology and the male contributions to female reproductive processes is still in its infancy (46). Here, we describe the expression of an *A. aegypti* ammonium transporter (AeAmt1) in the sperm during all stages of spermatogenesis, spermiogenesis, and egg fertilization, which is critical for fertility.

Results and Discussion

AeAmt1 Is Expressed during All Stages of Spermatogenesis and Spermiogenesis. Previous characterizations of ammonia transporters (AMT and Rh proteins) in reproductive tissues in the context of male reproductive biology and fertility are scarce and come from vertebrate models (47, 48). In the mosquito *Aedes albopictus*, Rh protein (*AalRh50*) transcript is expressed at modest levels in ovary tissue of adult females and is unaltered following blood feeding (15). Given our previous characterizations of AMT proteins (AeAmt1 and AeAmt2) in *A. aegypti* larvae which are expressed in organs important for ion and water balance and digestion (32, 33, 49), we speculated that AeAmt1 and AeAmt2 would play functionally similar roles in digestive and excretory organs in adults, particularly following ingestion of a protein-rich blood meal by females. To examine this hypothesis, we utilized immunohistological techniques with previously validated AeAmt antibodies (32, 33). To our surprise, our results demonstrate that AeAmt1 protein is immunolocalized almost exclusively to the reproductive organs of both male and mated, sugar-fed female *A. aegypti* (Fig. 1). In a longitudinal section of a mature testis follicle from male *A. aegypti*, AeAmt1 is expressed in primary spermatogonia (germ cells), primary spermatocytes, spermatids, and spermatozoa (Fig. 1*A*). In early stages of spermatogenesis, AeAmt1 localizes to regions consistent with mitochondria found near the centriole which in later stages stretches alongside axial filament formation within the flagella (50). Live cell staining of mitochondrial derivatives that localize to the full length of the flagella of mature spermatozoa (Fig. 1*B*) (51) shows a localization pattern similar to observed AeAmt1 staining along the length of the flagellum of a mature spermatozoa (Fig. 1 *C* and *D*). Interestingly, AeAmt1 immunostaining appears to be absent within the centriole adjunct (Fig. 1*D*, red arrow), a structure located between the nuclei and flagella of spermatozoa and implicated in proper flagellar development (51).

In mated, sugar-fed females, AeAmt1 protein was immunolocalized to the spermathecae (Fig. 1 *E–G*), and this immunostaining was absent when the AeAmt1 antibody was preabsorbed with 20× molar excess of AeAmt1 immunogenic peptide prior to application to tissue sections (Fig. 1 *H* and *I*). High-magnification images of spermathecae filled with spermatozoa reveal a flagellar-like AeAmt1 staining pattern similar to that observed in the mature spermatozoa within the male testis. The spermathecae are spherical reservoirs for spermatozoa in females, consisting of a cuticular lining surrounded by epithelial cells and glandular cells associated with the entrance of the spermathecal duct (52, 53). Our findings are consistent with those of other *Aedine* species in that only the largest, median spermatheca and one of the smaller lateral spermatheca are filled with sperm following copulation (46, 53, 54). AeAmt1 immunostaining localized to the nurse cells of the previtellogenic egg chambers within the ovary of sugar-fed females was also observed [*SI Appendix*, Fig. S1 *E* and *F* (55)]. AeAmt1 immunostaining is qualitatively absent in the midgut (MG) and Malpighian tubules (MT) (*SI Appendix*, Fig. S1 *A–D*) and the rectum (RM) epithelium and rectal pads (*SI Appendix*, Fig. S1 *E–H*), in contrast to AeAmt1 immunostaining of spermatozoa in recently inseminated female *A. aegypti* (*SI Appendix*, Fig. S1 *E–H*). *AeAmt1* messenger ribonucleic acid (mRNA) levels corroborate these findings, whereby transcript levels are higher in the reproductive organs (RP) (combined with hindgut [HG] tissue to extract sufficient RNA for qPCR), relative to MG and MT levels (*SI Appendix*, Fig. S2). *AeAmt1* mRNA is highest in fat body (FB) and carcass tissues (*SI Appendix*, Fig. S2), translating to only moderate AeAmt1 immunostaining in sections of fat body cells in comparison to the spermatozoa (*SI Appendix*, Fig. S1*F*, yellow dashed arrow). We suggest that carcass tissues (muscle, nervous, and hypodermis) may also be enriched in *AeAmt1* transcript, perhaps explaining the difference in transcript and protein levels but these carcass tissues have yet to be examined. Alternatively, translation efficiency by specific cells can greatly influence the correlation between mRNA and protein levels, in part due to untranslated mRNA that remains in the cell (56). By comparison, *AeAmt2* mRNA is enriched in the HG, RP, and FB (*SI Appendix*, Fig. S2), and we have immunolocalized AeAmt2 within the MG, MT, ovary, HG (including rectal pads), and the fat body cells of sugar-fed, mated adult females (*SI Appendix*, Fig. S3). From this, we postulate that AeAmt2 likely plays critical roles in ammonia detoxification and excretion during blood meal digestion, as this causes significantly elevated hemolymph [NH_4^+] within 48 h after blood meal (*SI Appendix*, Fig. S4) which corresponds to simultaneously elevated ammonia concentrations in the feces (57). Interestingly, *AeAmt2* mRNA and protein are also highly enriched in the antennae (*SI Appendix*, Fig. S3 *A–D*). In *Anopheles gambiae,* an ammonium transporter (*AgAmt*) sharing high sequence homology with *AeAmt2* (33) is expressed in chemosensory appendages with evidence that *AgAmt* functions in critical host-seeking behaviors involving ammonia sensing (22, 31).

