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Rising stars in insect physiology

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Rising stars in insect physiology

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Editorial: Rising stars in insect physiology

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Editorial on the Research Topic
Rising stars in insect physiology

We are excited to present the inaugural Frontiers in Insect Science 'Rising Stars in Insect Physiology' Research Topic. When envisioning this Research Topic in 2022, our goal was to highlight the recent research contributions of early career investigators (graduate students, postdoctoral researchers, assistant professors) in Insect Physiology, with an emphasis on research topics that are integrative and/or multidisciplinary in nature. We aligned this Research Topic with a new travel award opportunity in 2022 that was co-sponsored by Frontiers and the Physiology, Biochemistry, and Toxicology (PBT) section of the Entomological Society of America (ESA), which sought to enhance the diversity of PBT section membership by supporting the travel of graduate students from under-represented groups to the annual conference of ESA (PBT Graduate Student Travel Award). Based on their impressive credentials, several applicants to this travel award were invited as contributors to the current Research Topic, and we were pleased that one of the inaugural awardees of the PBT Graduate Student Travel Award (Nia I. Keeys-Scott) was the primary author on the first manuscript to be accepted for publication in this Research Topic (Keyes-Scott et al.).

The four original contributions in this Research Topic focus on molecular and/or biochemical insights into the physiology of mosquitoes. Keyes-Scott et al. demonstrated the role of two previously orphaned G protein-coupled receptors (GPCRs) in reproduction of *Aedes aegypti*. Bianco et al. found that diapause in *Culex pipiens* can be disrupted by feeding them royal jelly produced by honey bees (*Apis mellifera*), which is enriched with Major Royal Jelly Protein 1, or by knocking down the mRNA encoding the orthologous protein in *C. pipiens*. Picinic et al. characterized the localization of several aquaporin (AQP) proteins in the alimentary canal, fat body, and ovaries of *A. aegypti* and demonstrated that localization was impacted by blood feeding, providing insights into putative roles in water and/or metabolite transport in these tissues. Finally, Sajadi and Paluzzi characterized the molecular and immunochemical expression of an understudied insect neuropeptide (ion transport peptide, ITP) in *A. aegypti* and used RNAi to uncover putative roles in excretory physiology, reproduction, and blood feeding.

The six reviews/mini-reviews in this Research Topic cover a variety of topics with connections to Insect Physiology and Toxicology. Abendroth et al. review recent evidence suggesting a non-canonical role of odorant binding proteins (OBPs) in adaptation of insects to xenobiotics. Mack and Attardo discuss the relationship between thermotolerance

and insecticide resistance in mosquitoes. They uncover a novel intersection between these physiological pathways that may be mediated by heat shock proteins, which has potential implications for vector control in the current era of climate change. Luker provides a critical review on the laboratory assays used in the past two decades to discover and assess the efficacy of mosquito repellents. Weger and Rittschof review the diverse physiological roles of insulin and insulin-like growth factor signaling in adult insects, along with molecular and neural mechanisms connecting insulin signaling to nutrition and behavior. Dates and Kolosov review novel and emerging roles of voltage-gated ion channels in non-excitable tissues, such as epithelia, where these channels have been understudied and may play key roles in epithelial transport and cell signaling. Finally, Vinauger and Chandrasegaran review studies on *A. aegypti* that use laboratory, semi-field, and field experiments to elucidate potential interactions between mosquito physiology and behavior and highlight the complications of studying mosquito life history traits associated with variations in larval competition nutrition and competition.

Together, these articles reflect that the future of insect physiology is in good hands, with a talented and diverse group of early career scientists leading the way. Insect physiology research is becoming increasingly integrative, and the above papers highlight that trend. The primary studies in this Research Topic incorporate approaches such as organismal biology, organ physiology, molecular biology, and behavior to demonstrate the complex interplay of factors that shape insect function. The review articles also synthesize diverse topics to highlight the importance of looking across traditional disciplinary boundaries (e.g., endocrinology, nutrition, behavior, etc.) to reveal insights into insect biology. We also want to thank an excellent group of reviewers for providing timely, thorough comments in support of these early career scientists. Through the efforts of the authors and review community, these 10 papers showcase some of the rising stars in our discipline that will lead the field into the next quarter of the century.

Author contributions

PP: Writing – original draft, Writing – review & editing. NT: Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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RNAi-mediated knockdown of two orphan G protein-coupled receptors reduces fecundity in the yellow fever mosquito *Aedes aegypti*

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G protein-coupled receptors (GPCRs) control numerous physiological processes in insects, including reproduction. While many GPCRs have known ligands, orphan GPCRs do not have identified ligands in which they bind. Advances in genomic sequencing and phylogenetics provide the ability to compare orphan receptor protein sequences to sequences of characterized GPCRs, and thus gain a better understanding of the potential functions of orphan GPCRs. Our study sought to investigate the functions of two orphan GPCRs, AAEL003647 and AAEL019988, in the yellow fever mosquito, *Aedes aegypti*. From our phylogenetic investigation, we found that AAEL003647 is orthologous to the SIFamide-2/SMYamide receptor. We also found that AAEL019988 is orthologous to the Trapped in endoderm (Tre1) receptor of *Drosophila melanogaster*. Next, we conducted a tissue-specific expression analysis and found that both receptors had highest expression in the ovaries, suggesting they may be important for reproduction. We then used RNA interference (RNAi) to knock down both genes and found a significant reduction in the number of eggs laid per individual female mosquito, suggesting both receptors are important for *Ae. aegypti* reproduction.

KEYWORDS

insect physiology, GPCR, reproduction, insect endocrinology, vector biology

1 Introduction

Mosquitoes are a persistent threat to global health due to their ability to transmit pathogens among vertebrate hosts through blood feeding, which is required for many mosquito species to produce eggs. The events beginning with blood meal digestion and ultimately leading to egg production are coordinated by several reproductive hormones, including insulin-like peptide 3 (ILP3) and ovary ecdysteroidogenic hormone (OEH),

which are released shortly after a blood meal is consumed (1–3). Release of ILP3 from brain neurosecretory cells stimulates blood meal digestion, and ILP3 and OEH both stimulate secretion of 20-hydroxyecdysone (20E) from the ovaries (1–4). After 20E is released into the hemolymph, expression of yolk protein precursors (YPP) in the fat body is induced, initiating the production of yolk proteins, including vitellogenin, which are subsequently transported to the ovaries and packaged into oocytes resulting in egg formation (5, 6).

Hormone signaling pathways have been exploited to control insect populations. Insect chemical growth regulators (IGRs), such as 20E antagonists, target insect hormonal pathways and have been utilized to control insect disease vectors (7, 8). IGRs are attractive control measures due to their selective toxicity against insects and decreased rate of insecticide resistance developed against them relative to traditional pesticides (9, 10). IGR targets such as, JH and 20E and their receptors, are widely conserved in insects increasing the chances of negative effects on non-target species (7, 8, 11–13). An attractive alternative to IGRs that act on JH or 20E are compounds that selectively target hormones or hormone receptors that are not widely conserved across all insect groups. G protein-coupled receptors (GPCRs) and their ligands may present taxa-specific targets, as insect genomes often encode unique GPCRs, including many that bind peptide hormones that regulate important aspects of insect physiology (14–16).

Hormone-binding GPCRs are essential in modulating insect physiology, including in metabolism (17, 18), reproduction (19), behavior (20), immunity (21), and embryonic development (22), as they transduce systemic hormonal signals into target cells. In addition to modulating a diverse number of functions in insects, GPCRs are the largest class of receptor and bind a variety of ligands, including neurotransmitters (23) and peptide hormones (24, 25). Peptide hormones govern many physiological functions in insects including feeding (26–29), mating behavior (30), development (31–33), metabolism (1, 18, 34–36), immunity (37), diuresis (38–40), and reproduction (1, 19, 41). While the ligands of many GPCRs have been identified, even well-studied organisms still encode GPCRs whose ligands are unknown.

Comparative genomics and phylogenetic analyses are useful tools in the identification of ligands of former orphan receptors (3, 19). Phylogenetic placement of orphan receptors, such as in the case of the OEH receptor of *Aedes aegypti* mosquitoes, can provide insights into potential ligands. A Venus flytrap domain-containing receptor tyrosine kinase was found to be closely related to the mosquito insulin receptor, and also displayed the same species distribution pattern as neuroparsin peptide hormones including OEH. Subsequent biochemical and molecular studies determined that the gene in question was an OEH receptor (3). Tissue-specific expression patterns are also useful in determining the functional roles and ligands of hormone receptors. We identified that the neuropeptide CNMa and its receptor, CNMaR, which were first identified in *Drosophila melanogaster*, were specifically expressed in *Ae. aegypti* ovaries and hypothesized that it was likely important for reproduction (3, 19, 42). In Culicidae, the CNMa receptor underwent gene duplication, resulting in two receptors, CNMaR-1a and CNMaR-1b, which both actively bind CNMa *in vitro* (19). In *Ae. aegypti*, CNMa and CNMaR-1b are highly expressed in female ovaries and modulate the production of eggs (19, 43).

We chose to examine two orphan GPCRs of *Aedes aegypti*, AAEL003647 and AAEL019988. These orphan GPCRs were chosen for further investigation based on their expression in female reproductive tissues following a blood meal (43), suggesting a potential role in the modulation of reproductive physiology. We built phylogenetic trees to identify closely related receptors and provide insight into possible functions of the receptors. To understand the tissue tropism and temporal distribution of AAEL003647 and AAEL019988, we conducted a detailed expression analysis of both GPCRs in juvenile and adult mosquitoes. Using RNAi, we then investigated the functional consequences of silencing the GPCRs on fecundity. These results shed new light on the role of these orphan GPCRs on the reproductive physiology of *Ae. aegypti* mosquitoes.

2 Materials and methods

2.1 Mosquitoes

UGAL strain *Aedes aegypti* were used for all experiments. Mosquito colonies were maintained at 27°C on a 16:8h L:D cycle. Larvae were fed Cichlid Gold fish pellets (Hikari, USA, Hayward, CA), and adult mosquitoes were fed an 8% sucrose solution until 2 days post-emergence. Adult females were fed defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA, USA) by an artificial feeding apparatus warmed to 37°C.

2.2 Phylogenetic analysis

Putative AAEL003647 and AAEL019988 orthologs were identified using OrthoDB (44). Taxa were chosen to represent all possible insect orders with available genome sequences (Tables S1, S2). Protein sequences were aligned using hmmalign as implemented in HMMER (45) with the –trim option. Gaps in alignments were manually removed, and trimmed alignments were used to construct maximum likelihood phylogenies using PhyML (46) using the options "-d aa -m LG -f e -o tl -b -2". FigTree version 1.4.4 was used for visualization of trees and trees were rooted on the midpoint. Accessions of included sequences are given in file S1.

2.3 Expression profiles

Eight to ten-day old, non-blood fed mated females were collected and dissected into head, gut, fat body, abdominal carcass ("pelt"), and clean ovaries without bursa or accessory glands in sterile, nuclease-free, *Aedes* saline. Additional ovary samples were collected from females at 2-hour intervals post-feeding (pbf) until 12 hours, then at 24, 48, and 72 hours pbf. Four or more tissue samples were collected for each tissue and time point. After collection, tissue samples were stored at -80°C prior to RNA extraction. Tissue samples were thawed on ice and

homogenized with a rotor pestle. Total RNA was isolated from homogenized tissues using the RNeasy Mini kit (Qiagen, Venlo, The Netherlands) according to manufacturer instructions. DNA was removed from each RNA sample using the Turbo DNA-free kit (Ambion, Austin, TX, USA). One hundred nanograms of RNA was used as input to synthesize cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). cDNA templates were used for quantitative real-time PCR, with the Quantifast SYBR Green PCR kit (Qiagen) and gene specific primers (Table S3). Standard curves for each gene were generated by cloning qPCR products into the pSCA vector with the Strataclone PCR cloning kit (Agilent, Santa Clara, CA, USA), isolating plasmid DNA using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Vilnius, Lithuania), and preparing plasmid standards to a known copy number. Expression levels of ribosomal protein S7 were used as a housekeeping gene to normalize transcript abundance.

2.4 RNAi knockdown of receptors and bioassays

A 400-500 bp region of each gene was chosen as a target for dsRNA synthesis for AAEL003647 and AAEL019988, subsequently referred to as *ds3647* and *ds19988*, respectively. Primers including the T7 promoter sequence were used to amplify each target using cDNA synthesized from RNA isolated from whole body, non-blood fed females (Table S3). PCR products were cloned into the pSCA vector and plasmid DNA was extracted using methods listed above. Plasmid DNA from each target and an EGPF control were used as the templates for dsRNA synthesis. dsRNA was synthesized using the MEGAscript RNAi kit (Ambion, Vilnius, Lithuania), according to manufacturer instructions. Following dsRNA synthesis, dsRNA was precipitated in ethanol and resuspended in *Aedes* saline to a concentration of 2μg/μL.

Newly emerged (≤ 1d post eclosion) mated females were injected with 2 μg *ds3647*, *ds19988*, or *dsEGFP*. To validate receptor knockdown, whole body females were collected 7 days post-injection. qPCR was used to validate knockdown of each gene using the methods detailed above. Females were blood fed three days post-injection and separated into individual egg laying chambers consisting of a damp paper towel in a plastic cup with a lid and a dental wick with 8% sucrose solution, for yolk deposition and fecundity bioassays. For yolk deposition bioassays, females were collected at 24, 48, and 72 hours PBF. Ovaries were dissected and yolk deposition per oocyte was measured along the anterior-posterior axis using an ocular micrometer. Five oocytes were measured and averaged per female, and 5 females were used per time point and treatment. Egg laying was measured by providing females with a wet paper towel at 72 h post blood feeding to stimulate egg deposition. Females were given 48 h to deposit eggs. After 48 h hours, the number of eggs laid per individual female was counted. Another cohort of knockdown females were allowed to lay eggs then dissected and the number of retained, mature oocytes were counted. Eggs that were laid were separated by parent and allowed to hatch, and the proportion of hatched versus unhatched eggs was recorded for each treatment.

3 Results

3.1 Phylogenetic comparison of AAEL003647 and AAEL019988

Our phylogenetic analysis included diverse insect species to identify the closest receptor relatives across both holometabolous and hemimetabolous insects. Our results for AAEL003647 indicate this receptor groups in a strongly supported clade of receptors that are distinct from, but sister to, the SIFamide receptors (Figure 1). These receptors are found in the genomes of culicids as well as cockroaches (*Periplaneta americana* and *Blattella germanica*), termites (*Zootermopsis nevadensis*). This robustly supported sister clade to SIFamide receptors suggest an ancient split between SIFamide receptors and the orthologs of AAEL003647 which predates the split of hemi- and holometabolous insects. Orthologs of AAEL003647 appear to have been lost in many lineages. No orthologs were found in lepidopteran, coleopteran or hymenopteran genomes. In contrast, most sequenced hemipteran genomes contained orthologs, several of which have subsequently duplicated. In the order Diptera, AAEL003647 orthologs were found in most nematoceran genomes, but absent from many available brachyceran genomes, including sequences from all members of the genus *Drosophila*. This loss was not complete in Brachycera, as *Rhagoletis zephyria* and *Hermetia illucens* both encode AAEL003647 orthologs in their genomes. Within the Culicidae, each species examined has a single ortholog of AAEL003647 with the notable exception of *Anopheles maculatus*, which has five orthologous sequences in OrthoDB (44). Two sequences were identified as orthologs of the SIFamide receptor (AMAM023590 and AMAM011260), and two orthologs identified as orthologs of AAEL003647 (AMAM023042 and AMAM009506). All of these sequences are lacking the complete 7 transmembrane region of canonical GPCRs and it seems likely that these sequences do not reflect true orthologs but rather annotation artefacts, potentially fragments of a single ortholog to the SIFamide receptor and AAEL003647. An additional duplication in *Anopheles maculatus* groups with the SIFamide-like receptor of *Thrips palmi*. Further investigation of this ortholog suggests that it is unique to *An. maculatus*, and that its grouping with non-mosquito sequences is likely an artifact of the alignment. Improved sequencing of the *An. maculatus* genome will likely resolve this in the future.

Our analysis identified AAEL019988 as an ortholog of the *D. melanogaster* trapped in endoderm (tre1) GPCR with strong support (Figure 2). Tre1 appears to be highly conserved among holometabolous insects but is absent from many hemimetabolous lineages. Only the orders Blattodea, Odonata, Thysanoptera, and Grylloblattidae encode orthologs. The sister group to this clade includes both the GPCRs Moody and Moody-like, which are known to be important to blood-brain barrier in *Drosophila melanogaster* (47).

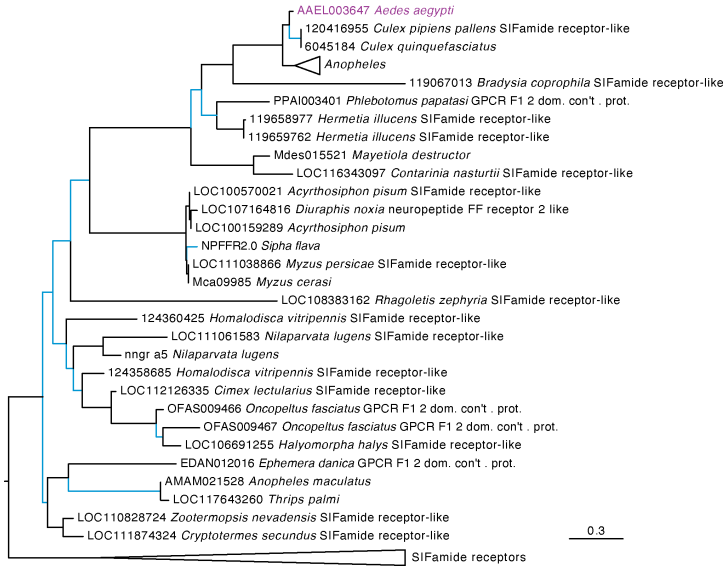

FIGURE 1
Maximum likelihood tree of AAEL003647 and its orthologs in other insects. Orthologs of AAEL003647 have been lost in many brachyceran taxa, including members of the genus *Drosophila*. AAEL003647 is most closely related to the SIFamide receptor. Sequences were downloaded from OrthoDB and aligned against a 7 transmembrane GPCR model (7tm-1.hmm) in hmmalign. Trees were built in PhyML. Support values are aLRT SH-like, and branches with support values < 0.95 are colored light blue. F1 2 domain containing protein is abbreviated as "F1 2 dom. con't. prot." Due to space constraints, orthologs of AAEL003647 in *Anopheles* species and SIFamide receptor sequences were collapsed. A full tree containing the *Anopheles* taxa is available in Supplementary Figure S1.

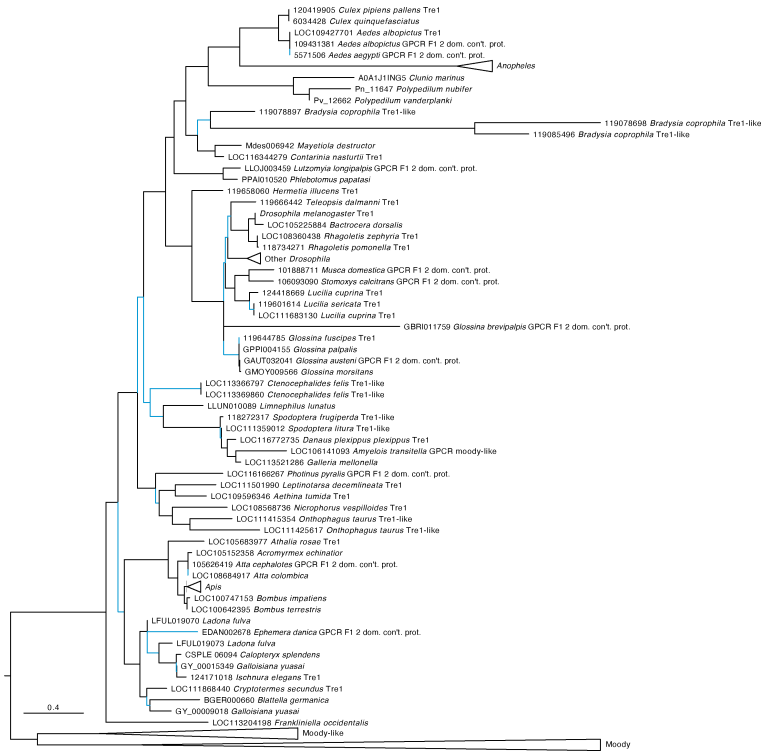

FIGURE 2
Maximum likelihood tree of AAEL019988 and its orthologs in other insects. AAEL019988 is absent in most but not all hemimetabolous insects and is conserved in most holometabolous lineages. The tree was rooted at the midpoint which formed two major clades, the orthologs of Trapped in endoderm 1 (tre1) and the orthologs of moody and moody-like. Sequences were downloaded from OrthoDB and aligned against a 7 transmembrane GPCR model (7tm-1.hmm) in hmmalign. Trees were built in PhyML. F1 2 domain containing protein is abbreviated as "F1 2 dom. con't. prot." Support values are aLRT SH-like and branches with low support (< 0.95) are highlighted in blue. Due to space constraints, sequences from *Anopheles, Drosophila*, and *Apis* species, as well as Moody and Moody-like sequences, were collapsed. A full tree with the expanded AAEL019988 orthologs is shown in Figure S2.