AeAmt1 Expression Is Only Detected in Spermathecae from Mated Females Containing Sperm. In light of the finding that AeAmt1 is expressed in the flagella of mature spermatozoa and this expression persists during storage of spermatozoa within spermathecae, we predicted that spermathecae of nonmated females should lack AeAmt1 expression. Indeed, AeAmt1 immunostaining is absent in the spermathecae of nonmated females (Fig. 2 *A* and *B*). Furthermore, Western blotting analysis confirmed these findings as AeAmt1 protein was not detected in spermathecae protein homogenate from nonmated females, in contrast to AeAmt1 protein detected in the spermathecae of mated female *A. aegypti* (Fig. 2*C*). A single band at ∼55 kDa was detected, the predicted monomer form (VectorBase), which is antibody specific based on previous peptide-blocking analysis in other *A. aegypti* tissues (32). In an effort to elucidate a functional role for AeAmt1 in spermatozoa during storage in spermathecae, NH_4^+-selective microelectrodes were used to measure the [NH_4^+] within the spermathecae of mated and nonmated female *A. aegypti*. Mated females exhibit significantly higher NH_4^+ levels released from spermathecae when punctured in a droplet of saline in comparison to nonmated females (Fig. 2*D*), and we estimate that nondiluted [NH_4^+] levels in intact spermathecae are ∼500 mM for mated females and 300 mM for nonmated females, consistent with levels measured along the gastrointestinal tract (58) and within buoyancy organs (7) of other invertebrates. Secretion of proteins by both male accessory glands and glandular cells of spermathecae is required for sperm viability during insemination and sperm storage in insects (59, 60); however, catabolism of these proteins would yield ammonia. These remarkably high levels in contrast to hemolymph [NH_4^+] (*SI Appendix*, Fig. S4) suggest that, at least in spermathecae, AeAmt1 in spermatozoa is likely functioning to mitigate ammonia toxicity. This is particularly important considering female *A. aegypti* undergo a single mating event with one male and sperm are stored in spermathecae for the duration of the female's life (46, 52, 53). While it is unclear how the spermathecal cells mitigate the effects of high ammonia stored within the spermathecal reservoirs, the morphology of the spermathecae is such that the epithelial cells lie externally to the thick cuticle that lines the reservoirs, and the glandular cells are present on the surface of cuticle-lined spermathecal ducts (52, 53). This suggests

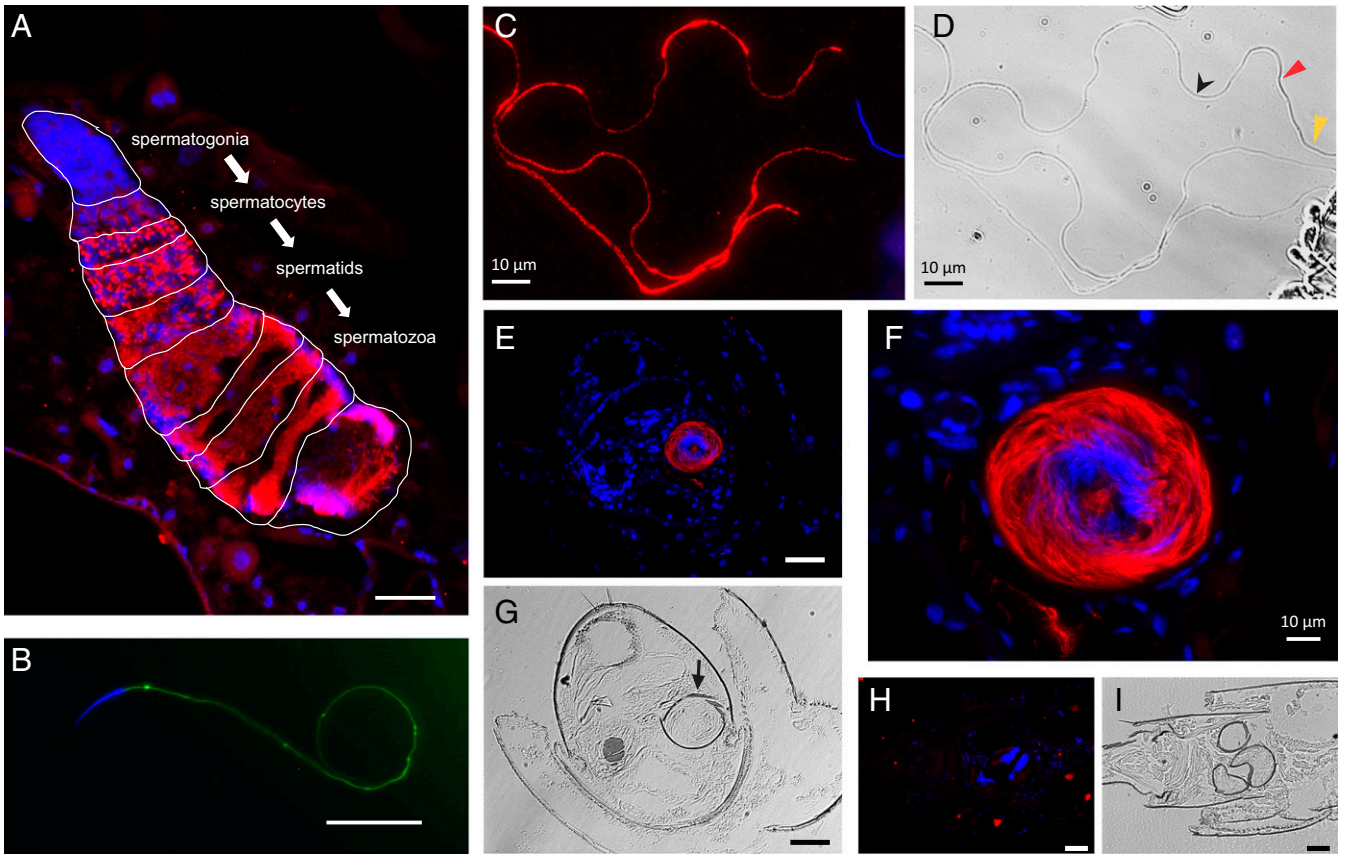

Fig. 1. AeAmt1 protein localization in male testes and spermatozoa stored within the spermathecae of female *Aedes aegypti*. (*A*) AeAmt1 (red) immunolocalization in a longitudinal section of a testis counterstained for nuclei (blue; DAPI). Zones of different stages of spermatogenesis, from primary spermatogonia (germ cells) to mature spermatozoa, are indicated (white labels). (*B*) High-magnification image of a single spermatozoon showing the nucleus (blue; DAPI) and the mitochondrial derivatives along the length of the flagellum (green; MitoTracker). (*C*) High-magnification image of AeAmt1 (red) localization counterstained for nuclei (blue; DAPI) in mature spermatozoa isolated from the testis and (*D*) bright-field image to corresponding *C* indicating the flagellum (black arrow), nucleus (yellow arrow), and region of the centriole adjunct (red arrow). (*E*) AeAmt1 (red) localization in a transverse section of spermathecae from mated female *A. aegypti* counterstained for nuclei (blue; DAPI) and (*F*) higher-magnification image of AeAmt1 (red) and nuclei (blue; DAPI) immunolocalization in spermatozoa from a single spermatheca of mated female *A. aegypti* from *E*. (*G*) The bright-field image corresponding to *E* indicating the sperm-filled spermatheca (black arrow). (*H*) Control slide (anti-AeAmt1 preincubated with 20x molar excess of AeAmt1-specific peptide) and (*I*) bright-field image corresponding to *H*. (Scale bars, 50 μm, unless indicated otherwise.)

that it is the cuticle that separates these cells from the high ammonia levels found within the spermathecae.

During ovulation and oviposition in female *A. aegypti*, spermatozoa are released from the spermathecae and traverse the spermathecal ducts, and this release is highly coordinated with ovulation of the descending egg for successful egg fertilization (46). At 48 h after blood meal, we found that AeAmt1 shows continued expression in sperm flagella as they exit the spermathecae (Fig. 3 *A* and *B*) and migrate through the spermathecal ducts (Fig. 3*C*). AeAmt1 immunostaining was also present in the follicular epithelium and within nurse cells surrounding the developing oocyte (*SI Appendix*, Fig. S5), paralleling our observation of AeAmt1 localization in previtellogenic egg chambers of sugar-fed females (*SI Appendix*, Fig. S1 *E* and *F*). The functional significance of AeAmt1 expression in the nurse cells of both previtellogenic and vitellogenic (post blood meal) ovarian follicles is not yet known; however, the high metabolic activities of the nurse cells to meet energy and nutrient demands mirrors that of spermatozoa. In this regard, metabolic processes in sperm cells occur in part through oxidative phosphorylation whereby ammonia (NH_3/NH_4^+) formation occurs as a by-product and requires regulation and, ultimately, excretion from the cell (61). This putative function for AeAmt1 in facilitating ammonia excretion from these highly metabolically active cells is most plausible considering the similar localizations of AeAmt1 and the mitochondrial derivatives (the site of oxidative phosphorylation) to the sperm flagellum. Findings from a comparative study using a murine model reported the production of potentially inhibitory levels of ammonium resulting from amino acid metabolism during embryonic development (62). Evidently, AeAmt1 likely has a critical function in protecting both spermatozoa and the developing oocyte against ammonia toxicity in reproductive organs in *A. aegypti*. Similarly, to sugar-fed females, AeAmt1 immunostaining within the midgut epithelium of females was not observed 48 h after blood meal (*SI Appendix*, Fig. S5*B*, white arrow).