3.2 Tissue tropism of orphan receptors

We investigated expression patterns of *AAEL003647* and *AAEL019988* among life stages, sexes, and tissues. Expression of *AAEL003647* was highest in females relative to males and immature stages (one-way ANOVA, $p < 0.0001$) (Figure 3A). Expression of *AAEL019988* was higher in adult females relative to 1st, 3rd, 5th instar larval, and pupal stage mosquitoes (one-way ANOVA, $p < 0.05$). There was no significant difference in expression between females and males (Figure 3B). We next examined tissue tropism of the receptors in females. The highest expression of *AAEL003647* and *AAEL019988* was observed in the ovaries (Figures 4A, B). We next measured receptor expression across a time series following a blood meal. Our results demonstrate that expression of *AAEL003647* was highest in non-blood fed, 2h, 4h, and 6h pbf female ovaries (Figure 4C). Expression of *AAEL019988* was highest in NBF ovaries (Figure 4D).

3.3 Effects of knockdown of AAEL003647 and AAEL019988 on female reproduction

The peaks of expression prior to feeding and nearing the time of oviposition informed our hypothesis that AAEL003647 and AAEL019988 may be important in regulation of egg production and/or oviposition. To understand the effects of both orphan GPCRs on oviposition, we injected newly eclosed female mosquitoes with 2 µg of *ds3647*, *ds19988*, or *dsEGFP*. For each receptor, we were able to achieve an 85% whole body transcript knockdown (one-way ANOVA, $p < 0.0163$, $p < 0.0163$, respectively; Figures 5A, B). Following dsRNA injection, females were allowed to mate and were fed 3 days post-injection. After feeding, females were separated into individual enclosures for oviposition assays. We found that *ds3647* and *ds19988* injected females laid significantly fewer eggs than *dsEGFP* injected females (one-way ANOVA, $p = 0.0184$, $p = 0.0393$, respectively; Figure 5C).

The observed reduction in egg laying by mosquitoes treated with *ds3647* or *ds19988* could be due to a disruption of egg maturation or egg laying. To disentangle this, we examined whether yolk deposition of *ds3647* and *ds19988* injected females was impaired, which would suggest that the receptors are important in post-vitellogenic egg development. We injected newly eclosed females with *dsEGFP*, *ds3647* or *ds19988*, fed females a blood meal at 3 days post injection, and dissected ovaries from blood fed females at 24, 48, and 72h pbf. Following dissection, we measured the packaged yolk in per individual oocyte with an ocular micrometer. We found no significant difference among oocyte yolk lengths in *ds3647*, *ds19988*, or *dsEGFP* injected females (one-way ANOVA, $p > 0.05$; Figure 5D), suggesting that the receptors mediate physiological events after egg maturation. We then examined the effect of receptor knockdown on the egg retention and egg hatching. Knockdown of *AAEL003647* did not result in retained eggs in females, but *AAEL019988* knockdown mosquitoes retained more mature oocytes than dsEGFP controls (Figure 6A, $p = 0.0476$; Wilcoxon rank-sum test). Of eggs that were laid, there was no difference in the proportion of eggs that hatched, suggesting that knockdown of the receptors does not interfere with fertilization (Figure 6B).

4 Discussion

Our phylogenetic analysis identified that ancestor of SIFaR underwent gene duplication early in arthropod evolution. This paralog is retained in several arthropod lineages including members of the Culicidae, *Ae. aegypti* (AAEL003647) and *Anopheles gambiae* (AGAP003335). The SIFamide receptor binds the peptide hormone SIFamide, which is localized to neurosecretory cells in the insect brain and central nervous system (29, 48, 49, 50). SIFamide is conserved among hemimetabolous and holometabolous insects and acts as a neurohormone to modulate appetitive behavior (28), feeding (29), heart contractions (29), and mating behavior (30). The phylogenetic

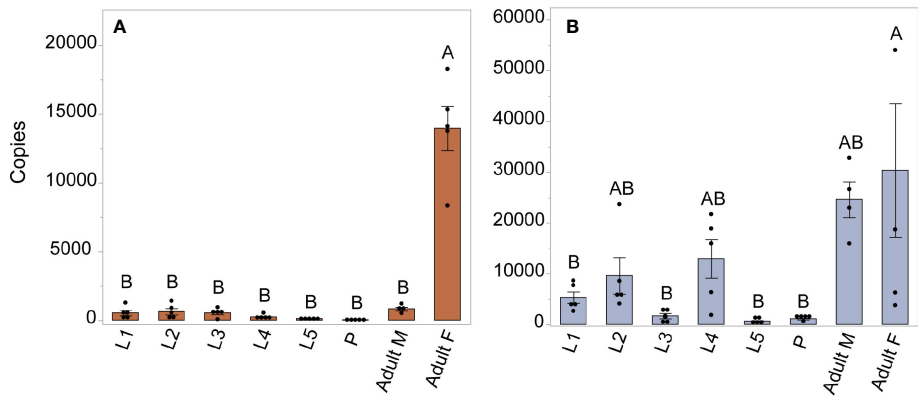

FIGURE 3
Expression profile of AAEL003647 and AAEL019988 in whole bodies of mosquitoes across life stages and sexes. The x-axis represents the number of copies of AAEL003647 and AAEL019988 per 100ng of RNA. **(A)** Expression of AAEL003647 is significantly higher in adult females (one-way ANOVA, $p < 0.0001$). **(B)** Expression of AAEL019988 was also significantly higher in adult females relative to 1st, 3rd, and 5th stage larvae and pupae (one-way ANOVA, $p < 0.05$). Treatments connected by the same letter are not significantly different ($p > 0.05$, one-way ANOVA). The letters above each bar/box in these figures indicate statistical significance. Different letters indicate statistical significance, and connected letters indicate no statistical significance.

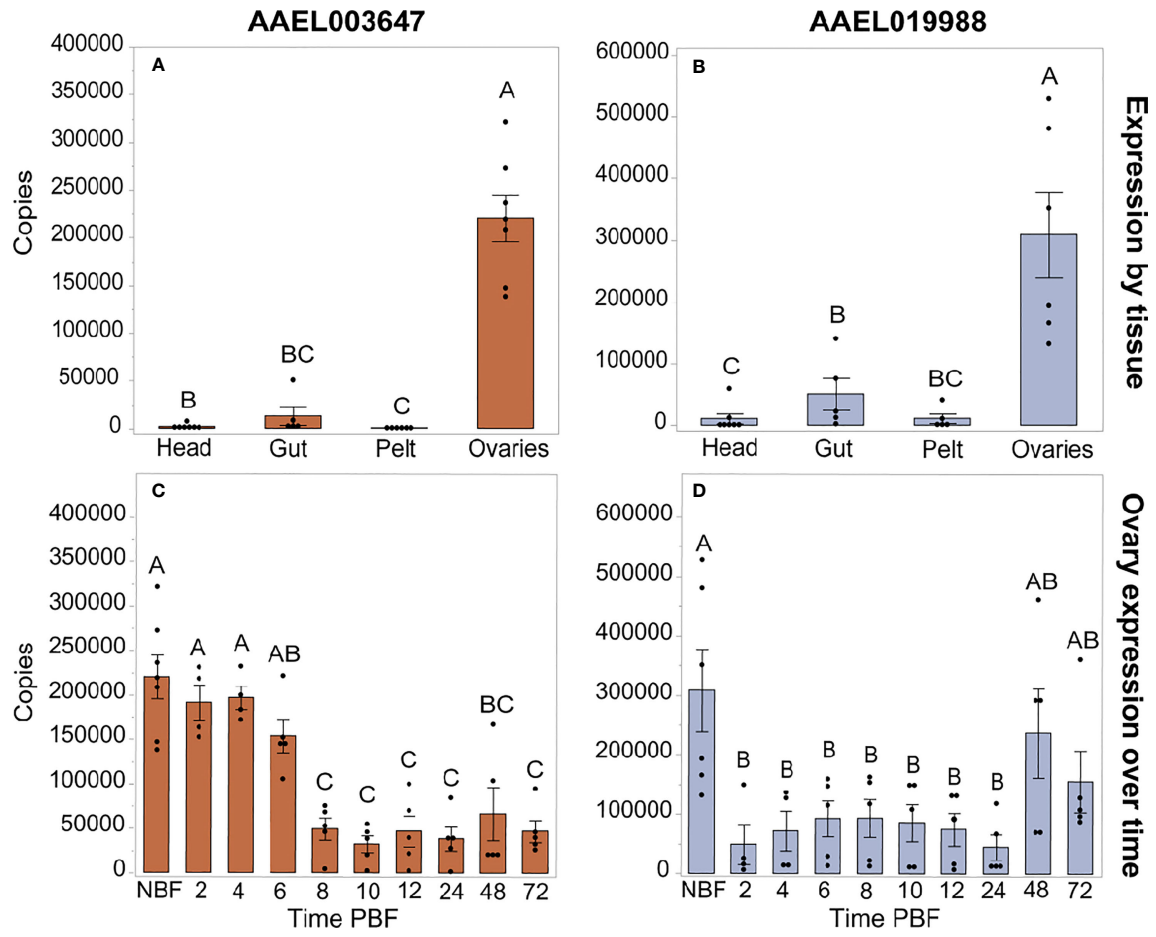

FIGURE 4
Expression profiles of AAEL003647 and AAEL019988 in NBF *Ae. aegypti* tissues **(A, B)** and in whole bodies following a blood meal **(C, D)**. Expression of AAEL003647 and AAEL019988 is highest in the ovaries for **(A)** AAEL003647 (one-way ANOVA, $p \leq 0.003$) and **(B)** AAEL019988 (one-way ANOVA, $p \leq 0.0092$). **(C)** Expression of AAEL003647 is significantly higher in the ovaries of NBF, 2h, 4h, and 6h pbf females (one-way ANOVA, $p < 0.05$). **(D)** Expression of AAEL019988 is significantly higher in the ovaries of NBF females (one-way ANOVA, $p < 0.05$). The letters above each bar/box in these figures indicate statistical significance. Different letters indicate statistical significance, and connected letters indicate no statistical significance.

relationships of insect SIFaR receptors indicate an ancient divergence early in arthropod evolution, as evidenced by the presence of two receptor genes in diverse insect species including aphids, cockroaches, and mosquitoes. Veenstra recently identified a novel peptide hormone, SMYamide, in the genome of the American cockroach *Periplaneta americana* (48). Phylogenetic analysis of the novel peptide revealed that it was sister to the *P. americana* SIFamide peptide, and though binding assays were not performed, the results suggest that SMYamide likely binds the protein encoded by the *SIFaR-2* gene of *P. americana*. Our expanded phylogenetic analysis indicates that the *P. americana* SIFaR-2 is an ortholog of AAEL003647, though we could not identify an ortholog of SMYamide in the *Ae. aegypti* genome. Future binding studies of AAEL003647 will focus on determining if the receptor binds SIFamide, a distant ortholog of SMYamide, or a novel peptide hormone.

The *Drosophila melanogaster* orphan GPCR, Trapped in Endoderm 1 (Tre1), was identified as an ortholog of AAEL019988 in our phylogenetic analysis. Tre1 is essential for the transepithelial migration of germ cells through the posterior midgut during embryogenesis (51–55). Tre1 is also important for the initiation of courtship behavior *D. melanogaster* (56). The role of Tre1 in germ cell migration and in courtship may have led to the co-option of this signaling system to regulate reproduction in *Ae. aegypti*. Interestingly, Tre1 is absent in most hemimetabolous insects.

Our expression profiles of *AAEL003647* and *AAEL019988* indicate that transcript abundance of both receptors is highest in adult females' ovaries, suggesting potential roles in egg production. To determine the potential roles of each orphan receptor in female reproductive physiology, we carried out a series of knockdown experiments which resulted in fecundity reduction in *ds3647-* and *ds19988-*injected females. Subsequently, we found that knockdown of both orphan receptors did not affect the amount of yolk packaged into oocytes, suggesting limited interactions with ILP3 and OEH, which are reproductive hormones that are known to modulate oogenesis (1–3). These results point to a role in oviposition rather than egg production.

The role of the SIFamide, a sister clade to AAEL003647, provides potential clues towards the mechanism of this receptor and its as-yet unknown ligand. SIFamide has been implicated in modulation of feeding and mating behavior in *Drosophila* (28, 29). SIFamidergic neurons are activated during starving conditions and

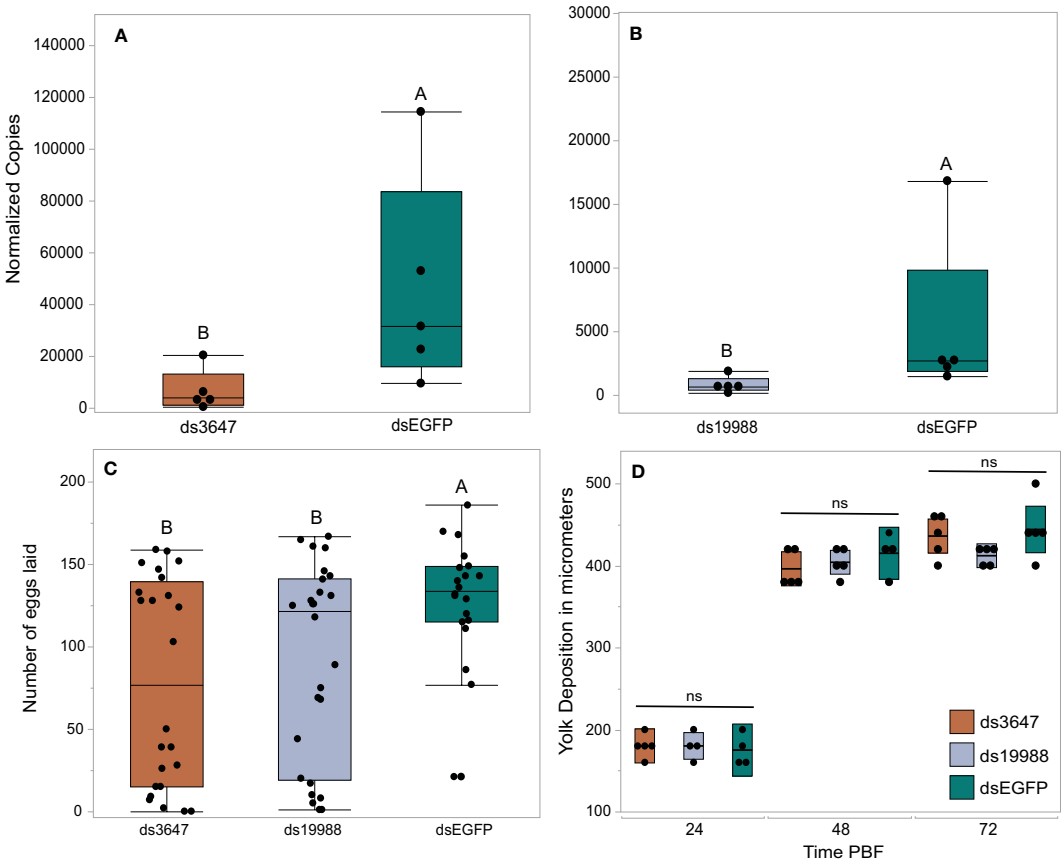

FIGURE 5
RNAi knockdowns **(A, B)**, oviposition bioassays **(C)**, and yolk deposition **(D)**. **(A, B)** Receptor knockdown validation. We achieved an 85% whole body transcript knockdown for AAEL003647 **(A)** (Wilcoxon rank-sum test, $p = 0.0163$) and AAEL019988 **(B)** (Wilcoxon rank-sum test, $p = 0.0163$). The x-axis represents the number of copies of AAEL003647 and AAEL019988 per 100ng of RNA. Transcripts were normalized by ribosomal S7 expression. **(C)** Knockdown of AAEL003647 and AAEL019988 resulted in a significant decrease in the number of eggs laid relative to dsEGFP controls (Wilcoxon rank-sum test, $p = 0.0184$, $p = 0.0393$, respectively). **(D)** Knockdown of AAEL003647 and AAEL019988 had no effect on yolk uptake (Wilcoxon rank-sum test, $p > 0.05$). ns = not significant, indicating average yolk length among each experimental treatment is not statistically significantly different. The letters above each bar/box in these figures indicate statistical significance. Different letters indicate statistical significance, and connected letters indicate no statistical significance.

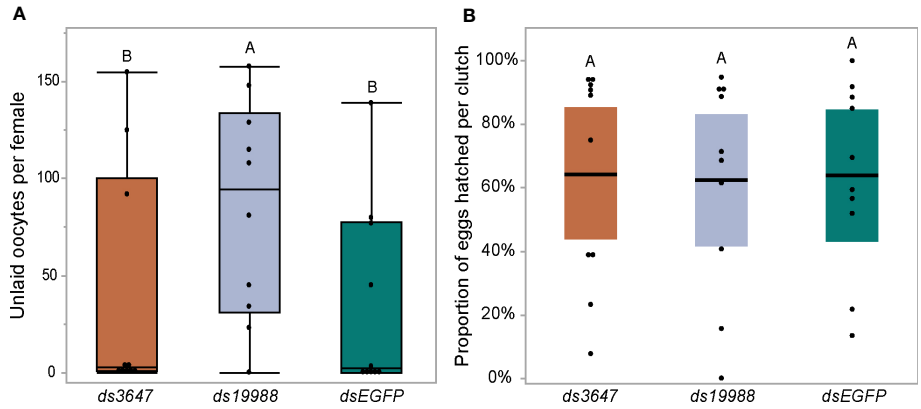

FIGURE 6
Effect of RNAi knockdown of AAEL003647 and AAEL019988 on egg retention **(A)** and egg hatching **(B)** of *Ae. aegypti*. Knockdown females were blood fed then allowed to lay eggs in individual cups. Females were then dissected and the number of unlaid, retained eggs were counted. Eggs were then allowed to hatch under standard conditions and successfully hatched larvae were counted. dsAAEL019988 females retained significantly more eggs than controls ($p = 0.0476$; Wilcoxon rank-sum test) while there was no significant difference between AAEL003647 knockdowns and controls. The letters above each bar/box in these figures indicate statistical significance. Different letters indicate statistical significance, and connected letters indicate no statistical significance.

are inhibited by the myosin inhibitory peptide (MIP) which modulates satiation (28). This SIFa/MIP neuropathway governs feeding behavior in *Drosophila*, but also directly affects mating behavior (28, 29). SIFa acts on *fruitless* in *Drosophila*, which modulates courtship behavior; upon inhibition of SIFaR, male flies exhibited bisexual mating behaviors (30). Although AAEL003647 and SIFaR belong to phylogenetically sister clades, it does not guarantee functional similarity. However, there is a possibility these receptors share similar functions, including modulation of oviposition by interaction with MIP.

AAEL019988 is an ortholog of Tre1, which in *Drosophila* regulates mating behavior. Luu et al., 2016 found that some *fruitless* expressing neurons also expressed *Tre1*, and that male and female flies exhibited expression of *Tre1* in a sexually dimorphic fashion (56). Female *Tre*1 expression was induced in males by generating transgenic males expressing the female Tre1 splice form, tra^f. This *Tre1* "feminization" in males resulted in latency in initiation of courtship behavior and complete absence of courtship initiation behavior in some males. However, there was no significant effect of *Tre1* feminization on the number of offspring per *Tre1* mutant male that mated with a female (56). We found that knockdown of AAEL019988 disrupts egg laying but not egg development, suggesting that it may have evolved alternative functions including but not limited to mating behaviors in *Ae. aegypti*. Future studies of AAEL003647 and AAEL019988 will examine the impacts of these orphan receptors on feeding and mating behavior, including through interactions with *fruitless* in *Ae. aegypti*.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

Conceptualization: KV. Methodology: NK-S and KV. Experimentation: NK-S, KS, LA, and KV. Writing—original draft preparation: NK-S. Writing—review and editing: KV, NK-S, KS, and LA. Project administration: KV. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Roles of insect odorant binding proteins in communication and xenobiotic adaptation

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Odorant binding proteins (OBPs) are small water-soluble proteins mainly associated with olfaction, facilitating the transport of odorant molecules to their relevant receptors in the sensillum lymph. While traditionally considered essential for olfaction, recent research has revealed that OBPs are engaged in a diverse range of physiological functions in modulating chemical communication and defense. Over the past 10 years, emerging evidence suggests that OBPs play vital roles in purifying the perireceptor space from unwanted xenobiotics including plant volatiles and pesticides, potentially facilitating xenobiotic adaptation, such as host location, adaptation, and pesticide resistance. This multifunctionality can be attributed, in part, to their structural variability and effectiveness in transporting, sequestering, and concealing numerous hydrophobic molecules. Here, we firstly overviewed the classification and structural properties of OBPs in diverse insect orders. Subsequently, we discussed the myriad of functional roles of insect OBPs in communication and their adaptation to xenobiotics. By synthesizing the current knowledge in this field, our review paper contributes to a comprehensive understanding of the significance of insect OBPs in chemical ecology, xenobiotic adaptation, paving the way for future research in this fascinating area of study.

KEYWORDS

xenobiotics, semiochemicals, adaptation, co-option, host location, pesticide resistance

1 Introduction

The ability to perceive and differentiate various chemical stimuli present in a set environment is paramount to an organism's success (1–4). Insects, the most successful group of animals on Earth, have developed a sophisticated olfactory system that has widely contributed to this success. Insect olfactory systems are known for their remarkable sensitivity and the ability to integrate odorant blends through distributed specificity of receptor tuning profiles (5–7). The classification and integration of these profiles in different portions of "odor

space" rely on structures like the mushroom body and lateral horn of the protocerebrum, enabling precise discrimination of pheromone blends or subtle differences in plant odor blends (5, 8). Insect olfaction is composed of several transmembrane receptors and soluble and insoluble proteins, which collaborate harmoniously to receive, process, interpret, and ultimately react to external stimuli (3). The key olfactory proteins involved in this process include odorant binding proteins (OBPs), odorant receptors (ORs), ionotropic receptors (IRs), odorant degrading enzymes (ODEs). and sensory neuron membrane proteins (SNMPs) (3). ORs form a heteromeric complex with a ubiquitous coreceptor coined odorant receptor co-receptor (Orco) that is omni-present in every functional OR complex and is highly conserved among all insects (3). In general, exogenous odorants or volatiles enter the sensillum lymph through cuticular pores and are subsequently bound and solubilized by OBPs, wherein this OBP-odorant complex is transported across the sensillum to a candidate OR for transduction (3, 9) (Figure 1). Once the OBP-odorant complex (or the odorant alone) is bound to a receptive OR, a transduction cascade is triggered, which leads to action potentials transmitting from olfactory receptor neurons to the higher integration centers within the protocerebrum. Odorants must be deactivated rapidly by ODEs or scavengers once this occurs, otherwise efficiency of olfactory processes will be impaired via prolonged exposure of the respective odorant inducing overstimulation. Numerous lines of evidence suggest that many ODEs such as cytochrome P450s, glutathione S-transferases (GSTs), carboxyl/cholinesterases (CCEs) are involved in degrading volatile molecules during the deactivation process (3, 10–13). Some studies indicate that prior to degradation by ODEs, pheromones undergo deactivation through their binding to OBPs (e.g., pheromone binding proteins, PBPs). Additionally, these OBPs serve as scavengers, contributing to the decline of the receptor potential after stimulus offset. This implies the existence of a broader molecular mechanism beyond enzymatic degradation (3, 14–17).

Within the realm of olfaction processing, OBPs play a vital role as the primary mediators connecting the external environment with ORs (7, 9). OBPs are frequently necessary for safeguarding exogenous hydrophobic volatiles against degradation prior to their interaction with the corresponding ORs. This protection occurs following the initial uptake, binding, and transportation of these volatiles within the aqueous sensillum lymph. The delivery of the exogenous volatiles to the OR triggers an elicited response, allowing for the recognition of volatiles from hosts or natural enemies and identification of pheromones of potential mates. Following the stimulation of ORs by exogenous molecules, OBPs may also participate as molecular traps, preventing neuron oversaturation (1–3, 17–20). In addition, evidence shows that OBPs may play essential roles in cleaning the perireceptor space from undesirable xenobiotics, including plant volatiles and pesticides. This function potentially contributes to host plant adaptation and pesticide resistance (20–27). Despite their primary role as olfactory proteins, recent research has identified OBPs to be involved in a variety of physiological roles in insects outside of olfactory tissues, owing in part to their structural variability and efficacy in the transporting, sequestering, and concealing of various hydrophobic molecules (2, 3, 9, 28–30).

Roughly half of insect species are phytophagous, forming a close relationship with the host plants they feed and interact with (31). During the coevolution of insects and plants over hundreds of millions of years, insects have evolved diverse mechanisms to adapt to numerous xenobiotics (12, 13, 32–34). Olfaction in insects may serve as an "Achilles heel" - a target for plant defense because of its remarkable sensitivity, critical importance, and vulnerability (22). OBPs serve as the primary point of contact for the insect olfactory system with xenobiotics, playing a principal role in modulating chemical communication and defense. Here, we initially summarize the classification and structural properties of OBPs in various insect orders. Then we focus on the variety of functional roles of OBPs in insect communication and adaptation to xenobiotics. Our review

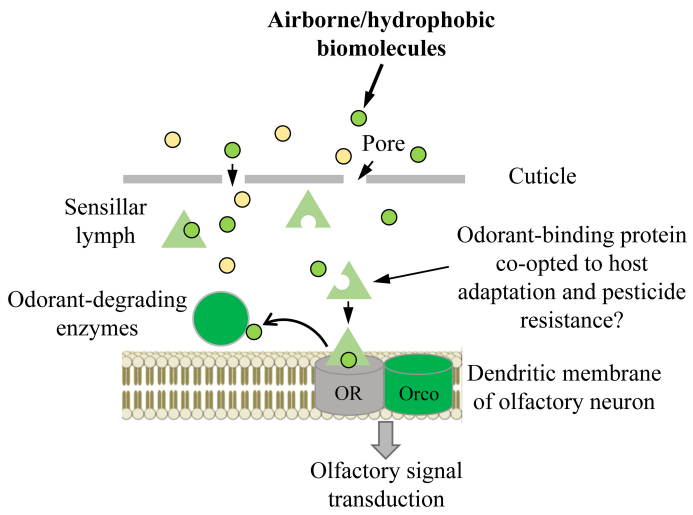

FIGURE 1
Schematic summary of the odor path. OR, odorant receptor (in some cases, it can involve other olfactory receptors, such as ionotropic receptors); Orco, co-receptor for OR.

concludes with prospective thoughts on future studies that could expand our knowledge of OBPs and their diverse functions in chemical ecology and xenobiotic adaptation.

2 Classification and structural characteristics of insect OBPs

Insect OBPs are small water-soluble extracellular proteins, ranging from between roughly 100 to ~200 amino acid residues, with very little sequence similarity within OBPs of the same species (1). Initially described in Lepidoptera (16), these proteins were categorized into three separate subfamilies based on the amino acid sequences and differential expression patterns: pheromone binding proteins (PBPs), general odorant binding proteins (GOBPs), and antennal binding proteins (ABPs) (1, 16). However, a primary challenge with this classification methods arises from the significant variation observed in the amino acid sequences, ligand binding affinity, differential expression, and functional roles beyond Lepidoptera, extending even to functions beyond chemosensation (35, 36). Therefore, there was a pressing need for a more comprehensive and flexible classification method to accurately characterize their diverse functional roles and implications. Currently, insect OBPs are generally divided into three primary groups based on the number of conserved cysteine residues and interlocked disulfide bridges: 1) Classic OBPs (e.g. *Chrysopa pallens* CpalOBP4, PDB ID:6JPM), which have six conserved cysteine residues that participate in three disulfide bridges; 2) Minus-C OBPs (e.g. *Apis mellifera* AmelOBP14, PDB ID:3S0A), featuring four or five conserved cysteine residues and two disulfide bridges; 3) Plus-C OBPs (e.g. *Anopheles gambiae* AgamOBP7, PDB ID:3R1P), which possess eight or more conserved cysteine residues, four or more disulfide bridges, and a conserved proline residue (Figure 2) (36). Among these groups, Classic OBPs are the most frequently identified type of OBPs in every insect genome (Table 1; Figures 2, 3). Phylogenic analysis of insect OBPs have shown that Classic OBPs seem to be the basal group, and other Minus-C and Plus-C groups of OBPs are subgroups of the Classic OBPs (39). This may suggest that Minus-C and Plus-C OBPs likely diverged from the Classic OBPs (39–41) (Figure 2). However, the relative composition of OBPs in an insect genome can vary greatly, as some OBP groups may feature a larger expansion in one group of insects as compared to others, as has been observed in certain beetle species (35, 42–47) (Figure 3A; Table 1). There is a group of OBPs that has been termed "atypical OBPs" characterized by 10 or more conserved cysteines, a long C-terminus, a conserved proline residue, and four or more disulfide bridges, which is recorded in several mosquito and locust species, suggesting this group of genes may be recently evolved in these species (36, 48–50). Additionally, groups of insect OBPs that exist outside of the three primary structural groups can be found in certain insects, such as double domain OBPs that are found exclusively in certain wasp species (51) and Dimer OBPs that are found in some species of dipterans and lepidopterans (Figures 3A–C; Table 1) (39, 51). In certain insect groups, there is a complete absence of an entire primary group of OBPs; for instance, honey bees lack of plus-C OBPs all together (Figure 3B; Table 1) (40). The amount of OBP genes in an insect genome can vary greatly among species, ranging from as low as 7 in *Ceratosolen solmsi* to as high as 111 in *Aedes aegypti* (Table 1). The reason why certain insect species possess a higher number of OBPs while others have relatively few remains unclear. However, this disparity can likely be attributed to the insects' unique lifestyles, evolutionary processes, and wide variety of environments (39).

Despite the high diversity and variation among insect OBPs, this group of proteins has some hallmark features. In addition to the extremely conserved cysteine residues, insect OBPs typically have two to four interconnected disulfide bridges (e.g., a pattern of C1-C3, C2-C5, and C4-C6) that play a vital role in stabilizing the protein (52–58) (Figure 2). Furthermore, six α-helices, which may vary in number in certain cases, synergistically work with the interlocked disulfide bridges to further enhance the protein's stability. Specific α-helices may be involved in forming a

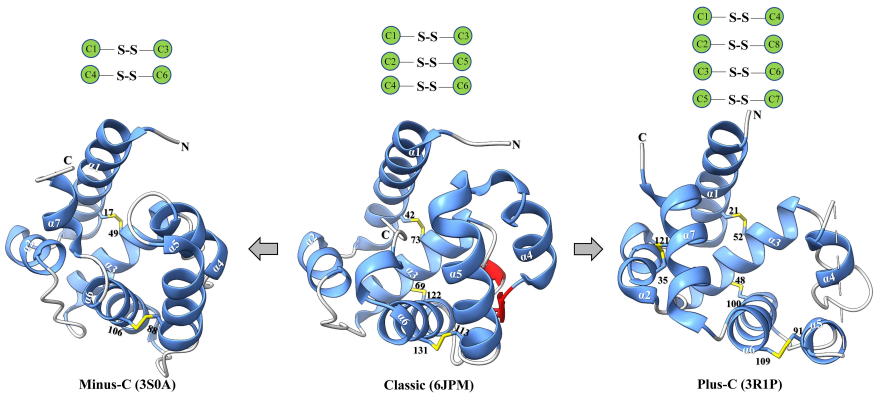

FIGURE 2
Major classes of insect OBPs. Beginning from the left, minus-C (e.g. *Apis mellifera* AmelOBP14, PDB ID:3S0A); Classic (e.g. *Chrysopa pallens* CpalOBP4, PDB ID:6JPM); Plus-C (e.g. *Anopheles gambiae* AgamOBP7, PDB ID:3R1P). Blue indicates α-helices; yellow indicates disulfide bridge; red indicates strands; and lastly grey indicates coils. Black text indicates a conserved cysteine residue, white text indicates an α-helix. Below each protein is the corresponding class of the odorant binding protein and the protein database reference used to generate the specific protein. Three-dimensional protein structures were constructed using the program ChimeraX.

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TABLE 1 Number of Odorant Binding Protein genes and classification in genomes or transcriptomes of 37 insect species.

Order	Species	Total	Classic	Minus-C	Plus-C	Other*	Reference [$]
Blattodea	*Blatella germanica*	109	38		71	0	(1)
	Periplaneta americana[†]	60	37	3	20	0	(2)
	Zootermopsis nevadensis	29	19	3	7	0	(2)
Coleoptera	*Anoplophora glabripennis*	52	20	31	1	0	(3)
	Dendroctonus ponderosae	31	18	12	1	0	(4)
	Holotrichia oblita[†#]	29	19	7	3	0	(5)
	Holotrichia parallela[†#]	25	15	6	4	0	(6)
	Leptinotarsa decemlineata[#]	59	14	43	1	1	(7)
	Tenebrio molitor[†]	19	10	8	0	1	(8)
	Tribolium castaneum[#]	49	20	21	1	7	(9, 10)
Diptera	*Aedes aegypti*	111	39	0	27	45	(11)
	Anopheles gambiae	69	29	0	20	20	(11)
	Anopheles stephensi	44	27	0	7	10	(12)
	Culex quinquefasciatus	109	69	0	12	28	(11)
	Drosophila melanogaster	52	28	7	15	2	(13-15)
Hemiptera	*Acyrthosiphon pisum*	15	13	0	2	0	(16)
	Adelphocoris lineolatus[†]	14	12	0	2	0	(17)
	Bemisia tabaci	8	5	1	2	0	(18)
	Riptortus pedestris	49	41	0	8	0	(19)
	Tropidothorax elegans[†]	19	14	0	5	0	(20)
Hymenoptera	*Aphidius gifuensis*[†]	14	12	2	0	0	(21)
	Apis florea[#]	22	13	9	0	0	(22)
	Apis mellifera[#]	21	13	8	0	0	(22, 23)
	Bombus terrestris[#]	16	16	0	0	0	(24)
	Ceratosolen solmsi	7	7	0	0	0	(25, 26)
	Cotesia vestalis	20	18	2	0	0	(27, 28)
	Nasiona vitripennis[#]	90	72	8	0	10**	(29)
Lepidoptera	*Bombyx mori*[#]	44	29	9	6	0	(29, 30)
	Danaus plexippus[#]	32	19	6	6	1	(31)
	Heliconius Melpomene[#]	51	23	22	6	0	(31)
	Manduca sexta[#]	49	24	18	7	0	(31)
	Plutella xylostella	39	39	0	0	0	(32)
	Spodoptera frugiperda	33	25	3	3	2	(33)
Orthoptera	*Locusta migratoria*	17	11	0	5	1	(34)
	Oedaleus asiaticus[†]	15	10	1	4	0	(35)
	Schistocerca gregaria[†]	14	9	0	3	2	(35)
Thysanoptera	*Odontothrips loti*[†]	7	5	1	0	1	(36)

[†]stands for the data collected from transcriptome studies; * "Other" corresponds to unidentified OBPs or OBPs that do not fall under the classic, minus-C, and plus-C classification; ** These OBPs are minus-C OBPs, but possess a double domain in their sequence, as compared to typical minus-C OBPs in other insect species; [$] These references are listed in the Supplementary Material; [#] OBPs from these species were used in the generation of the phylogenetic trees featured in Figure 3.

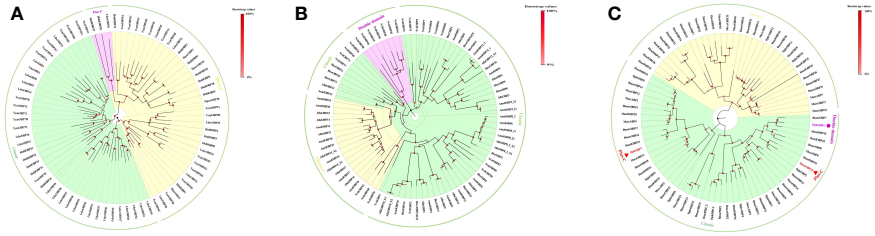

FIGURE 3
Phylogenetic analysis of insect OBPs in three major orders, and OBPs used in the analysis had been previously characterized through either proteomic or transcriptomic analyses. **(A)** Coleopteran insect OBPs from *Holotrichia oblita, Holotrichia parallela, Leptinotarsa decemlineata*, and *Tribolium castaneum*; **(B)** Hymenopteran insect OBPs from *Apis florea, Apis melifera, Bombus terrestris, and nasiona vitripennis*; **(C)** Lepidopteran insect OBPs from *Bombyx mori, Danaus plexippus, Heliconius melpomene*, and *Manduca sexta*. Phylogenetic trees were inferred by the neighbor-joining method (37) and were created using MEGA11 software (38). The trees were visualized using Figtree v1.4.4 software.

hydrophobic cavity crucial for ligand binding activity (52, 53, 58–60). The ligand binding specificity of insect OBPs exhibits significant variation, ranging from high specificity to remarkable broadness. This diversity is influenced by the overall size and shape of the binding pocket, as well as the specific amino acids lining it (54, 55). Previous studies have demonstrated that variability in amino acid identity and length of the C-terminal region can influence ligand binding affinity. For example, in a specific case, the rearrangement of amino acids within the C-terminus region of a *Drosophila melanogaster* OBP (LUSH) disrupted the formation of a salt bridge, resulting in impaired binding ability to the expected ligand 11-*cis* vaccenyl acetate, a conspecific male sex pheromone (56). The length variation in the C-terminal region also impacts the interaction of the C-terminus with the hydrophobic binding cavity. Insect OBPs with longer C-terminus regions possess a flap that can cover the entrance of the binding cavity, whereas those with shorter C-terminus regions leave their binding cavities exposed to bulk solvent (2, 61, 62). Additional research has demonstrated that pH-induced conformational changes can impact the ligand-binding capability of specific insect OBPs (52, 63–65). Notably, Lepidopteran OBPs AtraPBP1 from *Amyelois transitella* and ApolPBP from *Antheraea polyphemus* possess a C-terminal region that plays a crucial role in pheromone binding and release, triggered by changes in pH levels (66, 67). In AtraPBP1, the polar amino acid residues Glu132 and Glu141 create two salt bridges with protonated histidine residues His 80 and His95, respectively. These two salt bridges are induced by acidic conditions, promoting the formation of a seventh helix at the C-terminal region that can compete with the ligand and trigger its release (9, 66). In contrast to OBPs in Lepidoptera, the majority of Dipteran OBPs lack a sufficiently long C-terminal region to form an additional helix able to occupy the binding cavity (9, 52). Nevertheless, many Dipteran OBPs, such as AaegOBP1 (*Ae. aegypti*, PDB ID:3K1E), AgamOBP1 (*An. gambiae*, PDB ID:2ERB), CquiOBP1 (*Culex quinquefasciatus*, PDB ID:3OGN), undergo pH-depended conformation changes associated with loss of binding affinity, similar to what has been observed in Lepidopteran OBPs, indicating a distinct mechanism (9). The C-terminal region of these Dipteran insect OBP proteins instead function as a "lid" over the binding cavity, a characteristic not found in other insect groups. This lid was suggested to act as a pH-sensitive hinge, moving away from the binding cavity when pH is reduced, as the OBP-odorant complex approaches the dendritic membrane (9, 52). Moreover, the ligand binding ability of an OBP may be affected by its molecular volume. For example, in the Minus-C OBP DhelOBP21 of *Dastarcus helophoroides*, the ligand being either too small (<100 A^3) or too large (>185 A^3) can disrupt its facultative binding ability (68). Additionally, hydrophobic and hydrogen bond interactions can also influence binding efficacy of an OBP, and the absence of either can lead to substantial reductions in the binding affinity of an OBP towards a ligand (68, 69). Lastly, it is worth noting that the majority of determined crystal structures of insect OBPs reveal a tendency for dimerization upon ligand binding (59, 70–73) (Table 2). Insect OBP protein structures in both ligand-free apo forms and in complex with various ligands, have been determined using protein crystallography and nuclear magnetic resonance (NMR) spectroscopy (9, 74). A list of currently published insect OBP structures at the time of this publication has been provided in Table 2. The list includes 27 individual insect OBP structures across 17 insect species, including 10 OBP structures from species in Diptera and 7 OBP structures from species in Lepidoptera. Currently, our understanding is limited, as over half of the elucidated structures (17 out of 27) come from Dipteran and Lepidopteran insects (Table 2). Further research is crucial to comprehensively understand the relationship between the varied structures and functions of numerous OBPs from a wide range of insect species.

3 Diverse roles of insect OBPs in communication and xenobiotic adaptation

Insects encounter a diverse array of semiochemicals and xenobiotics in their environment, necessitating adaptive responses. These chemicals range from allospecific and conspecific pheromones, plant allelochemicals, volatiles, and a multitude of anthropogenic compounds, such as pesticides (34, 75–77). On one hand, insects use these chemical cues to detect their

TABLE 2 List of 27 three-dimensional crystal structures, classification, and function of insect Odorant Binding Proteins.