AeAmt1 Is Essential for Spermatozoa Viability and Male Fertility. Considering that AeAmt1 is highly expressed in spermatozoa during the entire course of development in males, insemination of females, and storage within spermathecae, we speculated that AeAmt1 plays an essential role in sperm viability. While information from insect models is certainly lacking, a Rh protein (*Rhcg*) in mice is highly expressed in the epididymis and late stage spermatids in the testes of males in which the epididymal luminal fluid is maintained acidic to immobilize sperm during maturation within this organ (47, 48, 63). Consequently, *Rhcg*

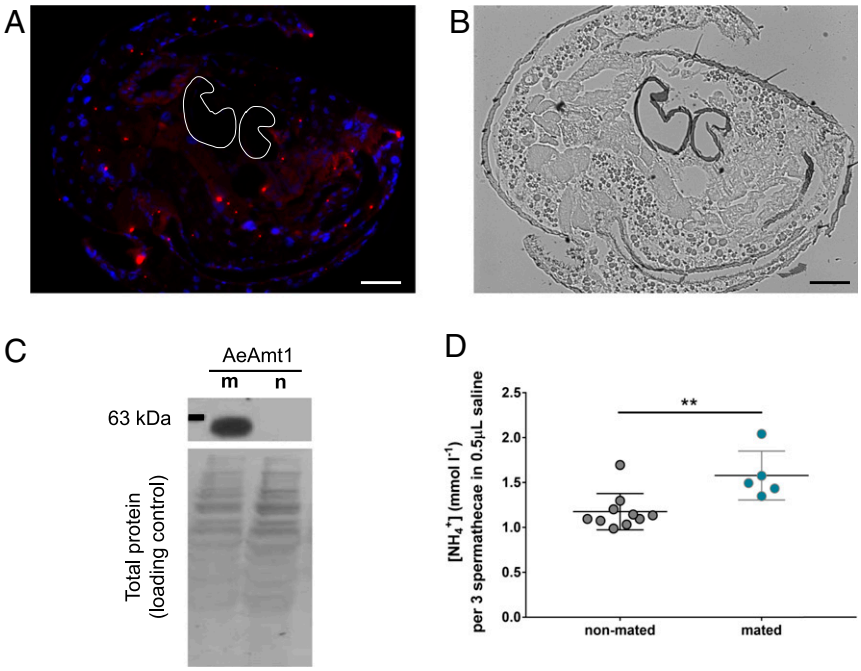

Fig. 2. Absence of AeAmt1 protein expression and localization in nonmated females and ammonium (NH_4^+) content in spermathecae of nonmated and mated female *Aedes aegypti*. (*A*) AeAmt1 (red) immunolocalization is absent in longitudinal sections of spermathecae (white outlines) from nonmated females counterstained for nuclei (blue; DAPI). (*B*) Bright-field image corresponding to *A*. (*C*) AeAmt1 protein expression (*Top*) in isolated spermathecae from mated (m) and nonmated (n) females and Coomassie total protein staining (*Bottom*) using Western blotting. (*D*) Diluted [NH_4^+] of pooled spermathecae (3 per animal) punctured in a 0.5 µL saline droplet from nonmated and mated female *A. aegypti* (Mann–Whitney *U* test, $^{**}P = 0.0077$). Data are shown as mean ± SEM. (Scale bars, 50 µm.)

mutant mice exhibit decreased fertility. Here, we employed RNA interference (RNAi) techniques whereby *AeAmt1* double-stranded ribonucleic acid (dsRNA) injection of males results in significant decreases in AeAmt1 protein in whole-body tissues within 24 h, which returns to normal levels within 36 h (Fig. 4 *A* and *B*). AeAmt1 protein knockdown at 24 h causes significant reductions in mature spermatozoa counts in the seminal vesicle (Fig. 4 *C* and *D*), a common storage unit for mature spermatozoa in male *A. aegypti* (64). We speculated that reductions in the number of spermatozoa are due to viability (i.e., sperm death) rather than delays in spermatogenesis and spermiogenesis, as the latter processes occur over ∼10 d in dipterans (50, 65). To examine if spermatozoa within the seminal vesicle were, indeed, dying or dead prior to when AeAmt1 protein is significantly decreased following dsRNA injection, we utilized a live/dead sperm viability assay. At 16 h after dsRNA injection, a higher proportion of cell death was observed in the AeAmt1 knockdown males, and total sperm counts were significantly reduced within the seminal vesicle (*SI Appendix*, Fig. S6), indicating that AeAmt1 knockdown begins earlier than 24 h after dsRNA treatment, demonstrated through Western blotting.