Order	Species Name	Name (PDB number)	Classification	Function	Reference*
Blattodea	*Leucophaea maderae*	PBP (1ORG)	Classic	Involved in recognition of sex pheromone components: 3-hydroxy-butan-2-on and butane-2,3-diol	(37, 38)
Coleoptera	*Tenebrio molitor*	THP12 (1C3Z)	Minus-C	N/A	(39)
Diptera	*Aedes aegypti*	OBP1 (3K1E)	Classic	N/A	(40)
		OBP22 (6OG0)	Classic	Potentially involved in the recognition of fatty acids	(41)
	Anopheles gambiae	OBP1 (2ERB)	Classic	Involved in host recognition	(42-45)
		OBP7 (3R1P)	Plus-C	N/A	(46)
		OBP20 (3VB1)	Classic	N/A	(47)
		OBP47 (3PM2)	Plus-C	N/A	(48)
		OBP48 (4KYN)	Plus-C	N/A	(49)
	Culex quinquefasciatus	OBP1 (3OGN)	Classic	Modulates ovipositional preference	(50, 51)
	Drosophila melanogaster	OBP28A (6QQ4)	Classic	Involved in the detection and mediation of sensitivity to fruit-like odors	(52)
		LUSH (OBP76A) (1T14)	Classic	Involved in host and pheromone recognition through mediation of alcohol compounds	(53-55)
Hemiptera	*Megoura viciae*	OBP3 (4Z39)	Classic	Potentially involved in the recognition of alarm pheromones	(56)
	Nasovonia ribisnigri	OBP3 (4Z45)	Classic	Potentially involved in the recognition of alarm pheromones	(56)
Hymenoptera	*Apis melifera*	ASP1 (OBP1) (3BJH)	Classic	Involved in the recognition of the queen pheromone	(57-60)
		OBP5 (3R72)	Classic	N/A	To be published
		ASP2 (GOBP2) (1TUJ)	Classic	Involved in non-sexual pheromone recognition	To be published, (61, 62)
		OBP14 (3S0A)	Minus-C	Binds with the highest affinity to citralva and eugenol	(63)
Lepidoptera	*Amyelois transitella*	PBP1 (2KPH)	Classic	Involved in the recognition and transport of non-polar pheromone	(64, 65)
	Antheraea polyphemus	PBP1 (1QWV)	Classic	Involved in the recognition of sex pheromone component (E, Z)-6,11-hexadecadienyl acetate (AC1)	(66-69)
	Bombyx mori	PBP1 (1DQE)	Classic	Modulates sensitivity to the sex pheromone bombykol	(70-72)
		GOBP2 (2WC5)	Classic	Involved in the recognition and discrimination of the sex pheromones bombykol and bombykal	(73, 74)
	Epiphyas postvittana	PBP3 (6VQ5)	Classic	Involved in recognition of sex pheromone components: E11-14: OAc and E9, E11-14: OAc	(75)
	Helicoverpa armigera	PBP1 (7VW8)	Classic	Involved in recognition of sex pheromone components: to Z11-16: Ald and Z9-16: Ald	(76, 77)
	Lymantria dispar	PBP1 (6UM9)	Classic	N/A	(78)
Neuroptera	*Chrysopa pallens*	OBP4 (6JPM)	Classic	Involved in the recognition of prey host plant volatiles	(79, 80)
Orthoptera	*Locusta migratoria*	OBP1 (4PT1)	Classic	N/A	(81)

PDB, protein database; N/A, not available; OaC, acetoxy functional group; Ald, aldehyde functional group. * These references are listed in the Supplementary Material.

food, mates, and other substrates critical for their survival and reproduction. On the other hand, insects must evolve adaptation strategies to cope with "delicious poisons", which are harmful compounds disguised as attractants. These chemical cues can be exploited by host plants as a defensive measure, posing survival challenges for insects (22, 78). Recent studies have demonstrated that insect OBPs play critical roles in the uptake or release of a diverse spectrum of molecules due to their stable and compact structure, high variability in binding affinity, and efficiency transportation of hydrophobic molecules (79–81). Additionally, many proteomic and transcriptomic studies focusing solely on olfactory organs, such as antennae or maxillary palps, may not identify all OBP-encoding genes within an insect genome. This suggests that certain OBPs could be exclusively expressed in non-olfactory organs and/or appendages (2, 82–85). Recently, there are many integrative reviews of insect OBPs discussing their diverse expression and functions in chemoreception and beyond (1, 2, 9, 36, 74). Therefore, in this section, our focus will be on the roles of insect OBPs in communication, host location, and their co-opted functions in pesticide adaptation.

3.1 Pheromone detection and release

Detection of conspecific and allospecific pheromones are essential to reproductive success, survival, and overall fitness of an insect (2, 86–88). Several studies have demonstrated the role and significance of OBPs in the detection and sensitivity to pheromones across a variety of insect orders (36, 89–94) since their initial discovery in the male silk moth, by Vogt and Riddiford in 1981 (16). For example, *Bombyx mori* BmorPBP1 was suggested to be essential for the activation of the receptor *B. mori* BmorOR1 to the female released sex pheromone bombykol rather than bombykal (95–97). In the absence of BmorPBP1, only low sensitivity to bombykol was detected in transgenic *drosophila* expressing BmorOR1, however, high sensitivity and ligand specificity towards bombykol was observed in mutants expressing both BmorOR1 and BmorPBP1 (96). The affinity of BmorPBP1 to bombykol is regulated by pH-dependent conformational changes in PBP, which lead to the release of pheromones under acidic environment surrounding the OR neurons (64, 65, 89, 98). Besides BmorPBP1, conformational changes that are integral to pheromone recognition were also observed in PBPs of several other insect species (66, 99, 100). For example, in *D. melanogaster*, it was observed that LUSH PBP detects and releases the male specific sex pheromone 11-*cis*-vaccenyl acetate (cVA) to activate *D. melanogaster* OR67d neurons, linking pheromone-induced behavior with PBP-dependent activation of olfactory neurons (56, 101, 102). Additional studies demonstrated that *D. melanogaster* OBP56h influences male courtship behavior. It plays a dual role in the production of precursors to cuticular pheromones, as its expression level is linked to the expression levels of several biosynthesis enzymes (1, 103, 104). One of these cuticular pheromones, 5-tricosene, is highly expressed in males and can decrease copulation latency at high levels, potentially preventing incidences of male-male courtship (1). In *Ap. mellifera*, brood pheromone (β-ocimene) and death pheromone (oleic acid) are strong ligands for two OBPs, AmelOBP16 and AmelOBP18. Expression levels of both OBPs were found to be linked with the degree of hygienicity displayed in bee colonies, suggesting these two OBPs may play important roles in triggering honey bee hygienic behavior (105, 106). Additionally, it was found that *Ap. mellifera* AmelASP1 and *Ap. cerana* AcerOBP1 are involved in the recognition of honeybee queen pheromone (107, 108). Recently, conserved insect OBPs were identified from various aphid species and their eavesdropping predators, such as ladybird beetles, lacewings, and the marmalade hoverfly, demonstrating the potential functions of OBPs in predator-prey interactions (109–112). These OBPs play roles in detection of (E)-β-farnesene (EBF), which is the primary alarm pheromone active component in many aphid species (Hemiptera: Aphididae) and is used as chemical cue to signal danger (113–117). For example, in *Acyrthosiphon pisum*, knockdowns of *ApisOBP3* and *ApisOBP7*, that are known to bind EBF, led to the disappearance of repellent behavior caused by EBF (110, 115). The functions of related ApisOBP3 and/or ApisOBP7 proteins in EBF detection were also characterized in other aphid species by using behavioral assays, ligand-binding assays, or X-ray crystal structure examination (110, 111, 114, 118). In *Rhopalosiphum padi*, both RpadOBP3 and RpadOBP7 bound EBF and additionally, RpadOBP3 showed affinity for the ligands, EBF and several other plant volatiles, while RpadOBP7 was specific to EBF (114). Most recently, four antennae specific OBPs were functionally characterized in the aphid natural enemy, *Harmonia axyridis*. Among these OBPs, HaxyOBP15 showed a broader binding profile among various substances, including EBF and other volatiles (117). Similarly, two lacewing species OBPs, *Chrysoperla sinica* CsinOBP1 and *Chrysopa pallens* CpalOBP10, were also found to bind to EBF (112, 119).

It has been demonstrated that besides the antennae, OBPs can also be expressed in the sex glands and various other organs, participating in both the uptake and release of various pheromones. A study performed in the diving beetle *Cybister japonicus* found two OBPs specifically expressed in the foreleg and testis of male beetles, which are used for holding a female during courtship and mating, suggesting potential roles of these OBPs in chemical communication (120). The sex pheromone for this species is still unknown, therefore, further research is required to confirm the functions of these OBPs in pheromone recognition and secretion (120). Several studies have also found the presence of OBPs in the seminal fluid of a wide range of insect taxa, that are transferred to females during mating or are potentially used as oviposition deterrents on fertilized eggs (121–126). Interestingly, fruit flies possess OBPs in the seminal receptacle along with an odorant receptor, displaying the highly adaptable nature of OBPs in the insect body (121, 127). In a Lepidopteran species, *Helicoverpa armigera*, *HarmOBP10* was expressed in antennal and reproductive organs of both sexes, binding to 1-dodecene, a compound reported as an insect repellent as well as several volatile compounds, suggesting its dual roles in chemical detection and a carrier for oviposition deterrents (125).

3.2 Host location and adaptation

Recognition of odorants that are associated with an insect's host is essential for locating nutrients and ultimately reproductive success (128–130). A living host of a particular insect can vary greatly based on its life history and feeding guilds, ranging from plants to other animals or humans. Insect OBPs involved in the recognition of host semiochemicals are mainly expressed in the sensillum lymph of the antennae and assist in the adaptation of an insect to their hosts, which has been demonstrated across a diverse range of taxa (131–133). For example, it was found that *An. gambiae* AgamOBP1 is involved in the recognition and sensitivity of indole and 3-methyl indole in the antennae, the former aiding in the location of a human blood host and the latter acting as an oviposition attractant (20, 134–136). Female *A. gambiae* subjected to RNAi mediated silencing of *AgamOBP1* caused a significant reduction in the ability to perceive indole, some individuals even exhibiting a complete loss of perception (136). Another study demonstrated that *Drosophila sechellia* OBP57d and OBP57e are involved in modulating the differences in taste perception and behavioral response towards its host plant *Morinda citrifolia* (28). The characteristic odor of the ripe fruit is due to the compounds hexanoic acid and octanoic acid, that have been shown to induce a repellent effect and cause mortality in other *Drosophila* species (137). After inducing the knockdown of *OBP57d* and *OBP57e* in *D. melanogaster*, it was found that the prior repellent behavior towards ripe fruit was replaced with attraction, suggesting that both OBPs participate in the adaptation of *Drosophila* to a toxic host (28, 138). In another study, it was found that *Nilapavarta lugens* NlugOBP11 is secreted during feeding on rice and alters upregulation of the plant phytohormone salicylic acid in the brown planthopper (139). Silencing of *NlugOBP11* expression resulted in a decrease in feeding performance and eventual death, but overexpression of *NlugOBP11* in the protoplast of rice suppressed the expression of salicylic acid genes, suggesting the contribution of NlugOBP11 in host plant adaptation. In contrast to prior reports, a recent study has shown that host semiochemicals can induce an opposite effect in an insect in the absence of certain OBPs (140). After RNAi-mediated silencing of *D. helophoroides DhelOBP4*, compounds that previously elicited a strong attractant response induced a sexually dimorphic inverse effect in this ectoparasitic insect (140). Adult males no longer elicited a behavioral response and adult females exhibited a strong repellent to the herbivore induced plant volatiles, γ-terpinene and p-cymene. Although the molecular mechanism was not determined, these results may indicate the involvement of DhelOBP4 in host plant volatile recognition and/or protection of olfactory processes from potential damage by plant volatiles (140).

During the evolution of plants and phytophagous insects, plant volatiles were used as a defensive strategy to repel these insects and/or attract their respective parasitoids and predators (141). For phytophagous insects, plant volatiles are essential cues for food and oviposition (22). There is increasing evidence suggesting that plant volatiles can also function as mate-finding cues and/or stimulate sex pheromone release, which assist insects to find their mating partners (142, 143). Recently, more functional studies suggested it is a common phenomenon that insect OBPs can bind both sex pheromone components and plant volatiles, including green leaf and floral volatiles (80, 144–150). Competitive fluorescence binding assays, for instance, have shown that in the rice leaffolder, *Cnaphalocrocis medinalis*, CmedPBP4 could selectively recognize three sex pheromones and eleven rice plant volatiles (145). In the geometrid moth *Ectropis obliqua*, EoblPBP1 bound three sex pheromone components and several green leaf volatiles that had been demonstrated to attract virgin male *E. obliqua*, indicating that green leaf volatiles may act as synergists to enhance the efficacy of sex pheromones (147). It has also been found that some non-PBP OBPs play roles in sex pheromone recognition and plant volatile identification (144, 149–152). For example, the electroantennogram and competitive fluorescence binding assays revealed that a Classic OBP in *Phthorimaea operculella*, PopeOBP16 was involved in recognizing and binding several plant volatiles and sex pheromone components (150). In the Eastern Honeybee, *A. cerana*, two Classic OBPs, AcerOBP6 and AcerOBP11 as well as one Minus-C OBP, AcerOBP15, have been characterized and been linked to recognition of bee pheromones and floral volatiles, indicating these OBPs may play a dual-role in sensing various bee pheromones and host odorants (80, 146, 152).

3.3 Pesticide adaptation

Despite the remarkable sensitivity of the insect olfactory system to detect and differentiate critical odorant cues even at minute concentrations, it also can act as an attractive target for harmful plant compounds and environmental toxins (22, 24). Plant volatiles or anthropogenic toxins pose potential risks to terrestrial insects, as they can impair the processing of odorant molecules or even cause physiological damage at high doses (24). Recently, a substantial amount of evidence emerged, indicating that the gene expression of certain OBPs undergo changes in response to pesticide exposure. These OBPs may play a role in pesticide adaptation by binding, buffering, or sequestration of pesticides that have penetrated the cuticle (2, 25–27, 79, 153–159). Investigating the mechanisms underlying OBP-mediated pesticide adaptation will open new avenues to broaden our understanding of how insects adapt to their xenobiotic environment and evolution of pesticide resistance (13, 33, 160).

One of the first studies to demonstrate the potential of insect OBPs to be involved in insecticide adaptation was conducted in the diamondback moth, *Plutella xylostella* (21). The study exposed *P. xylostella* larvae to two separate selection treatment regimens: Low concentrations of permethrin (LC_5 of prior generation) only applied to the upper and center portion of the host cabbage plants and high concentrations of permethrin (LC_{50} of prior generation) uniformly applied across the entire canopy of the cabbage plant (21). It was found that upon comparing the F_1 parental generation to the selected G_{25} generation, *PxylOBP13* was upregulated in the low concentration of permethrin treatment group, implying a possible role in resistance. Lin et al. in 2018 reported that the gene expression of *SlituOBP9* in the tobacco cutworm *Spodoptera*

litura, was increased in response to chlorpyrifos and emamectin benzoate (25). After injection of dsRNA targeting *SlituOBP9*, the survival of tobacco cutworm moths exposed to chlorpyrifos for 48 hours was decreased to 7.7%, as compared to 50% in the control moths, indicating that SlituOBP9 could play a role in chlorpyrifos adaptation (25). Similarly, it was found that exposure to the herbicide butachlor caused reduced susceptibility to chlorpyrifos in the tobacco cutworm in a separate study (156). Gene silencing of one general OBP, *S. litura SlGOBP2*, decreased larval tolerance to chlorpyrifos, suggesting that olfactory recognition of butachlor by SlGOBP2 may contribute to enhanced chlorpyrifos resistance by induction of ecdysone synthesis and regulating expressions of detoxification genes (156). In the Asian citrus psyllid, *Diaphorina citri*, the expression of *DcitOBP2* was induced in response to imidacloprid exposure. When *DcitOBP2* was silenced via RNAi, susceptibility to imidacloprid was increased in *Di. Citri* adults, suggesting that DcitOBP2 is involved in imidacloprid resistance (161). Similarly, *N. lugens* NlOBP3 was associated with nitenpyram and sulfoxaflor resistance in the brown planthopper (157). Two PBPs in *Athetis lepigone*, AlepPBP2 and AlepPBP3, had high binding affinities to an organophosphate insecticide, phoxim, indicating that these two PBPs may play roles in the phoxim adaptation of this polyphagous pest (155). Similarly, a recent study demonstrated that a G protein coupled receptor, latrophilin may contribute to insecticide resistance through regulating the expression of *Tribolium castaneum TcOBPC01* and one other chemosensory gene (27). Additionally, it was also reported that an increase in larval mortality to dichlorvos and carbofuran was observed when *latrophilin* or *TcOBPC01* was silenced.

Other than acute effects on target insect pests, chemical insecticides cause serious negative effects on nontarget insects, such as parasitoid wasps and pollinators (162). Several studies reported that the OBP either showed high binding affinity to insecticides (154, 158) or the binding of OBP to floral volatile was significantly affected by insecticides (163). These studies implied that OBPs may contribute to olfaction based behavioral response to insecticides. In addition to synthetic pesticides, insect OBPs play roles in adaptation to biopesticides (e.g. essential oils) that are derived from natural materials, including plants, microorganisms, and other biological sources. For example, the *TCOBPC11* (*T. castaneum*) gene expression was induced in response to the essential oils of *Artemisa vulgaris* in the late instar larvae (26). Gene silencing of *TCOBPC11* by RNAi led to higher mortality in larvae compared with the control larvae treated with essential oils, suggesting that TCOBPC11 may play a role in resistance by sequestrating of plant essential oils and masking the toxic effects.

Host plant and pesticide adaptation might be linked due to chemical, evolutionary, and ecological evidence in detoxification and chemosensory pathways (22, 33, 34, 77, 164–166). It is possible that the capability associated with OBP-mediated pheromone or host plant adaptation in herbivorous insects has been co-opted for pesticide adaptation when they are exposed to pesticides. Most recently, research reported that insect OBPs can bind sex pheromone components, plant volatiles and pesticides (79, 153, 159). An OBP (AlepGOBP2) that was functionally characterized in the polyphagous insect *A. lepigone* showed high binding affinity to two conspecific sex pheromones ((Z)-7-dodecenyl acetate and (Z)-9-tetradecenyl acetate), two maize plant volatiles (Ocimene and (E)-β-Farnesene), and two organophosphate insecticides (chlorpyrifos and phoxim) (79). These results indicated AlepGOBP2 may facilitate recognition and adaptation to sex pheromones, plant volatiles, and insecticides all together.

In summary, current studies suggest that insect OBPs contribute to pesticide adaptation through sequestration and subsequent masking of the harmful effects of toxic compounds, or by acting as phase 0 transport proteins and shuttling toxic compounds across the cell membrane to phase I and/or phase II enzymes for further processing (27, 167–169). Whether this is accomplished solely by insect OBPs or through the assistance of other proteins, such as detoxification enzymes, remains to be elucidated.

4 Conclusion

While our understanding of insect OBPs was initially centered on olfaction, recent research conducted over the past decade has unveiled their involvement in diverse physiological processes, including communication, host location and adaptation, pesticide resistance, and reproduction. However, our comprehension of the molecular mechanisms governing OBP functions beyond olfaction remains limited due to their substantial diversity across various taxa. Recent advances in whole genomic sequences, RNA interference, gene editing, X-ray crystallography, and fluorescent competitive ligand binding assays, promise to enhance our understanding on the roles of insect OBPs towards communication and xenobiotic adaptation. This cutting-edge research will also contribute to unraveling the intricate and multifaceted mechanisms underpinning the evolutionary relationship between insects and their environment.

Author contributions

JA: Methodology, Visualization, Writing – original draft, Data curation, Investigation, Software. TM: Investigation, Software, Visualization, Resources, Writing – review & editing. HW: Resources, Software, Visualization, Writing – review & editing, Data curation, Methodology. FZ: Methodology, Resources, Visualization, Writing – review & editing, Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/finsc.2023.1274197/full#supplementary-material

SUPPLEMENTARY TABLE 1
Summary of odorant binding proteins (OBPs) used in the creation of the phylogenetic trees (Figure 2). Sequences without a complementary accession number were adapted from prior literature, where sequences were referenced but lacked an accession number.

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A critical review of current laboratory methods used to evaluate mosquito repellents

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Pathogens transmitted by mosquitoes threaten human health around the globe. The use of effective mosquito repellents can protect individuals from contracting mosquito-borne diseases. Collecting evidence to confirm and quantify the effectiveness of a mosquito repellent is crucial and requires thorough standardized testing. There are multitudes of methods to test repellents that each have their own strengths and weaknesses. Determining which type of test to conduct can be challenging and the collection of currently used and standardized methods has changed over time. Some of these methods can be powerful to rapidly screen numerous putative repellent treatments. Other methods can test mosquito responses to specific treatments and measure either spatial or contact repellency. A subset of these methods uses live animals or human volunteers to test the repellency of treatments. Assays can greatly vary in their affordability and accessibility for researchers and/or may require additional methods to confirm results. Here I present a critical review that covers some of the most frequently used laboratory assays from the last two decades. I discuss the experimental designs and highlight some of the strengths and weaknesses of each type of method covered.