When nonmated females were inseminated by AeAmt1 knockdown males, significant reductions in the number of eggs laid (Fig. 5*A*), the number of larvae hatched (Fig. 5*B*), and, ultimately, the percentage of viable eggs laid per female (Fig. 5*C*) were observed. Although moderate levels of spermatozoa remain in the seminal vesicle following AeAmt1 protein knockdown at 24 h postinjection, we cannot discount the possibilities that sperm death continues to occur prior to, during, and after mating and insemination of the female or that physiological defects exist

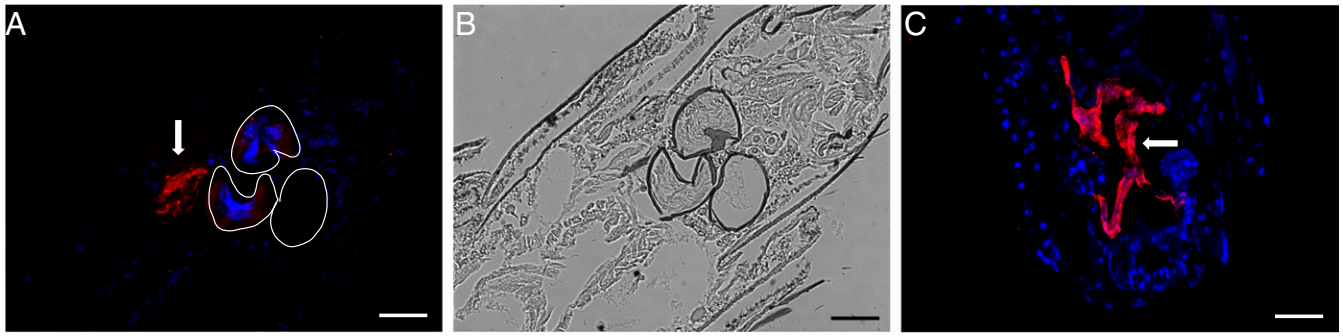

Fig. 3. AeAmt1 protein localization in migrating spermatozoa within spermathecal ducts from blood-fed (48 h post blood meal, pbm) adult female *Aedes aegypti*. (*A*) AeAmt1 (red) immunolocalization in a longitudinal section of spermathecae (white outlines) counterstained for nuclei (DAPI, blue), (*B*) bright-field image corresponding to *A*, and (*C*) AeAmt1 (red) immunolocalization within spermathecal ducts containing migrating spermatozoa (white arrow) counterstained for nuclei (DAPI, blue) in a blood-fed female *A. aegypti*. (Scale bars, 50 µm.)

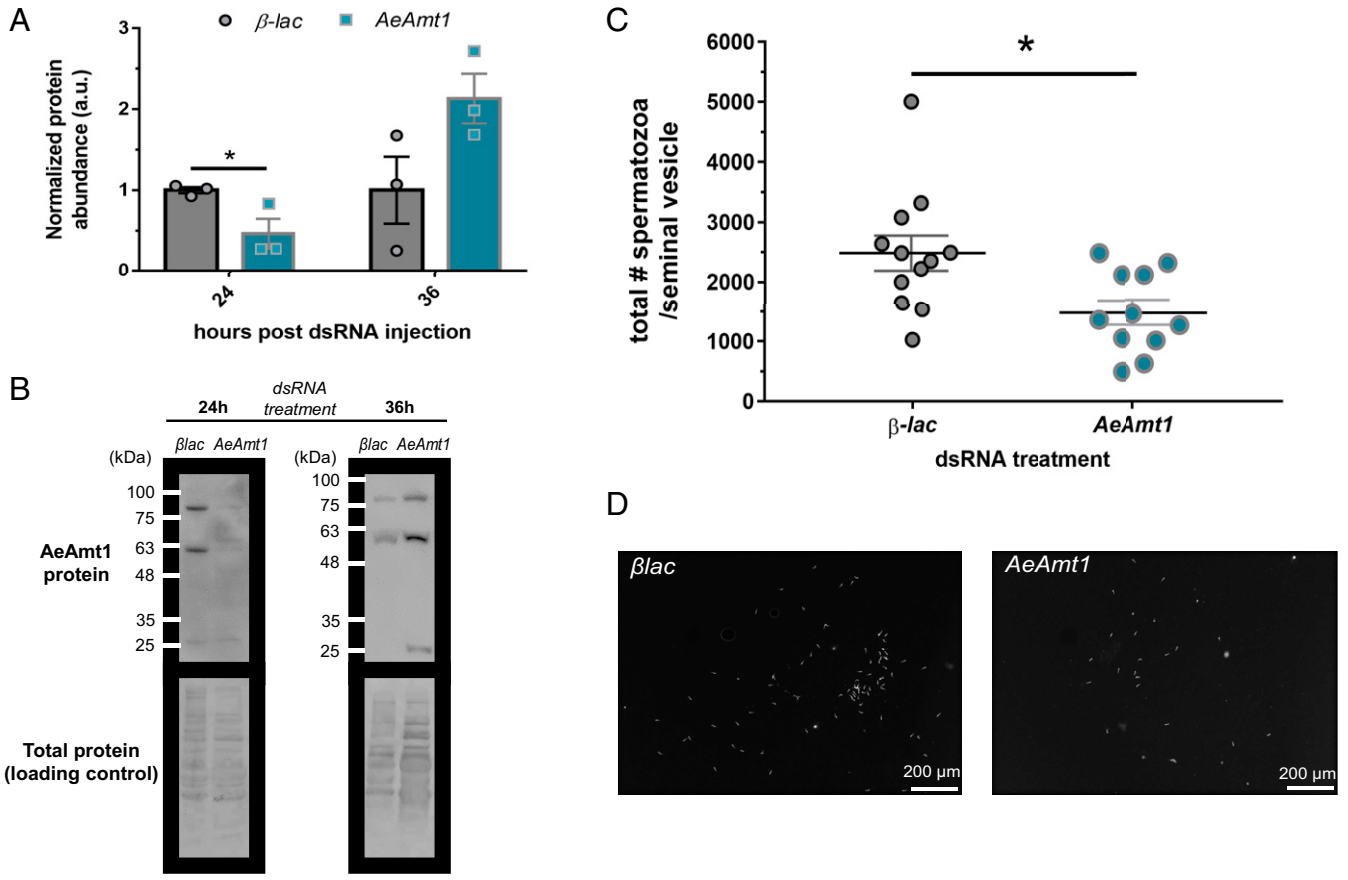

Fig. 4. Total spermatozoa in the seminal vesicle of male *Aedes aegypti* following RNAi (dsRNA)-mediated knockdown of AeAmt1. (*A*) AeAmt1 protein abundance in male *A. aegypti* at 24 and 36 h postinjection with *AeAmt1* ($n = 3$) or control *β-lactamase* (*β-lac*; $n = 3$) dsRNA (*$P = 0.0467$ for 24 h, unpaired Student's *t* test, two tailed; $P = 0.100$ for 36 h, Mann–Whitney *U* test). (*B*) Representative Western blot images corresponding to *A* demonstrating AeAmt1 protein abundance (*Top*) and Coomassie total protein staining (*Bottom*) at 24 and 36 h after *AeAmt1* and *β-lac* dsRNA injection. (*C*) Total spermatozoa number within the seminal vesicle of males at 24 h after *AeAmt1* and *β-lac* dsRNA injection (*$P = 0.0073$; Mann–Whitney *U* test; each point represents an individual replicate value). (*D*) Representative images of immunofluorescent nuclei (grayscale, DAPI) from fixed spermatozoa in a 1 µL droplet of PBS from the seminal vesicles of *AeAmt1* and *β-lac* dsRNA-injected males at 24 h postinjection. Data are shown as mean ± SEM.

in these remaining sperm as a result of AeAmt1 knockdown, which would impact the ability of sperm to fertilize the egg. Mated female *A. aegypti* injected with *AeAmt1* dsRNA produce amounts of eggs and hatched larvae similar to control females, and no significant reduction in the percentage of hatched larvae per female was observed (*SI Appendix*, Fig. S7 *A–C*), which may