KEYWORDS

mosquito repellents, laboratory assays, spatial repellency, contact repellency, methods review, mosquito attractants, standardized methods, repellent efficacy

1 Introduction

Mosquito-borne diseases pose a massive threat to public health. Rising temperatures worldwide expand the geographical range of many key vector species, increasing the number of people at risk of contracting these diseases (1–5). Mosquitoes can transmit human pathogens that cause malaria, dengue, and West Nile, to name a few. Pathogen transmission occurs due to the blood-feeding constraint of anautogenous mosquitoes to complete their life cycle. When an infected mosquito takes a blood meal from a host, the host can become infected and vice versa (6–8). Integrated vector management (IVM) is an approach to mitigate pathogen transmission on a global scale (9–12). Some strategies of this approach, that target mosquito pathogen transmission, are controlling mosquito

populations using pesticides, reducing larval habitat near human infrastructure, and educating communities on the mosquito life cycle and the risks that they pose (13–16). When used in tandem with others, a powerful strategy for individuals to implement regularly to prevent mosquito bites is the proper use of effective repellents (17–21).

Understanding the mode of action of mosquito repellents has been a large topic in the vector biology community for decades and has only been partially explained for some mosquito repellent active ingredients (22–28). The literature on this topic is vast and can easily fill several review papers, therefore I will only briefly touch on this topic (17, 29–34). To provide a general description, mosquito repellents target chemoreceptors associated with olfactory and/or gustatory organs, as well as other appendages that have chemoreceptive sensilla like the wings and tarsi (35–41). Mosquito repellents can act on chemoreceptors in various ways that elicit a repellent response in mosquitoes. Some of these ways include overstimulating or blocking specific chemoreceptors, or by masking odors (23, 42–46). In this review, I will be covering laboratory methods that test the behavior of mosquitoes in response to potentially repellent treatments.

There are two major categories of mosquito repellents commonly found in the literature (47–52):

- Spatial repellents.
- Contact repellents.

A mosquito repellent can convey either one type of repellency or both (27, 38, 53). Spatial repellents can be applied in several different forms including topical treatments like lotions and sprays or as devices that aerosolize repellent molecules into the proximal area. Spatial repellency is typically observed by the absence of mosquitoes in the vicinity or by the significant decrease in mosquitoes physically touching a treated object or individual (54, 55). The other category of repellency is contact repellency. Contact repellents repel mosquitoes that come into direct contact with the product and are usually applied topically through sprays or lotions. Contact repellency is typically observed when mosquitoes land on a treated host or object without proceeding to initiate feeding behaviors, like probing, and instead promptly fly away (41).

There are hundreds of commercially available mosquito repellent products available on the market worldwide (56–58). These products often contain active ingredients such as DEET (N,N-diethyl-meta-toluamide), Picaridin, IR3535, or para-menthane-diol (PMD) (59, 60). However, there is still a continuous search for effective alternatives to these products (61). One reason for this continued search is the general negative consumer opinion regarding the safety of the synthetically-derived active ingredients found in long-lasting mosquito repellent products (58, 62). This concern persists even with reports that conclude these active ingredients are safe when used as directed (63–70). The search for alternative products that do not utilize synthetic active ingredients is also amplified by the underlying premise that "natural" active ingredients are safer for human health and better for the environment (71).

Another reason for this continued search is because of individuals with skin allergies or sensitivities to some of the commonly-used active ingredients found in mosquito repellents (72, 73). It is challenging to formulate novel, effective mosquito repellents that can compete with the top-performing products on the market. New products should be scientifically tested for their repellent efficacy, before becoming commercially available, which can be done in a variety of ways (74, 75).

I present a literature review on common laboratory methods, from the last two decades, that can be used to measure the repellent efficacy of novel and established treatments on mosquitoes. For this review, the term "treatment" is defined as any material, chemical, or device that may elicit a response in mosquitoes.

2 Laboratory methods to test mosquito repellency

Laboratory assays used to measure the repellent efficacy of a specific treatment on mosquitoes vary greatly in:

- How repellency is measured.
  - Repellency is typically determined by either recording changes in mosquito location, host-seeking behavior, or feeding behavior.
- The type of repellency being tested.
  - Spatial or contact.
- The parameters of the experiment.
  - Type of treatment being tested, dimensions of assay, presence of an attractant source, number of mosquitoes being tested, etc.

To organize this literature review, I divided laboratory assays that can be used to test the repellent efficacy of a treatment into two categories. Assays that measure mosquito behavior not related to host-seeking are categorized as "repellency assays without an attractant source". Assays that measure mosquito movement and behavior during host-seeking by either incorporating a living host or a synthetic attractant are categorized as "repellency assays with an attractant source". The assays that utilize attractant sources were further separated into two different groups: spatial repellency assays and contact repellency assays.

2.1 Repellency assays without an attractant source

These laboratory assays measure changes in mosquito behavior in the presence or absence of a treatment and are independent from mosquito host-seeking behavior. The assays covered in this category generally have the following common element in their experimental design: an apparatus that contains adult female mosquitoes that are monitored for changes in behavior. Mosquitoes can either fly towards or away from a treatment. Mosquito behavior in response to each treatment is recorded. Treatments that induce avoidance behaviors in mosquitoes, demonstrated by them relocating away from the treated area, are repellent.

The general strengths of these types of laboratory assays are that they can be easily reproduced and can rapidly screen many different treatments due to simple and straightforward experimental designs. Another strength is that these assays don't involve mosquito host-seeking and feeding behavior. Mosquito feeding behaviors can vary widely between species and are influenced by variables like circadian rhythms, mosquito age, seasons, temperature, etc. (76). Assays that rely on mosquito feeding behaviors to test repellency require frequent control testing to assure mosquitoes are actively host-seeking during experiments. While not relying on mosquito feeding behavior to test repellency is a strength, it is also a weakness of these types of assays. A mosquito's behavior in the presence of a treatment might be very different depending on if the mosquito is actively host-seeking or not. Additional tests using different types of assays are necessary to confirm if a treatment will actually protect humans from mosquito bites. Repellency assays without an attractant source are very useful, most notably in their ability to screen numerous treatments in a short amount of time and identify potentially effective mosquito repellents for a low cost and with relative ease.

Below I describe some common and useful laboratory assays that test the mosquito repellent activity of treatments in the absence of mosquito attractants (50, 77–91).

2.1.1 Tube assays

Overview: The Tube assay is a simple and low-cost technique that uses a hollow cylinder apparatus to measure mosquito behavior and location (see Figure 1A) (77–80).

Experimental design: This assay consists of a transparent plastic or glass tube with removable caps on both ends. A treated filter paper is placed in the lining of one of the caps and mosquitoes are transferred into the tube. The tube is divided (not physically) into two parts representing a treated and an untreated side. Control experiments typically consist of filter paper on both sides that are treated with a solvent like acetone or ethanol.

Calculating repellency: The repellent efficacy of treatments can be measured by recording the location or behavior of mosquitoes at specific times throughout the experiment and comparing data from treatment and control experiments to determine repellency. The repellent efficacy of a treatment can be calculated as a repellent ratio (77) or as a percentage by using the following equation:

$$\frac{(\#\ of\ mosquitoes\ in\ untreated\ half) - (\#\ of\ mosquitoes\ in\ treated\ half)}{(Total\ \#\ of\ mosquitoes)}\ x\ 100$$

Example from scientific literature: This type of apparatus has been used in several publications to measure mosquito repellency of new and/or established treatments (77–79). In 2006, Schultz and colleagues tested four treatments, catnip essential oil, Osage orange essential oil, elemol, and DEET at different concentrations using a version of a Tube assay, they called "Static-Air Repellency Chamber" (80). They performed two different experiments using this assay, one to screen for repellency in the treatments and another to test the residual repellency, how long a treatment repels mosquitoes. The first experiment tested each treatment at three different concentrations (0.1%, 0.5%, and 1%) using hexane as

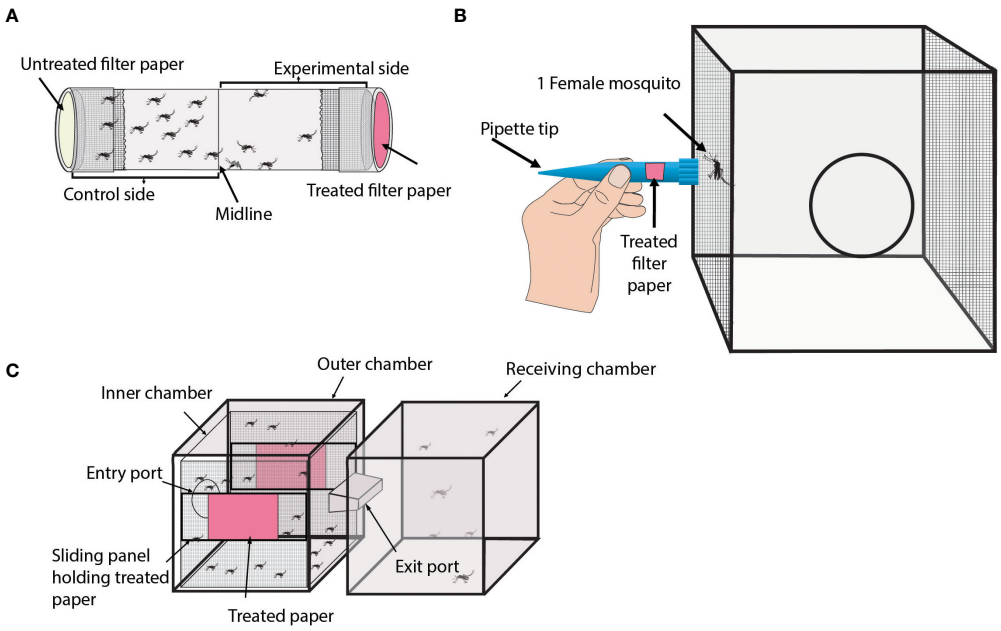

FIGURE 1
Laboratory repellency assays without an attractant source. **(A)** Diagram of the general format of a Tube assay. Shown is a transparent tube containing female mosquitoes. Both ends of the tube are capped to prevent mosquitoes from escaping the assay. The interior side of each cap contains a filter paper. The beige cap represents the untreated filter paper, and the pink cap represents the treated filter paper. **(B)** Diagram of the Close Proximity Response assay. Shown is a mesh-sided cage containing one female mosquito. A modified pipette tip containing a filter paper is shown being held up against the mesh region of the cage the mosquito is resting at. The pink box in the pipette tip represents a treated filter paper. **(C)** Diagram of the Excito-Repellency Test Chamber assay. Shown are two connected cages referred to as chambers. The left chamber is the main chamber and contains female mosquitoes. The main chamber has two treated papers shown in pink. The right chamber is the receiving chamber where repelled mosquitoes can relocate.

the solvent. They identified Osage orange essential oil to be an ineffective mosquito repellent at all concentrations tested. Next, they determined the residual repellency of the other three treatments (catnip essential oil, elemol, and DEET). They found that the repellency of elemol and DEET remained constant over 180 minutes, but catnip essential oil lost some repellency over time.

Variations: There are several different variations and ways to modify the experimental design of this type of assay to test unique and/or specific hypotheses. These variations include the orientation of the tube (horizontal or vertical), the dimensions (width and length), and material of the tube (opaque, glass, plastic) (77–80). Variations in tube assays are very easy to standardize and these assays can be used to test for repellency, attraction, olfactory desensitization, or toxicity of treatments. Another variation described by the World Health Organization (WHO) is called the Resting Site Choice Test, where mosquitoes are placed in a tube that has two cages on either end, one cage contains a treatment or pesticide and the other has no treatment or pesticide (92, 93).

Strengths:

- Flexibility for variations and modifications.
- Affordable.
- Practical for most laboratories.
- Great tool for initial screening of treatments.
- Easy to establish.

Weaknesses:

- Lack of mosquito attractants.
- Challenging to distinguish between spatial or contact repellency.
- Treatments are not applied to human skin, which may not reflect the real-world application of a mosquito repellent product.
- Needs additional assays to support findings.

2.1.2 Close proximity response assays

Overview: The Close Proximity Response assay is another simple method that can be used to test mosquito repellency to a treatment (see Figure 1B) (50, 81).

Experimental design: This assay involves testing individual mosquitoes in a mesh-lined cage. The test mosquito is allowed to acclimate to its surroundings until it rests on one of the mesh walls of the cage for a specified amount of time. Upon this requirement being met, the wide portion of a modified 1000 µl pipette tip is held against the exterior side of the mesh where the mosquito is resting. The modified pipette tip contains a treated filter paper. The pipette tip is held up to the mosquito for a specified amount of time. If the mosquito flies away from the treatment within this time, the time of flight is recorded. The same mosquito can be used to test more than one treatment. Typically, a large number (>30) of individual mosquitoes are tested for each treatment. A filter paper treated with paraffin oil is used as a control.

Calculating repellency: The repellent efficacy of treatments is measured by recording the time points that a mosquito flew away from the treatment. The proportion or percent of mosquitoes that did not fly away from the treatment is calculated and compared to the control.

Example from scientific literature: This assay has been used in a couple scientific papers to screen for mosquito repellent treatments (50, 81). One of these studies was conducted in 2020, by Afify and Potter to test variations in the behavior of different mosquito species after exposure to the same treatments. In their experiment six established mosquito repellents (IR3535, DEET, Eugenol, Picaridin, PMD, and Lemongrass oil) were tested on three different species of mosquito (*Anopheles coluzzii*, *Aedes aegypti*, *Culex quinquefasciatus*). Afify and Potter found clear differences in the behavioral responses to treatments among the different mosquito species tested. They found that only lemongrass oil repelled all three species at a similar rate. PMD repelled *A. coluzzi* and *C. quinquefasciatus*, but only slightly repelled *A. aegypti*. Eugenol significantly repelled *A. aegypti* and slightly repelled *C. quinquefaciatus*. DEET slightly repelled *A. aegypti* and *C. quinquefasciatus*, but it did not repel *A. coluzzi*. IR3535 and picaridin did not show evidence for mosquito repellency in this assay.

Variations: Some variables that can be altered are the size of the cage and the concentration of treatments used. This assay could be used to test if mosquitoes develop an olfactory blindness to treatments that they are exposed to for a certain duration of time.

Strengths:

- Affordable.
- Practical for most laboratories.
- Great tool for initial screening of treatments.
- Tests for spatial repellency.
- Easy to establish.

Weaknesses:

- Lack of mosquito attractants.
- Cannot test for contact repellency.
- The presence of an experimenter's hand and overall experimenter presence may add an unaccounted-for variable.
- The movement of the experimenter may cause mosquitoes to fly away which may be confused for repellency of the treatment.
- Requires several repeats to compensate for random mosquito flight or experimenter influence.
- Treatments are not applied to human skin, which may not reflect the real-world application of a mosquito repellent product.
- Needs additional assays to support findings.

2.1.3 Excito-repellency test chamber assays

Overview: The Excito-Repellency Test Chamber (ER) assay is an effective technique to evaluate the repellency of a treatment by measuring the number of mosquitoes that escape from a treated chamber to an untreated one (see Figure 1C) (82–90).

Experimental design: This apparatus is a box-shaped chamber with an escape port that leads to an untreated receiving chamber.

The main chamber contains treated fabric or paper. Mosquitoes are transferred into the main chamber and acclimatize for a specified amount of time before the escape port is opened. Mosquitoes can either escape from the main chamber into the receiving port or remain in the main chamber. At specified intervals, the number of escaped mosquitoes is recorded throughout the duration of the study. The treated paper or fabric is situated in the main chamber. It can be exposed to landings (direct contact) or covered to prevent landings. An untreated fabric or paper is used as a control.

Calculating repellency: The repellent efficacy of a treatment is measured by recording the number of mosquitoes that remained in the main chamber and the number of mosquitoes that escaped into the untreated receiving chamber at the end of each experiment. To calculate escape rates, the data can be analyzed using a Kaplan-Meier survival analysis where escaped mosquitoes are counted as deaths and remaining mosquitoes are survivals (88). Using this analysis, mosquito escape rates when exposed to treatments are calculated and this value is used to compare mosquito repellency between treatments.

Example from scientific literature: This type of assay has been developed and modified in several studies starting as early as 1973 (82). A standardized experimental design called the "Excito-repellency test" was developed in the early 90s (83–87, 89). An example of a publication that uses the ER assay comes from Boonyuan and colleagues (88). In this 2014 study, the repellent efficacy of essential oil extracts from five different plants (hairy basil, ginger, lemongrass, citronella grass, and plai) was tested. Each essential oil treatment was tested at different concentrations, 2.5%, 5%, and 10%, using ethanol as a solvent. Treatments were applied to filter paper. Boonyuan and colleagues tested each treatment in a 30-minute contact and a 30-minute non-contact experiment. They found the hairy basil essential oil extract to have the strongest repellent effect at a 2.5% concentration, followed by 5% lemongrass oil, 5% citronella oil, and 5% ginger oil.

Variations: A variation of the ER assay called the "High-Throughput Screening System" or HITSS, was developed by Grieco and collaborators in 2005 (91) and utilizes a cylinder-tube shaped apparatus instead of a box-shaped chamber. The experimental design is similar, mosquitoes are placed in a treated region and allowed to escape into an untreated area. The number of mosquitoes escaped or knocked down is recorded and the data is used to infer the repellency or pesticidal activity of treatments.

Strengths:

- Consistency in experimental design.
- Can test either contact or spatial repellency.

Weaknesses:

- Lack of mosquito attractants.
- May not be feasible for all labs.
- Treatments are not applied to human skin, which may not reflect the real-world application of a mosquito repellent product.
- Needs additional assays to support findings.

2.2 Repellency assays with an attractant source

These laboratory assays measure changes in mosquito host-seeking behavior towards an attractant source in the presence of a treatment. There are several different variations and methods used to test mosquito repellents that include an attractant component. The assays covered in this category have the following common elements in their experimental designs: an apparatus that contains test mosquitoes and a mosquito attractant located in conjunction with or proximal to a treatment.

Attractant sources used for these assays can range from, or be a combination of, synthetic odors, CO_2, heat, animal hosts, or human volunteers. Mosquito repellent treatments, tested in this category of assays induce a reduction or complete mitigation of mosquito host-seeking behavior, displayed by either changes in mosquito location, mosquito landing, or mosquito biting and feeding, compared with controls.

A strength of assays that have an attractant component is the ability to gather compelling evidence on the mosquito-repelling properties of a treatment. If the treatment conveys repellency, the data collected from these assays generally show clear differences between the host-seeking behavior in mosquitoes exposed to a control or treatment. A general disadvantage of these assays is the wide variations in mosquito host-seeking behavior and the impact of variables that, at times, can be difficult to account for, predict, or control. Some of these variables include environmental variations such as time-of-day, lighting, temperature, humidity, and season. Others concern organismal variations, such as mosquito species, age, different stressors, larval and adult densities, and natural variations in attraction to different attractant sources. In these types of assays, it is crucial to frequently run control tests to assure that mosquitoes are actively host-seeking during and between experiments.

For this review, I split laboratory repellency assays with an attractant source into two groups: spatial repellency assays and contact repellency assays.

2.2.1 Spatial repellency assays

Spatial repellency assays measure changes in mosquito location relative to an attractant source that is proximal to, or coated by, a treatment. In spatial repellency tests, mosquitoes do not directly contact the treatment and can either fly towards or away from it. These types of assays generally calculate the "reduction in mosquito attraction" relative to the mosquito attraction measured in a control. A treatment that does not repel mosquitoes will result in a relatively high number of mosquitoes flying towards an attractant source. While a treatment that is an effective mosquito repellent will have less or a complete reduction in the number of mosquitoes flying toward the attractant source.

Below are some common and useful assays that use an attractant source to measure the spatial repellent activity of treatments on mosquitoes (52, 94–113).

2.2.2 Y-tube olfactometer assays

Overview: The Y-tube Olfactometer assay evaluates the spatial repellency of a treatment by measuring the number of mosquitoes that fly toward an attractant in the presence or absence of a treatment (see Figure 2A) (52, 94–104).

Experimental design: This apparatus is a "Y" shaped tube with chambers at each end that can be opened or closed. A fan located at the base of the "Y" is used to create an airflow through the tube. Mosquitoes are transferred into a holding chamber located at the base of the "Y" and are given a specified amount of time to acclimate. A volunteer's natural odors, body heat, and carbon dioxide are used as an attractant source for control and treatment experiments. The volunteer and treatment never come into direct contact with the test mosquitoes. For treatment experiments, the volunteer's hand is either coated with the treatment or the volunteer holds a container containing the treatment in their palm. After the acclimation time, all chambers are opened, and the mosquitoes can fly throughout the apparatus. Mosquitoes can either remain in the holding chamber, fly toward the volunteer's hand, or fly toward the blank chamber. After a specified amount of time, the chambers are closed, and the number and location of all mosquitoes are recorded. An untreated hand is used as a control.

Calculating repellency: The repellent efficacy of a treatment is measured by recording the number of mosquitoes in each location to calculate the percent attraction using the following equation:

$$\frac{(\#\ of\ \ mosquitoes\ \ located\ \ in\ \ the\ \ hand\ \ chamber)}{(Total\ \ \#\ \ of\ \ \ mosquitoes)}\ x\ 100$$

Example from scientific literature: Since the last couple of decades, the Y-tube Olfactometer assay has become a staple test for measuring mosquito spatial repellency and attraction (94). The design of the Y-tube olfactometer apparatus has varied significantly, however the general experimental design has remained consistent. This assay has been frequently used in many scientific research papers (95–103) and has been recommended by the World Health Organization (WHO) in their "Guidelines for efficacy testing of spatial repellents" (52). To promote a more standardized apparatus and experimental design, the WHO published specifications on dimensions and shape for the Y-tube. Rodriguez and colleagues used these specifications in their study in 2015 to test seven commercially available mosquito repellents, a perfume, bath oil, and a vitamin B patch (104). In this study, a volunteer's hand was treated and tested at initial treatment application, and 30-, 120-, and 240-minutes post-treatment. Rodriguez and colleagues tested all treatments on both *A. aegypti* and *A. albopictus*. They measured a 61% and a 41% mosquito attraction to the untreated volunteers' hands with *A. aegypti* and *A. albopictus*, respectively. They found that of the commercially available repellents tested on *A. aegypti*, products containing DEET displayed a significant reduction in attraction at all time points tested. DEET-free products conferred various levels of reduction in attraction. They found no spatial mosquito repellency when testing the vitamin B patch.

Variations: Variables that can be modified in the Y-tube assay include the speed of airflow in the tube, the species of mosquito tested, the type of attractant source, and the duration of replicates. A similar assay to the Y-tube is the Uniport Olfactometer. The Uniport assay is a cylinder-shaped apparatus where mosquitoes are placed in one location and can move towards or away from an attractant source in the presence or absence of a treatment (81, 114). The major difference between these two assays is that in the Y-tube assay mosquitoes can make a decision between two branched chambers, this allows to address different questions such as competition between two treatments (115).

Strengths:

- Flexibility for variations and modifications.
- Presence of a mosquito attractant.
- Tests for spatial repellency.
- Can test one treatment or competition between two treatments.

Weaknesses:

- Variation in attractant sources.
- Cannot test for contact repellency.
- Needs frequent control testing.

2.2.3 Taxis Cage assays

Overview: The typical Taxis Cage assay evaluates the spatial repellency of a treatment by recording the changes in mosquito location relative to the location of a human volunteer in the presence or absence of a treatment (see Figure 2B) (105–113).

Experimental design: The Taxis Cage is an apparatus, consisting of three boxy cages. Adjacent cages are connected by a port that can be opened or closed. When all ports are open, mosquitoes can move towards an attractant source that is typically located outside at a specified distance from the apparatus. The sides of each cage are mesh to allow for air flow throughout the taxis cage. The cage can be set up with a fan used to create an airflow through the cages or can be placed in a large wind tunnel environment (see Figure 2C). A volunteer's natural odors, body heat, and carbon dioxide are used as an attractant source. The volunteer sits at a specified distance from the taxis cage. Mosquitoes are transferred to the center cage and are left to acclimate. After the acclimation period, the ports connecting all three cages are opened and the mosquitoes can travel between cages for the duration of the experiment. At the end of the experiment, the ports are closed and the numbers of mosquitoes in each cage are counted. Treatments tested in this type of study can be topical treatments or free-standing products meant for outdoor use, such as candles or incense burners. Treatments are applied to or placed in front of the volunteer. In the presence of an effective mosquito repellent treatment, less mosquitoes will fly toward the volunteer compared to the control, and vice versa. An untreated volunteer is used as a control.

Calculating repellency: The repellent efficacy of a treatment can be measured by calculating the percent of mosquitoes attracted to the volunteer in the presence or absence of a treatment by using the following equation:

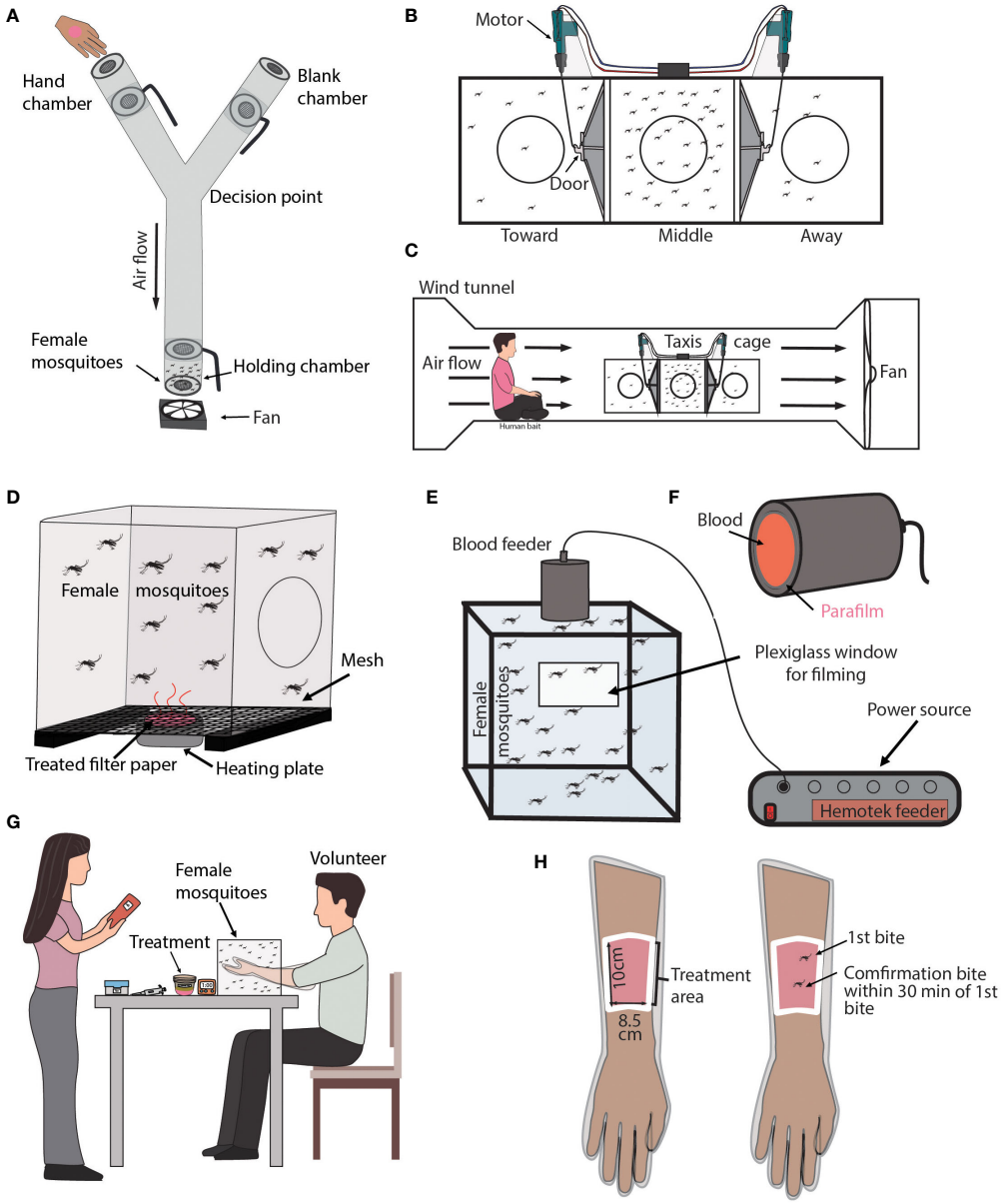

FIGURE 2
Laboratory repellency assays with an attractant source. **(A)** Diagram of Y-tube Olfactometer assay. Shown is a transparent Y-tube that is laid down horizontally with a small fan placed at the base of the "Y". Each chamber has a small door that can be rotated open or close. The holding chamber contains acclimating mosquitoes. The pink circle on the hand represents a treatment. The location of the hand alternates between chambers for each replicate. **(B)** Diagram of a Taxis Cage. Shown are three cages connected by doors that can be opened or closed by the remote-controlled motor. Each cage is labeled either toward, middle, or away in relation to the volunteer's location. **(C)** Diagram of a Taxis Cage located in a Wind Tunnel. Shown is a volunteer sitting near a Taxis Cage. The volunteer's pink shirt represents a treatment. **(D)** Diagram of a Surface Landing assay. Shown is a mosquito-infested cage with a heated plate located underneath it. The pink area of the heated plate represents a treatment. **(E)** Diagram of a Feeding assay. Shown is a cage filled with mosquitoes. A plexiglass window is used to film mosquito feeding and behavior. A heated feeder filled with blood is located at the top of the cage. **(F)** Diagram of a blood feeder used in the Feeding assay. The red region represents the blood within the feeder and the parafilm is treated, indicated by the pink font. **(G)** Diagram of the Arm-In-Cage assay. Shown is a volunteer with their arm inserted in a mosquito-infested cage. **(H)** Diagram of the volunteer's arm that is used in the Arm-In-Cage assay. Shown is a sleaved arm. The white border represents the cutout region where the volunteer's skin is exposed to mosquitoes. The exposed skin is treated which is shown in pink.

$$\frac{(\#\ of\ mosquitoes\ located\ in\ cage\ closest\ to\ attractant)}{(Total\ \#\ of\ mosquitoes)}\ x\ 100$$

Example from scientific literature: Using box-shaped apparatuses for olfactometer tests to identify mosquito attractants or repellents has been a prominent method over the past couple of decades. There have been several complex and thoroughly designed apparatuses that use this type of experimental design to test different treatments (105–109). However, more simplified designs have been developed and published (110–112). A current Taxis Cage was designed by Lorenz and colleagues in 2013. In their study, the movement of *Anopheles gambiae* mosquitoes was measured in

response to three different attractant olfactory cues using the taxis cage (113). These olfactory cues were carbon dioxide, synthetic odor blend combined with carbon dioxide, or a human volunteer. Lorenz and colleagues performed two experiments using the taxis cage, one in a semi-field environment and one in an open field environment. The three cues were placed 20-, 50-, 70-, or 100-meters away from the taxis cage. They found that at a 20-meter distance all three cues significantly attracted approximately 60% of the mosquitoes tested. At a 50-meter distance the carbon dioxide, and the synthetic odor blend combined with carbon dioxide, attracted mosquitoes, but the human volunteer did not. At a 70-meter distance only the synthetic odor blend combined with carbon dioxide significantly attracted mosquitoes, and at 100-meters none of the olfactory cues attracted any mosquitoes.

Variations Some variations that can be applied to the Taxis Cage are the location the experiment is conducted in, the species and number of mosquitoes, and the distance of treatments to the Taxis Cage. An alternative assay that has a similar methodology is the WHO Tunnel Test, where a live animal is placed inside the apparatus serving as the attractant source (92, 93). This variant to the Taxis Cage is designed specifically to test insecticide-treated bed nets, where mosquitoes must cross through holes in a treated net to approach the attractant source.

Strengths:

- Can be placed in several different environments (a room with a fan attached, a wind tunnel, in a semi-field site, and in a field site).
- Presence of mosquito attractant.
- Tests for spatial repellency.
- Can test free-standing devices and mosquito repellent methods meant for outdoor-use.

Weaknesses:

- Variation in attractant sources.
- Cannot test for contact repellency.
- Needs frequent control testing.
- May not be feasible for all labs.

2.2.4 Contact repellency assays

Contact repellency assays measure changes in mosquito host-seeking behavior relative to an attractant source that is proximal to, or coated by, a treatment. In contact repellency tests, mosquitoes can make direct contact with the treatment and can either initiate feeding behaviors like landing, probing, and engorging, or fly away from it. These types of assays generally calculate repellency by measuring either the time of or the number of landings, probing, or blood meals in the presence of a treatment compared to the control. A treatment that does not repel mosquitoes will have a relatively high number of mosquitoes quickly initiating feeding behaviors towards the attractant source. While a treatment that is an effective mosquito repellent will have less or a complete reduction in the number of mosquitoes conveying these behaviors toward the attractant source for a longer period of time.

Below are some common and useful assays that use an attractant source to measure the contact repellent activity of treatments on mosquitoes (41, 116–135).

2.2.5 Surface landing assays

Overview: The Surface Landing assay evaluates the repellency of a treatment by measuring the number of mosquito landings on an attractive platform that has been treated (see Figure 2D) (116–119).

Experimental design: The typical apparatus is a mesh cage with a heated element and an attractive odor blend located on one of the cage walls. The heat element is usually set to 36-37°C. Mosquitoes are transferred into the cage and given time to acclimate. After the acclimation period, mosquito landings or probing on the heated element are recorded over time. Treatments are applied to the heated element, along with the attractive odor blends, either by using a treated fabric or paper.

Calculating repellency: Repellency is measured by recording the number of mosquito landings or probing on the mesh region of the cage directly above the heated platform. The number of landings or probing on this platform in the presence or absence of a treatment is compared to calculate mosquito repellency.

Example from scientific literature: The Surface Landing assay has been used to test mosquito repellents for a little over a decade (116–118, 136). In 2014, Menger and collaborators used this assay to compare nine prospective mosquito repellent compounds to DEET with *A. gambiae* (119). In their study, they used an odor mixture that mimics the scent of a human foot as an attractant source in addition to the heated platform and pulses of carbon dioxide. The odor mixture and treatments were applied to separate nylon strips. The nine different compounds that were tested in this study were 1-dodecanol (1DOD), 2-nonanone (2NON), 6-methyl-5-hepten-2-one (6MHO), 2,3-heptanedione (23HD), 2-phenylethanol (2PHE), eugenol (EUG), δ-decalactone (dDL), δ-undecalactone (dUDL) and linalool (LNL). They found that application of DEET, PMD, 2NON, 6MHO, LNL, dDL, and dUDL resulted in significantly less landings from *A. gambiae* mosquitoes compared to the controls (no treatment and ethanol treatment). dDL and dUDL had not been previously shown to repel mosquitoes, so Menger and colleagues continued their study focusing on these two compounds. They performed the same assay using *A. aegypti* mosquitoes and tested only DEET, PMD, dDL, and dUDL. They found again that dDL and dUDL performed similar to the positive controls (DEET and PMD).

Variations: This type of assay can vary in the location of heat elements and attractant sources inside or outside the apparatus, the type of attractant source used, the carrier materials, and the mosquito species (24, 108, 137).

Strengths:

- Presence of standardized mosquito attractant.
- Tests for contact repellency.
- Easy to establish.

Weaknesses:

- Treatments are not applied to human skin, which may not reflect the real-world application of a mosquito repellent product.

- Cannot test for spatial repellency.
- Needs frequent control testing.

2.2.6 Feeding assays using artificial feeding systems

Overview: Feeding assays can evaluate the repellent efficacy of a treatment by measuring the feeding behavior and engorgement rates of mosquitoes in the presence of a treatment (see Figures 2E, F) (41, 120–123).

Experimental design: This design is straight forward, consisting of a test cage that has a heated feeding unit pressed alongside one of the cage's meshed sides. A treated membrane or fabric is placed in between the feeder and the mesh. The feeder is typically filled with defibrinated blood and the number of mosquitoes that probe the feeder or engorge on blood is recorded.

Calculating repellency: Repellency is measured by recording mosquito probing or the rate of mosquito engorgement in the presence or absence of a treatment.

Example from scientific literature: Feeding assays are a common and frequently used assay in scientific research papers (120–123). This type of assay can be used to measure the efficacy of mosquito repellents or attractants, or to study mosquito behavior under different conditions. In 2019, Dennis and colleagues performed a study to further investigate the mechanism of action of DEET (41). They conducted two separate experiments. The first experiment was designed to test the anti-feedant properties of DEET by mixing DEET into the blood used in the artificial feeder. The second experiment was designed to test contact repellency of DEET by treating the membrane of the artificial feeder. They found that DEET mixed into blood strongly deterred mosquito blood feeding and that DEET applied to the feeding membrane completely deterred mosquito contact.

Variations: There are several variations that can be applied to these types of assays. These variations include the material that treatments are applied to, and the type of nutrients in the feeder, such as sugar solutions, Skitosnack, different types of blood (24, 138, 139). Other variations can be the status of the mosquitoes used (age, health, genetic modifications, species, etc.), and the treatment's location in the apparatus. For instance, there is a variation to this type of assay where the treatment is located only at the perimeter of the feeding unit on a cylindrical shaped filter paper (140, 141).

Strengths:

- Alternative to using human volunteers.
- Flexibility for variations and modifications.
- Presence of standardized mosquito attractant.
- Tests for contact repellency.

Weaknesses:

- Membranes used on the feeders may be dissolved by certain treatments like essential oils.
- Treatments are not applied to human skin, which may not reflect the real-world application of a mosquito repellent product.
- Cannot test for spatial repellency.
- Needs frequent control testing.

2.2.7 Arm-In-Cage assays

Overview: Arm-In-Cage assays evaluate the repellent efficacy of a treatment by measuring mosquito probing or landing on a human volunteer's treated or untreated skin (see Figures 2G, H) (124–135).

Experimental design: This type of assay consists of a mosquito-infested cage in which a volunteer inserts their arm for a specified period. The volunteer's arm is typically protected in an elbow-length glove that mosquitoes cannot penetrate, such as a plastic food-serving glove. The glove has a cutout at the inner-forearm region where the volunteer's skin is exposed to host-seeking mosquitoes. The cutout is secured using fabric tape, and an exposed patch of skin is either treated or left untreated. The volunteer then places their arm in the mosquito-infested cage and continuously observes the exposed region of skin for mosquito landings or probing for a specified amount of time. An untreated control is used to confirm that the test mosquitoes are attracted to the volunteer's arm.

Calculating repellency: Repellency can be measured by calculating the complete protection time (CPT) of a treatment. CPT is calculated by averaging the times of the first event (landing or probing) on the volunteer's skin. The first event is the only data point used to calculate CPT. Typically, a second event is necessary for the first event to be validated and used. The second event must occur within a specified amount of time from the first event. Repellency can also be calculated in different metrics, such as by measuring percent bite protection for a treatment, using a "Biting Deterrence Index", or measuring the minimum effective dosage of a treatment (136, 142).

Example from scientific literature: The Arm-In-Cage assay has been one of the most common and heavily relied-on assays to measure the repellent efficacy of treatments (124–131). This assay first took shape almost a century ago (132) and since then has been standardized and recommended by the Environmental Protection Agency (US-EPA) and the World Health Organization (133, 134). In 2023, Luker and colleagues used a version of this assay to test 20 active ingredients from the EPA's Minimum Risk Pesticides List on *A. aegypti* mosquitoes (135, 143, 144). 19 of these active ingredients were oils or essential oils, and one was a terpene compound. They tested 10% emulsions in an organic lotion base for each treatment and found that of the 20 treatments tested, four provided CPTs of over 60 minutes. 10% clove oil protected from mosquito bites for almost 2 hours, while 10% Cinnamon Oil protected for about 1 hour and 30 minutes, and 10% 2-Phenylethyl Propionate and 10% Geraniol provided protection for about 1 hour.

Variations: There are many variations to the Arm-In-Cage assay in the current literature; besides the dimensions of the mosquito cage and the number of mosquitoes in the cage. These include using the hand or leg of a volunteer rather than the forearm, pressing the volunteer's arm, hand, or leg against the mesh of the cage instead of inserting their arm, or using a treated cloth placed on the volunteer's arm instead of applying treatment directly onto the volunteer's skin (46, 81, 86, 89, 120, 145–150).

Strengths:

- Presence of mosquito attractant.
- Tests for contact repellency.
- Treatment used on human skin.

Weaknesses:

- Variation in volunteer attraction.
- Cannot test for spatial repellency.
- Needs frequent control testing.
- Uses human volunteers.

3 Conclusion and future directions

In this review, I covered several different laboratory methods that can be used to measure the repellent efficacy of specific treatments on mosquitoes (see Table 1). These assays were placed into two broad categories: assays without an attractant source and assays with an attractant source. While I covered many common and current laboratory methods and their variations, there are several more to be found in the published literature.

It is apparent that there are a multitude of ways to test the repellency of treatments on mosquitoes in a laboratory setting. Each method has its own strengths and weaknesses that I attempted to highlight throughout this review. Some can be used to screen multiple treatments in short periods of time, others are more refined and can be used to answer specific and unique questions. Assays can be used in conjunction to produce thorough and extensive research on the repellent efficacy of novel or established treatments.