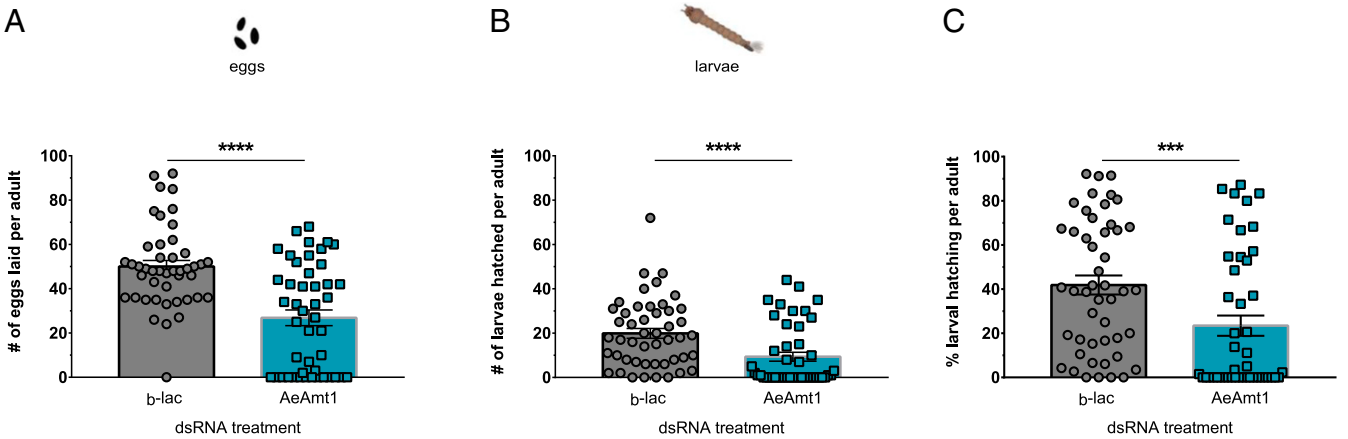

Fig. 5. Effects of *AeAmt1* protein knockdown in males on egg laying and larval hatching (egg viability) of mated female *Aedes aegypti*. (*A*) Number of eggs laid (****$P < 0.0001$; Mann–Whitney *U* test), (*B*) number of larvae hatched (****$P < 0.0001$; Mann–Whitney *U* test), and (*C*) percentage of larval hatching (***$P = 0.0006$; Mann–Whitney *U* test) by individual females mated with males that were injected with *AeAmt1* and *β-lac* dsRNA ($n = 45$ for β-lac; $n = 46$ for AeAmt1). Data are shown as mean ± SEM (each point represents an individual replicate value).

be due to adequate amounts of spermatozoa surviving in the spermathecae following AeAmt1 knockdown. It should be noted that high proportions of male and female *A. aegypti* survive following *AeAmt1* dsRNA injection, with gradual decreases in female survival following blood feeding and decreases in the proportion of females that lay (viable) eggs occurring, to a greater extent in AeAmt1 knockdown groups (*SI Appendix*, Fig. S8).

Taken together, these multiple lines of evidence demonstrate that AeAmt1 expression in spermatozoa is important for sperm survival and successful egg fertilization in *A. aegypti*. AeAmt1 expression within the developing follicles of the ovaries merits further investigation because there was no effect on fertility when females were injected with *AeAmt1* dsRNA, and thus, the functional role of AeAmt1 in this respect is unclear. While our findings provide an initial description of a member of the ammonia transporter (Amt/MEP/Rh) protein family that is localized within reproductive organs in a single mosquito genera, we posit that this feature may be conserved, at the very least across insect taxa, as a method of protecting sperm from ammonia toxicity as they navigate the reproductive tract. The use of spermathecae for sperm storage and maintenance of viability until fertilization is a general characteristic of insect reproductive biology but also extends to other animal phyla, including mollusks, annelids, nematodes, and some vertebrates (e.g., amphibians) (66, 67). In this regard, protection of the spermatozoa against ammonia toxicity is a challenge that likely many animals are tasked with, and our current work highlights the importance of an AMT protein function in this aspect of insect reproduction. These findings also contribute to our understanding of *A. aegypti* reproductive biology, which is still in its infancy but is of medical importance because of their frequent feeding on human hosts and their high vectorial capacity for arboviral pathogens. In this context, AeAmt1 protein expression during the early stages of sperm development, maturation, and later insemination and fertilization of the egg is necessary for male fertility and, more broadly, the reproductive fitness of *A. aegypti*, which is significant in terms of the development of novel vector control methods.

Materials and Methods

Immunolocalization and Western Blotting of AeAmt1. For immunohistological studies, whole adult male and female *A. aegypti* (7 to 10 d postemergence) were fixed in Bouin's solution at room temperature overnight. Fixed animals were further processed (dehydrated), paraffin embedded, and sectioned (5 μm thick) following an established protocol (68). Tissue sections were placed on VistaVision HistoBond adhesive microscope slides (VWR International). Immunohistochemical localization of AeAmt1 was carried out using a 1:40 (7.46×10^{-04} μg μL^{-1}) dilution of a custom-made polyclonal antibody produced in rabbit, which has been outlined in detail (32). For control slides, anti-AeAmt1 antibody was incubated with 20× molar excess of immunogenic peptide for 1 h at room temperature prior to application. To visualize the mitochondrial derivatives that are found along the entire length of the flagellum of isolated spermatozoa, seminal vesicles dissected from adult males were incubated in MitoTracker Red CMXRos at a 100 nM dilution (Invitrogen) in phosphate-buffered saline (PBS) for 2 h at room temperature (51). Seminal vesicles were then fixed in 4% paraformaldehyde for 2 h and were then gently pressed onto a microscope slide using a coverslip to release the spermatozoa. Slides were mounted using ProLong Gold antifade reagent with DAPI (Life Technologies). Western blotting of AeAmt1 was carried out following a previously published protocol (32). For assessment of AeAmt1 dsRNA-mediated protein knockdown (see *dsRNA Synthesis and Microinjection*) in males, three biological replicates consisting of whole animal tissues from 10 males per replicate were processed. To examine AeAmt1 protein abundance in spermathecae from nonmated and mated females, isolated spermatheca (three per animal) were pooled from 15 to 20 animals per replicate.

dsRNA Synthesis and Microinjection. *AeAmt1* and control *β-lactamase* dsRNA were synthesized using the Promega T7 RiboMAX Express RNAi kit (Promega) following a published protocol (32). Isolated male and female (2 to 3 d for males, and 5 to 7 d for females, postemergence) were briefly anesthetized using CO_2. Males were injected with 150 nL (510 ng) and females were injected with 200 nL (680 ng) of *AeAmt1* or control *β-lactamase* dsRNA diluted in PCR-grade water using a Nanojet III Programmable Nanoliter Injector (Drummond Scientific Company). dsRNA was injected dorsally into the thorax of mosquitoes.