Mosquito repellents can convey repellency in different ways, including spatially and/or through contact. Laboratory assays like the Excito-Repellency Test Chamber (non-contact version), Y-Tube, and Taxis cage are effective techniques to specifically measure for spatial repellent properties of a treatment, while the Surface Landing, Feeding, and Arm-In-Cage assays are effective to measure the contact repellent properties of a treatment. Other assays covered in this review can measure mosquito repellency to a treatment, but it may be difficult to distinguish between spatial and/or contact repellency when only using these assays alone.

This review also unveils how basic and straightforward many of these laboratory methods are. Nearly all of them are relatively low-tech with the most technical aspects being the optional use of cameras to record data or the use of computers to run statistical analyses.

Every year technology becomes more accessible, affordable, and user-friendly in the field of scientific research. In the next decade, I predict a surge of novel methods to test mosquito repellents that will revolutionize current screening methods. Some of the most promising technology is already prevalent in research such as video tracking. Video tracking has been used to graph arthropod behavior in an arena or container (151–153). Significant advancements in video tracking technology enables the development and implementation of specialized mosquito behavior studies (154). Another type of technology that can advance the study of mosquito repellents is the use of artificial intelligence (AI). AI can be used to predict novel mosquito repellent active ingredients based on molecular structures of known mosquito repellents and their targets (155). AI can also be used to collect and analyze large amounts of complex real-time data.

In conclusion, there are numerous established laboratory assays to test the repellent efficacy of treatments on mosquitoes and each has unique strengths and weaknesses. Due to technological advancements and new perspectives entering this field of research, there is continuous development of novel laboratory methods to test mosquito repellents. As time progresses, these novel methods may replace or improve the assays frequently used today.

TABLE 1 Summary Table.

Attractant source?	Assay	Type of repellency tested	Variable measured	Human volunteers/ live animals
No	Tube assay	General - cannot distinguish contact vs spatial	Mosquito location	No
No	Close Proximity Response assay	Spatial repellents	Time to mosquito flight	No
No	Excito-Repellency Test Chamber assay	Contact or spatial repellents	Mosquito escape rate	No
Yes	Y-tube Olfactometer assay	Spatial repellents	Percent mosquito attraction	Yes
Yes	Taxis Cage assay	Spatial repellents	Percent mosquito attraction	Yes
Yes	Surface Landing assay	Contact repellents	Mosquito landings	No
Yes	Feeding assay	Contact repellents	Mosquito probing or engorging	No
Yes	Arm-In-Cage assay	Contact repellents	Mosquito landing or probing	Yes

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Heat shock proteins, thermotolerance, and insecticide resistance in mosquitoes

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Mosquitoes transmit pathogens that pose a threat to millions of people globally. Unfortunately, widespread insecticide resistance makes it difficult to control these public health pests. General mechanisms of resistance, such as target site mutations or increased metabolic activity, are well established. However, many questions regarding the dynamics of these adaptations in the context of developmental and environmental conditions require additional exploration. One aspect of resistance that deserves further study is the role of heat shock proteins (HSPs) in insecticide tolerance. Studies show that mosquitoes experiencing heat stress before insecticide exposure demonstrate decreased mortality. This is similar to the observed reciprocal reduction in mortality in mosquitoes exposed to insecticide prior to heat stress. The environmental shifts associated with climate change will result in mosquitoes occupying environments with higher ambient temperatures, which could enhance existing insecticide resistance phenotypes. This physiological relationship adds a new dimension to the problem of insecticide resistance and further complicates the challenges that vector control and public health personnel face. This article reviews studies illustrating the relationship between insecticide resistance and HSPs or *hsp* genes as well as the intersection of thermotolerance and insecticide resistance. Further study of HSPs and insecticide resistance could lead to a deeper understanding of how environmental factors modulate the physiology of these important disease vectors to prepare for changing climatic conditions and the development of novel strategies to prevent vector-borne disease transmission.

KEYWORDS

HSPs (heat shock proteins), mosquito, insecticide resistance, thermotolerance, heat shock protein genes

Introduction

Mosquitoes contribute to the deaths of up to 1 million people each year globally. Across the diverse 3000 species of mosquitoes, only 9.3% have been identified as vectors of various pathogens (1). These pathogens include the malaria parasite and dengue virus. While mosquito control and disease treatment have improved significantly over the last century, the previous 20 years have seen increases in the transmission of these pathogens.

According to the World Health Organization (WHO), malaria cases steadily decreased from 2000 to 2015 from 245 million to 230 million, though since 2016, cases have again risen to 245 million. Deaths decreased from 2000-2015, from 897,000 to 577,000, but rose again to 619,000 in 2022 (2). Dengue virus has also seen massive increases in cases in the last 20 years. Since 2000, dengue cases increased from 500,000 to 5.2 million in 2019. Therapeutics for dengue are lacking (3).

Much of the increase in these diseases has been attributed to habitat expansion due to climate change and widespread insecticide resistance (4). Insecticide resistance is arguably one of the most significant challenges for pest control professionals today and plagues the public health, agricultural, and urban pest control sectors (5, 6). Resistance is developed through target-site modifications, metabolic mechanisms, cuticular modification and behavioral changes, typically with multiple mechanisms occurring within the same population (7, 8).

Mosquitoes are poikilotherms, meaning they do not regulate their body temperature and are subject to the ambient temperature in their environment. For this reason, ambient temperature influences all aspects of mosquito physiology, including metabolic rate, an important consideration for the metabolism of insecticide products (reviewed in (9)). Stresses associated with exposure to high temperatures results in initiation of a suite of responses by mosquitoes to mitigate negative impacts on physiological processes, fitness, and survival (10). This is referred to as the heat shock response and is generally facilitated by a family of stress responsive proteins called heat shock proteins (HSPs). Many genes coding for proteins within this family are associated with thermal stress, including *hsp70, hsp26* and *hsp83* (10–12).

In recent years, improvements in the quality of and access to high throughput sequencing technologies has made it easier to understand gene expression responses to external stressors, such as the xenobiotic response. This response includes a suite of detoxification and stress response enzymes including cytochrome p450s (CYPs), glutathione-s-transferases (GSTs), catalases, ATP-binding cassette (ABC) transporters, and heat shock proteins (HSPs) (13). Many of the proteins involved in this response are associated with facilitating insecticide resistance, either through mutations improving their binding efficiency for these chemicals or improving the rate at which they can breakdown insecticide (7).

Most of the research in the context of insecticide resistance has focused on ABC transporters, GSTs, and CYPs, though other stress response associated factors are likely playing a role (14). One such group is the HSPs, which act as molecular chaperones aiding in proper folding of proteins or in the repair of damaged proteins (15). Insecticide exposure induces oxidative damage through the generation of free radicals, which may be something these proteins help compensate for via a variety of mechanisms. These include protein refolding and prevention of aggregation, stabilization of reactive oxygen species, facilitating degradation of damaged molecules, and prevention of apoptosis (15–18).

The induction of HSPs by insecticides and other xenobiotics as well as meteorological factors such as temperature and humidity is a particularly important phenomenon to investigate in today's warming climate. In California, a major center for agricultural production, new agricultural and public health pests are spreading, and the state has experienced dramatic changes to the environment over the last 20 years (19, 20). Developing a holistic understanding of the overlapping physiological processes mediating the responses to the changing climate and xenobiotic exposure will be crucial to prediction and mitigation of issues with arthropod vectors of animal and plant pathogens. Awareness of these complex interactions will provide space in which to modify existing practices that exacerbate these issues and assure adequate preparation for and mitigation of the associated public health and agricultural issues.

Research demonstrates that sublethal insecticide doses induce *hsp* expression in mosquitoes and other insects, though few functional analyses have been performed (21–26). Additionally, research has shown cross-tolerance between increased temperatures and insecticides, though the direct causes of this cross-tolerance are not understood (summary in Figure 1) (27). Here, we review this literature and discuss future directions for research within this realm. HSPs may be an interesting target for the development of novel synergists, though further research on the physiology and direct mechanism by which these proteins are improving tolerance to insecticides is necessary.

A brief introduction to HSPs

HSPs are named for their initial discovery as heat shock responsive genes. They have a wide variety of roles (reviewed thoroughly in (15)). They are well known as molecular chaperones, assisting in folding new, misfolded, or damaged proteins. They also serve important functions in cell processes like cell cycle regulation, signal transduction, and stress responses. In insects, there are 4 primary families of HSPs: small HSPs, HSP60, HSP70, and HSP90 (summarized in Figure 2). These are so named for their respective molecular weights in kDa. HSPs have been associated with many types of stress in insects, including temperature, hypoxia/anoxia, and oxidative stress (reviewed in (16)). In particular, their role in oxidative stress tolerance is relevant to insecticide stress and likely why they have been observed to respond to insecticides in some mosquito strains. HSPs are thought to function by stabilizing and assisting with the folding or degradation of proteins damaged by oxidative stress (reviewed in (18). Briefly, members the HSP family of proteins function through nucleotide exchange or other substrate binding measures via ATP hydrolysis in concert with various cochaperones. The commonality of oxidative stress with both insecticide exposure and heat stress may explain the cross tolerance observed in heat and insecticide resistant insects.

Heat shock protein gene induction by insecticide

Multiple high-throughput studies of gene expression in mosquitoes have revealed increased expression of genes coding for HSPs following exposure to insecticide or in comparative studies

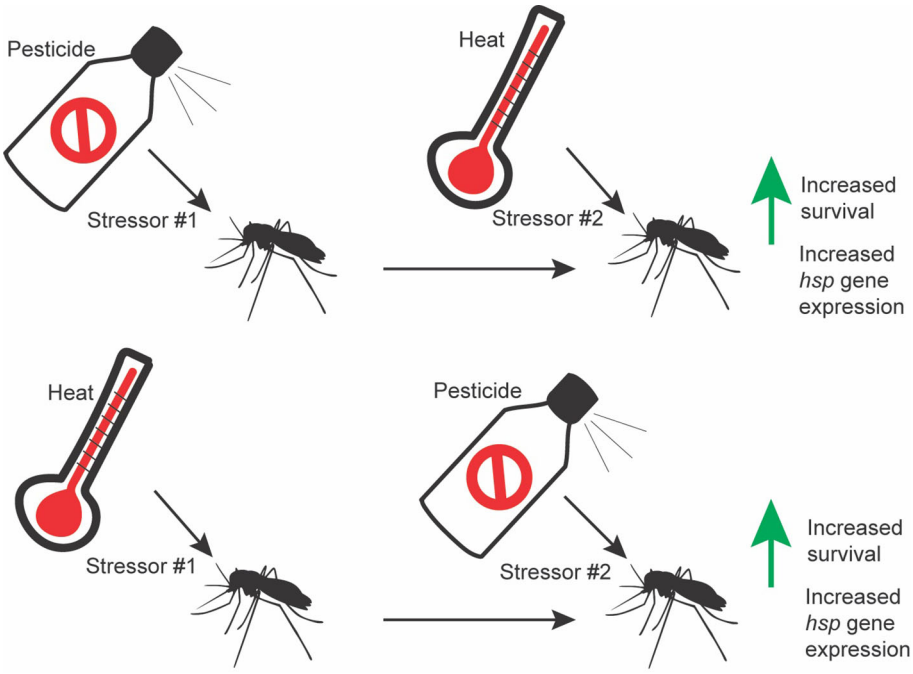

FIGURE 1
Graphical abstract representing cross tolerance between heat and insecticides.

of resistant and susceptible strains (22, 23, 30–33). The results are strain specific across studies and can vary both in the responsive genes and the dynamics of the response. A recent study in *Aedes aegypti* from California found that *hsp60A* contained a significant number of single nucleotide polymorphisms in comparison to other genes (34). This is particularly interesting, as this species of mosquito did not establish populations in California until 2013 (20). Throughout the state, mosquitoes are highly resistant to pyrethroids (35–37), and the overall variation observed in this *hsp* may be indicative of adaptation to hot, dry California weather and/or insecticide use, though further investigation is necessary.

A high throughput gene expression study examining differences between resistant field populations and a susceptible lab strain of *Anopheles sinensis*, found several *hsps* differentially expressed in the resistant groups (23). Among the 3 resistant populations tested, different *hsps* had significant changes. Interestingly, *hsp70* was downregulated in 2 populations, while *hsp90* was upregulated in all 3. The downregulation of *hsp70* may be indicative of its role as a negative regulator of the heat shock factor transcription factor (38). However, this gene displays downregulation in populations of *Drosophila* that previously experienced heat shock or a maintained heat stress, which may indicate a fitness cost of sustained expression of this gene (39). Alternatively, in a study of *Culex pipiens* examining gene expression profiles of lab selected resistant and susceptible strains, 3 *hsp70* genes and 1 *hsp83* gene were upregulated while *hsp67* was downregulated (40). No further interpretation was given to these results by the authors. Differences between the results of these two studies may be indicative of species specific, or strain specific responses.

In an RNA-seq study of resistant and susceptible strains of *Anopheles gambiae*, researchers discovered increases of *hsp* expression in mosquitoes they deemed resistant (30). Here, they used the WHO bioassay and divided mosquitoes into susceptible and resistant groups based on survival during the assay: susceptible being those that knocked down early and resistant as those who survived the 24-hour rest period. *Hsps* with increased expression included 2 *hsp20s*, 4 *hsp70s*, and 3 *hsp90s*, along with *DnaJ*. Authors attributed accumulation of these genes to an overall stress response experienced by mosquitoes after exposure to pyrethroids. Another comparative transcriptome study utilizing the WHO resistance assay for partitioning resistance groups in *Aedes albopictus* identified *hsps* that were differentially expressed in their data, but were not mentioned by gene ID, or functionally discussed (32).

An RNA-seq study characterizing the temporal genetic response of pyrethroid resistant *Ae. aegypti* from California to permethrin exposure found that 8 of the 20 genes with the largest fold change across 24 hours after exposure were *hsps* (21). These included 4 *hsp70s* and 4 *hsp20s (alpha-crystallin* and *lethal (2)-essential-for-life)*. The expression of these genes also increased in the handling controls as well as the permethrin treated mosquitoes, however, expression remained elevated through 24 hours after exposure while the controls returned to baseline by 24 hours. This suggests these genes are responsive to general stressors as well. However, in *Anopheles stephensi* larvae, 7 *hsps* were shown to be downregulated across a 48-hour time period after deltamethrin exposure, which may indicate that results are strain, species, or life stage dependent (41). A study using microarrays to observe changes in gene expression over time found upregulation of *DnaJ*, 2 *alpha-*

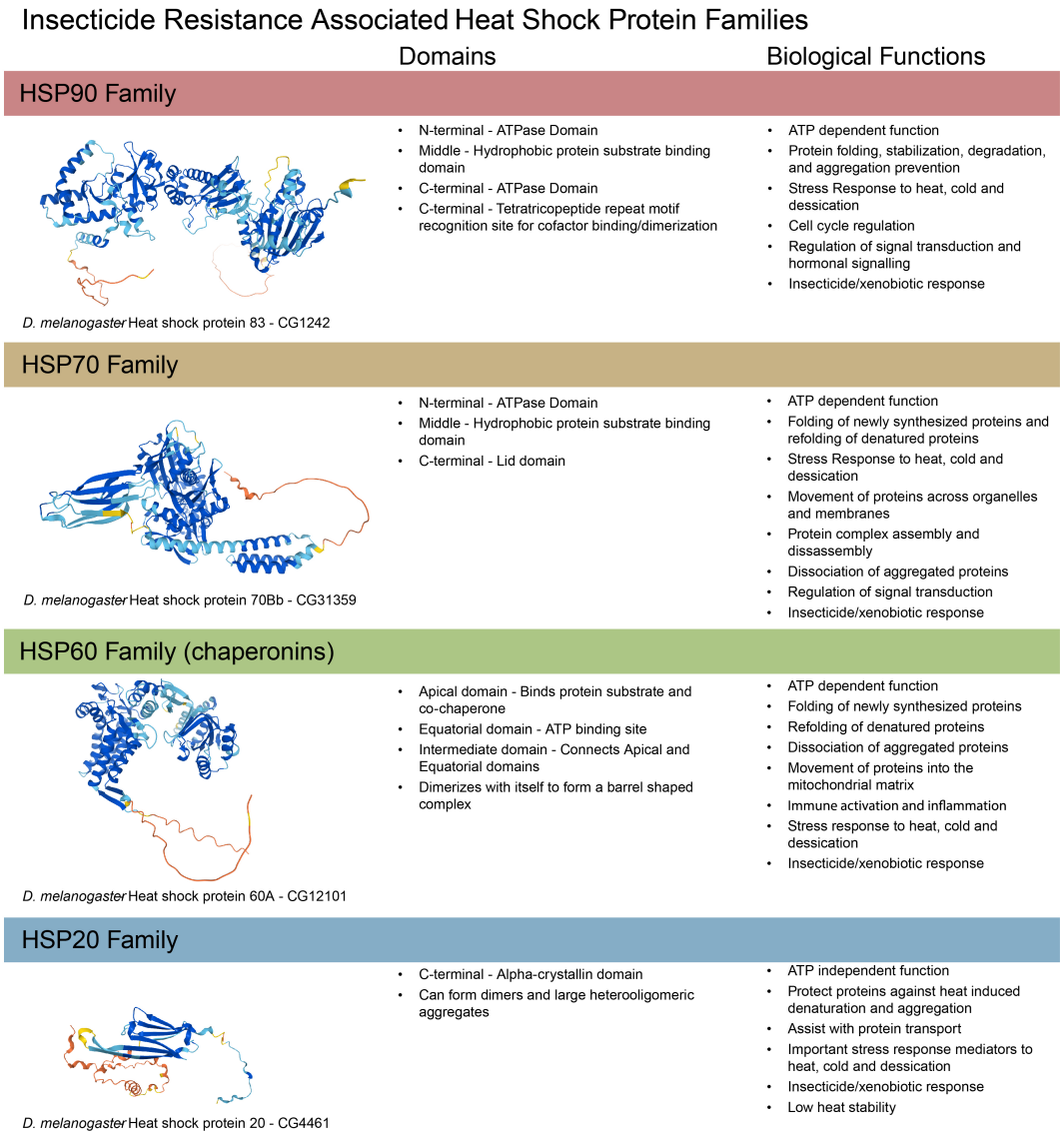

FIGURE 2
Summary of structure and function of insect heat shock protein families. Insects have have 4 families of heat shock proteins: HSP90, HSP70, HSP60, and HSP20, functionally reviewed in (15–18, 28) and summarized here. Protein structures created using Alphafold (29).

crystallin A chain, and 1 *hsp70* (42). Unrelated resistant and susceptible strains were used to assess fold changes in resistant versus susceptible mosquitoes. Again, researchers attributed these changes to general stress response induction due to permethrin exposure.

In larval *Ae. aegypti*, investigators identified 9 *hsp20* genes and 3 *hsp70* genes which increased in transcript abundance after treatment with a eucalyptus derivative (31). Interestingly, the 9 *hsp20* genes were clustered on chromosome 2, suggesting these genes are co-regulated. Their results were consistent with those found in Ingham et al., 2018, a comparative analysis of insecticide resistance and the associated transcriptional response in several *Anopheles* species, where 4 *hsp20* genes located on the same chromosome exhibited significant upregulation in resistant strains suggesting co-regulation (22).

Ingham et al. is also one of the few studies to complete functional analyses of heat shock proteins in resistance. The 4 *alpha-crystallin* genes were silenced and mortality assessed when exposed to deltamethrin, with no difference in mortality. However, RNAi knockdown of AGAP007159, an alpha crystallin B chain that was not upregulated in the microarray assay, resulted in a significant increase in mortality.

In a study examining the 2La chromosomal inversion and its role in thermotolerance and pyrethroid resistance, researchers discovered a correlation between the heterozygote form of this inversion and increased heat and pyrethroid resistance in 1 population studied (43). Additionally, researchers investigated 9 genes associated with heat tolerance and/or insecticide resistance including 6 heat shock proteins. Three *hsps (hsp70, hsp83, and hsp90)* were highly overexpressed in heat-hardened, pyrethroid

exposed, and unexposed controls compared to a susceptible lab colony, suggesting these genes are associated with heat tolerance, pyrethroid response, and pyrethroid tolerance.

While the studies discussed thus far focus on *hsp* gene expression shifts in relation to insecticide exposure or resistance, two studies have examined changes to HSP protein expression in relation to bacterially derived toxins from *Bacillus thuringiensis*. In a proteomic analysis of *Aedes aegypti* larval midguts, 1 heat shock protein, HSP90, was found downregulated after exposure to the LC50 dose of Cry11Aa toxin (33). When silenced, larvae were much more tolerant of the toxin, indicating an interesting assistive effect of this protein with the toxin. However, another study found that 2 HSP70 proteins were upregulated in larvae treated with nanoparticles to deliver the Cry4Aa toxin (44). This may reflect differences in response to the specific toxins.