Sperm Quantification and Ammonium [NH_4^+] Measurements in Spermathecae. Sperm quantification in the paired seminal vesicles of male *A. aegypti* at 24 h after dsRNA injection was performed following a published protocol, with minor modifications (69). The seminal vesicles were isolated from individual males, placed in 100 μL PBS, and gently torn open using extrafine forceps under a dissecting microscope (Carl Zeiss Canada) to release spermatozoa. An additional 10 μL PBS were used to rinse the forceps, and the PBS with spermatozoa was mixed thoroughly using a P100 pipette. Immediately after mixing for each animal, five 1 μL droplets of the mixture were spotted onto a microscope slide, were allowed to air dry, and were fixed with 70% ethanol. Slides were mounted using ProLong Gold antifade reagent with DAPI (Life Technologies), and nuclei of spermatozoa within each 1 μL droplet were imaged under 4× magnification using an Olympus IX81 inverted microscope (Olympus Canada). The nuclei of spermatozoa in each 1 μL droplet were counted using ImageJ software (ImageJ Version 1.51J8, NIH), averaged across all five droplets for each animal, and multiplied by the dilution factor to determine total spermatozoa counts within the seminal vesicle. Free ammonium (NH_4^+) concentrations were measured using a liquid membrane ion-selective microelectrode that was constructed using a detailed protocol (70). The three spermathecae from female *A. aegypti* were isolated under physiological saline (71), pooled together and transferred to a 0.5 μL droplet of saline submerged in hydrated paraffin oil, and carefully punctured with extrafine forceps to release contents into the droplet. Droplets were held in paraffin oil for no longer than 15 min before NH_4^+ measurements were made. Estimations of undiluted [NH_4^+] in spermathecae stated in the text (see *AeAmt1 Expression Is Only Detected in Spermathecae from Mated Females Containing Sperm*) were calculated by taking into account the volume of the spermathecae (60 μM diameter for the lateral lobes, 70 μM diameter for the large, median lobe) and the dilution factor of the three spermathecae when punctured (in 0.5 μL physiological saline) and subtracting the background [NH_4^+] concentration in droplets of physiological saline submerged under paraffin oil (~0.538 mM).

Mating and Egg-Laying Assays. Pupae of *A. aegypti* (Liverpool) were acquired from a colony reared in the Department of Biology, York University, and were used for all experiments (32). Female *A. aegypti* of this colony were blood fed twice weekly with sheep blood in Alsever's solution (Cedarlane Laboratories). Groups of male and female *A. aegypti* were separated at the pupal stage (identified by sexual dimorphisms in pupal size) and placed in BugDorm-5 insect boxes (MegaView Science Co.) with 20% sucrose solution where the adults emerged. For experimental analyses of mated females, a 1:1 ratio of male and female pupae was collected and reared in the same insect boxes as described above. This study employed two different mating assays protocols: 1) *AeAmt1* dsRNA knockdown males that were mated with nonmated females and 2) AeAmt1 dsRNA knockdown females that were mated with nonmated males prior to dsRNA injection. For protocol 1, dsRNA-injected males were placed with nonmated females (2:1 ratio of male:female per insect box) between 18 and 24 h after dsRNA injection. After 24 h, females were provided a blood meal. For protocol 2, mated females were separated from males and injected with dsRNA. After 48 h, females were provided a blood meal. For both mating protocols 1 and 2, females were given 20 min to blood feed, and all blood-fed females were subsequently isolated individually in inverted 25 cm^2 cell culture flasks (Corning) containing 3 mL of distilled water (dH_2O) from larvae rearing containers (to entice egg laying) lined with filter paper (51). Laid eggs were collected after 4 d and were semidesiccated for 48 h and then counted. Spermathecae were dissected from females and viewed under a microscope to confirm insemination occurred, and any nonmated females were excluded from experimental analyses. Eggs were placed in 20 mL of dH_2O with 0.5 mL larval food (1:1 ratio of liver powder and inactive yeast), and hatchlings were counted after 48 h.

Statistics. All statistical analyses were performed using the GraphPad Prism software, and the critical level was taken to be $P = 0.05$. The appropriate statistical test used for each experimental analysis is indicated in the figure caption with corresponding P values. Power analyses were performed on all datasets which yielded a minimum statistical power of 0.80 with the given sample sizes and variances of each group.

Data Availability. All study data are included in the article and *SI Appendix*.

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