Overall, the *hsp70* class of genes and their respective proteins are the most well-documented in association with the response to insecticide challenge or tolerance. Not only does *hsp70* act as a molecular chaperone, but it also plays a direct role in reducing stress induced apoptosis (45). *Hsp70* may be assisting with insecticide response in both contexts. *Hsp20* is also represented in a variety of studies, often with multiple members of this gene family responding. These small heat shock proteins are characterized as chaperones, maintaining cellular processes and homeostasis. They are also ATP-independent, which could be beneficial in an energy intensive state like insecticide response (17).

Mosquitoes have a unique relationship with heat due to their use of blood as a food source. The HSP70 protein has a protective effect and positive association with fertility in *Aedes aegypti, Culex pipiens*, and *Anopheles gambiae* (11). Upon blood feeding, the mosquito body increases in temperature quickly, resulting in increased production of HSP70 in the midgut (11). Knock down of this gene resulted in a 25% reduction in egg production, indicating the importance of this protein in fertility (11). The innate ability of mosquitoes to cope with rapid shifts in temperature may make them uniquely primed to adapt to both heat stress and insecticides.

Cross-tolerance between heat and insecticides

Generally, cross-tolerance between heat and insecticides has been studied without the addition of molecular investigation, however these studies portray the functional implications of temperature and insecticide resistance and are important to briefly review.

The first study to observe cross-tolerance between heat and insecticides in mosquitoes was published in 1996, where the authors found that larvae that experienced a heat shock event prior to exposure to propoxur, a carbamate insecticide, experienced a 50% reduction in mortality (27). Additionally, pre-treatment with propoxur prior to heat exposure proved almost as protective. In another study, researchers exposing adult *Anopheles stephensi* mosquitoes to permethrin at varying temperatures, found that those undergoing exposure between 22-28°C experience the greatest resistance ratios compared to either 16°C or 37°C (46). This differs from the previous study, as these mosquitoes did not experience a heat shock event followed by a period of recovery, so did not experience the protective effects as observed in the first mentioned study.

Larvae experience similar improvements to tolerance when adapted to high temperatures. A study in *An. stephensi* which larvae were adapted to high temperatures and then exposed to malathion as adults found an increase in survival of 2.4 to 3.1 fold at 37°C and 0.96 to 1.1 fold increase at 39°C, so it seems that the benefits of heat adaptation may be temperature dependent (47). However, in a study examining *Culex pipiens* raised at 20°C or 24°C, larvae raised at the higher temperature demonstrated higher mortality when treated with chlorpyrifos (48). Alternatively, *Cx. pipiens* that experienced a heat shock (30°C for 48 hours) prior to exposure to chlorpyrifos had much lower mortality than those raised at a constant temperature (49). The disparities in outcomes between these studies may be due to the significant differences in experimental temperature challenges or could potentially reflect strain specific adaptations.

Reciprocally, insecticide resistant strains demonstrate tolerance of higher temperatures. Another study considering differences between thermotolerance in susceptible and resistant *Cx. quinquefaciatus* found that resistant mosquitoes were more tolerant to high temperatures than their susceptible counterparts (50). The same group found that *Cx. quinequefaciatus* resistant to deltamethrin or lambda-cyhalothrin were more resistant to their respective chemicals after a 3 hour exposure to a high temperature (51). The use of resistant populations is important for these types of studies, as it provides information on the intersection of resistance mechanisms and heat tolerance mechanisms.

Future directions

Many gaps in knowledge remain about the connection between heat shock proteins/genes, thermotolerance, and insecticide resistance. Mounting evidence suggests that mosquitoes experience cross-tolerance between heat and insecticides, and heat shock proteins could be the reason for this. Further studies are necessary to unravel the intricacies of this multifaceted response and to improve vector control decisions in the context of rising temperatures.

Understanding the relationship between HSP gene expression and protein levels is an important first step in elucidating how these genes respond to various stimuli. Mechanistic follow-up experiments, involving either the knockdown or overexpression of specific HSPs, will be crucial to assess the role of these proteins in insecticide tolerance. Such studies should be conducted across multiple species, using a range of insecticides, and at varying temperatures. The evidence suggests that there is a strong relationship between insecticide resistance and thermotolerance. However, there is significant variance in the results of the studies reviewed here, underscoring the need for further research under comparable conditions and/or targeting specific factors to provide breadth and depth to these findings. Unraveling the mechanisms and factors contributing to the relationship between environmental stress responses and insecticide resistance is essential to predict and

understand the challenges facing vector control practices in the context of a warming climate.

Author contributions

LM: Writing – original draft, Writing – review & editing. GA: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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The diverse roles of insulin signaling in insect behavior

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In insects and other animals, nutrition-mediated behaviors are modulated by communication between the brain and peripheral systems, a process that relies heavily on the insulin/insulin-like growth factor signaling pathway (IIS). Previous studies have focused on the mechanistic and physiological functions of insulin-like peptides (ILPs) in critical developmental and adult milestones like pupation or vitellogenesis. Less work has detailed the mechanisms connecting ILPs to adult nutrient-mediated behaviors related to survival and reproductive success. Here we briefly review the range of behaviors linked to IIS in insects, from conserved regulation of feeding behavior to evolutionarily derived polyphenisms. Where possible, we incorporate information from *Drosophila melanogaster* and other model species to describe molecular and neural mechanisms that connect nutritional status to behavioral expression via IIS. We identify knowledge gaps which include the diverse functional roles of peripheral ILPs, how ILPs modulate neural function and behavior across the lifespan, and the lack of detailed mechanistic research in a broad range of taxa. Addressing these gaps would enable a better understanding of the evolution of this conserved and widely deployed tool kit pathway.

KEYWORDS

nutrition, developmental plasticity, fat body, foraging, fecundity, mating, social insect, genetic tool kit

Introduction

Nutritional state is a universal factor that alters behavioral expression in animals including insects (1, 2). Adult insects must accrue sufficient energy to support things like somatic maintenance, mate search, egg development, nest construction, oviposition, and parental care (3–7). To do this, individuals combine complex information about their own nutritional state with environmental information like resource and mate availability (8) in order to make prudent decisions about energy acquisition and use.

Insulin/insulin-like growth factor signaling (IIS) is one of the most well-recognized pathways that contributes to the organization and expression of energy-sensitive behaviors (1, 9). This pathway, particularly its satiety signaling function, is conserved across vertebrates and invertebrates (9). However, presumably because of the diverse connections between nutritional state and behavioral expression, IIS has been co-opted

to regulate phenotypes like egg production, reproductive tactics, and courtship behavior across taxa (10–12). It thus offers fertile ground for studies that investigate the physiological links between nutritional state and nervous system processes, and how these relationships evolve.

In this mini review, we explore the variety of roles for IIS in regulating behavioral expression in adult insects. One of our major goals is to describe links between IIS activity and the modulation of nervous system function, highlighting knowledge gaps in these areas. To do so, we use known mechanistic examples from *Drosophila melanogaster* (13, 14), and draw parallels and distinctions with other species where possible. To emphasize the expansion and diversification of IIS over evolutionary time, we focus on behaviors ranging from most conserved (e.g., feeding behaviors) to derived (e.g., social behaviors and polyphenisms).

Insulin/insulin-like growth factor signaling pathway fundamentals

IIS activity is dynamic throughout life. Here we focus on how IIS modulates adult behaviors, but we include some developmental processes that give rise to adult polyphenisms. IIS involves the action of insulin-like peptides (ILPs), which are produced in the brain and peripheral tissues and operate either as circulating hormones or neuromodulators (15–17). These peptides fall into three categories based on their shared homology with their vertebrate counterparts: insulin-like, insulin growth factor-like (IGF), or relaxin-like (18). Most ILPs are insulin-like (18, 19). Studies in some taxa differentiate insulin-like, IGF-like, and relaxin-like peptides, but many others refer to all types collectively as ILPs (18, 20, 21). In keeping with the convention set by *D. melanogaster*, we will generally refer to ILPs but note IGF and relaxin-like peptides where possible.

Insulin-like and IGF-like peptides activate the tyrosine kinase insulin receptor (InR) causing insulin receptor substrate (IRS) phosphorylation and downstream activation or inhibition of effectors via two major pathways, the phosphoinositide 3-kinase/protein kinase b (PI3K/Akt) pathway, which is associated primarily with cellular energy metabolism (22, 23), and the mitogen-activated protein kinase (MAPK) pathway, which is involved in cell and organismal growth, typically during development, via ecdysone signaling (4, 20). Notably, these pathways can have overlapping effects that are difficult to differentiate (18, 24–26). With PI3K/Akt, IRS binds to PI3K, activating Akt, which phosphorylates and inhibits a class O of forkhead box transcription factor (FOXO) and its downstream targets (27, 28), including developmental growth and differentiation regulators in conserved pathways such as hedgehog signaling (29–31, see 32 for an example of FOXO activity in adults). Akt can also activate the cAMP-response element binding protein (CREB, involved in memory formation) and inactivate glycogen synthase kinase 3 (GSK3), promoting glycogen synthesis and energy storage (23, 33–35). Alternatively, IRS can interact with growth factor receptor bound protein-2 (Grb2), ultimately initiating MAPK signaling (23).

While the identity of insulin-like peptides and IGFs are well-established in a variety of insect species, less is known about relaxin-like peptides outside of *D. melanogaster* (36). In *D. melanogaster*, relaxins activate G-protein coupled receptors (GPCRs), specifically leucine-rich repeat-containing GPCRs 3 and 4 (Lgr3 and Lgr4) during metamorphosis and oviposition, respectively (14, 18, 37–40). Recent studies are beginning to investigate relaxin-like peptide GPCRs in other taxa (24, 37, 41, 42).

IIS activity is often manipulated and/or measured using changes in ILP, InR, or IRS mRNA or protein levels. FOXO mRNA levels are also commonly used to infer PI3K/Akt pathway activity (43); other downstream effects of InR and the effects of relaxin-like peptides are less studied. To understand the role of IIS in coordinating nutritional state and behavior, it is necessary to know the location of ILP production and action in the periphery and brain. These are best understood in *D. melanogaster* (reviewed in 15), although characteristics are likely to be similar in other species (9, 44). In *D. melanogaster*, some ILPs are released by insulin-producing neurosecretory cells (IPCs) in the brain, where they act locally (45). IPCs respond directly and indirectly to peripheral signals including fat body produced ILPs, hemolymph glucose content, adipokinetic hormone, and other peptides and biogenic amines that can also act independently of nutritional state (17, 18, 37, 46, Figure 1). IPCs project to the heart, corpora cardiaca, and the midgut, stimulating ILP release from those tissues (8, 18, 50). Peripheral ILPs are also produced by ovarian follicle cells and regions of the gut. Some of these ILPs act locally, and others circulate (15, 51, 52). Notably, ILP production and inhibition are impacted by circulating hormones including juvenile hormone (JH) and ecdysteroids, and in turn, ILPs can affect the synthesis of these hormones (38, 47, 53–56). Many details regarding the coordination of ILP production and release among tissues, and the interaction of IIS with other behaviorally relevant pathways, are still under study.

IIS regulation of feeding behavior

Perhaps the most universal function of IIS is in satiety signaling, telling an individual they do not need food (46). IIS activity has been implicated in feeding behaviors in diverse species, including fruit flies (*D. melanogaster*), locusts (*Schistocerca gregaria*), and mantids (*Tenodera sinensis*9, 44, 57). ILPs produced in brain IPCs or peripherally, e.g., in the fat body, alter sensitivity to food cues or food acquisition behaviors through changes in sensory physiology, activity levels, nutrient preferences, and learning and memory processes (1, 9, 58–61). For example, in *D. melanogaster*, elevated circulating ILPs following food intake inhibit short neuropeptide F (sNPF) expression in the olfactory sensory neurons, reducing sensitivity to food odors and inhibiting food searching behavior (45). Similarly, starvation, and decreased production of ILPs by IPCs, induces hyperactive food search (62), while locomotion inhibits IPC ILP production, increasing sensitivity to food cues (63).

Data from other insects indicate that at least some IIS-mediated satiety mechanisms are generally conserved, although locations of

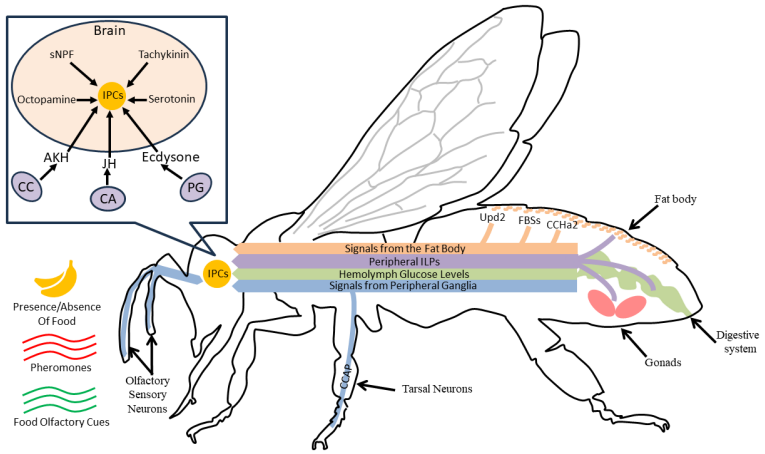

FIGURE 1
For a hypothetical adult insect, we show various IIS mechanisms that coordinate activity in the brain and periphery to give rise to behavioral variation. In the head (box insert), IPCs release locally acting ILPs to modulate nervous system processes like sensory responses and locomotor activity. Nearby glands such as the corpora cardiaca (CC), corpora allata (CA), and the prothoracic gland (PG) produce hormones that can alter ILP production and release from IPCs (18, 47). IPCs are activated by peptides (sNPF, tachykinin) or biogenic amines (octopamine, serotonin) released by other neurons in the brain in response to neural or peptide signaling from peripheral sensory systems (navy blue lines, (48, 49), or peripheral signals like hemolymph glucose levels (green line, 46); these are modulated by social and nutritional cues and nutritional status (indicated by navy blue lines, 18). The fat body also releases several types of uncharacterized fat body signals (FBSs), as well as Upd2 and CCHa2 in response to changes in available nutrients, and these ultimately stimulate IPC ILP production through unknown mechanisms (17). The fat body, and other tissues including the midgut and gonads (e.g., ovaries), also produce ILPs, shown in purple (15). These ILPs, some of which are also produced by the IPCs, can act on the brain as well as ganglia or other peripheral tissues (15). Notably, although we have depicted all relationships with a directional arrow, various signals can activate or inhibit IPCs depending on environmental context and the specific taxa.

ILP production, signaling relationships, and neural mechanisms giving rise to behavioral variation may differ. For example, in the desert locust *Schistocerca gregaria*, IIS via both MAPK and PI3K/Akt pathways increases sNPF expression in the optic lobe, leading to decreased feeding (57). Parasite infection at the time of a bloodmeal increases mosquito (*Anopheles stephensi*) olfactory sensitivity to hosts due to changes in midgut ILP mRNA expression (59). Female mosquitoes alternate between nectar and blood feeding as their nutritional needs change with egg production. In the mosquito *Aedes aegypti*, nutrient-specific hormone dynamics stimulate different sets of ILPs in the brain and peripheral tissues to synchronize metabolism and reproductive stage (47), as well as activate digestion of blood meals along with the target of rapamycin (TOR) pathway (64). In Western honey bees (*Apis mellifera*), IIS in the fat body modulates neural sensory systems via unknown mechanisms to cause a preference for lipid and protein-rich pollen over nectar in foragers (65). In this species, increased expression of brain InR mRNA is also correlated with spatiotemporal memory formation and anticipation of encountering known food resources (66), possibly through the MAPK pathway (67). Similarly, in *D. melanogaster*, IIS has been linked to cAMP-dependent memory formation and aversive learning in both adults and larvae (34, 68–71). In mantids, injection of mammalian insulin causes decreased movement, but rather than decreasing foraging activity, this causes a shift from active prey stalking to a more sedentary ambush strategy (44). It is largely unknown how ILPs modulate nervous system processes in these diverse species and contexts, but clearly IIS is involved in many types of behaviors and preferences related to foraging and diet choice.

IIS regulation of courtship, mate choice, and oviposition

In insects, IIS reflects nutrient availability for reproduction, and as such it affects vitellogenesis and the number of eggs a female produces (43, 72). However, because reproductive individuals perform suites of behaviors required to successfully mate and lay eggs, IIS is also more broadly involved in courtship and mate choice (49, 73). For example, in *D. melanogaster* males, tarsal contact with pheromones from male competitors or heterospecific females leads to the release of an ILP from the IPCs, inhibiting the P1 neurons that promote courtship (49). Relaxin-like ILPs and associated downstream mechanisms in male glial cells and abdominal ganglion neurons are also required for mating, sexual receptivity, and mate attraction in *D. melanogaster* (74–76). Similarly, in females, IIS in olfactory sensory neurons responsive to male sex pheromones mediates a starvation-induced decrease in sexual receptivity (12, 48). IIS seems to incorporate individual mating history in the context of mating decisions: inhibiting ILP production in unmated females increases sexual receptivity (77) while following a mating event, decreased InR expression or ILP production reduces willingness to remate (78).

Peripheral IIS activity in females also alters attractiveness to males through cuticular hydrocarbon (CHC) profiles. In *D.*

melanogaster, increased ovary IIS with decreased fat body IIS alters CHC production in fat body oenocytes (73) and increases mate attraction (52, 79). Because diet and nutrition influence IIS and CHC production, CHCs are honest signals of female quality (80). CHCs can indicate female mating status, fertility, and mating compatibility in many other insect species, suggesting this connection between IIS and mating cues may be broadly conserved in species from hymenopterans to coleopterans (81–83).

IIS mediates maternal offspring provisioning and oviposition site selection, combining the classic role of IIS in feeding behavior with its more elaborated reproductive functions. *D. melanogaster* females use gustatory cues to choose oviposition sites based on substrate sucrose concentrations (40). Interestingly, these decisions are not mediated by IPC-produced ILPs, but rather via relaxin-like ILP7 activity in neurons in the thoracic-abdominal ganglia (15), which have projections to the sub-esophageal ganglia and the female reproductive tract (40). The other *D. melanogaster* relaxin peptide, ILP8, is expressed in follicle cells and binds to receptors on abdominal ganglia cells, enabling the oviduct muscle to perform the needed oviposition movement (84). Ovary IIS may also modulate provisioning behaviors in social species where sterile workers feed offspring: in honey bees, workers with larger ovaries show a preference for pollen (used to make larval food) over nectar; genetic studies assessing variation in pollen preference have implicated the IIS pathway (85, 86).

IIS regulation of adult polyphenisms

Eusocial insect castes

IIS activity plays a critical developmental role across insects, affecting both juvenile and adult phenotypes (4). Here we highlight the developmental role of IIS in the context of adult polyphenisms, which are well-studied examples of nutrition-mediated behavioral variation in adult insects. For example, across independent evolutionary origins of eusociality, there is a common role for nutrition and IIS in caste determination, although the pathway is implemented differently among taxa (87–91). In honey bees, where colonies contain a single reproductive queen and thousands of sterile female worker bees, the queen larval diet increases IIS and leads to a spike in juvenile hormone (JH) production necessary for queen development (92–94). Later in development, queen IIS drops to worker-like levels (95), suggesting a transient increase in IIS/JH in queens gives rise to persistent effects at multiple levels of biological organization (93). While JH is produced in the corpora allata, it is unclear which tissues are involved in producing the upstream IIS signal and responding to IIS/JH (96).

IIS/JH signaling during larval stages could directly impact the development of the brain and/or other tissues that communicate with the brain throughout adulthood. In honey bees, IRS expression during development is responsible for differentiating queen and worker ovaries, but additional variation in IRS expression throughout adulthood also underpins behavior-relevant variation in ovary size among workers (85, 86, 97). For example, among workers, there is evidence that ovary size modulates the response to social pheromones (98). Enlarged ovaries are associated with increased octopamine signaling in the brain (98); octopamine activates the IPCs and thus could modulate olfactory sensitivity through IIS (8). A similar mechanism appears in the clonal raider ant *Ooceraea biroi*, where adults can switch between ovary activated (reproductive) and ovary suppressed (brood care) phases. Larval pheromones suppress reproduction and promote brood care by inhibiting ILP expression in adult IPCs (89).

While it is unknown whether or how IIS/JH signaling impacts brain development, differences in IIS expression continue into adulthood in honey bees; queens have decreased brain IIS compared to workers (87). Other social species also show caste differences in brain IIS, but patterns vary. Reproductives have higher brain IIS compared to workers in a wasp (*Polistes candensis*99), termite (*Cavitermes tuberosus*100), earwig (*Forficula Auricularia*101), and many ant species (20, 89, 102–106). IIS could be linked to different, specific functional outcomes in these diverse social species, for example, species-specific trade-offs among egg production, queen behavior, and lifespan (87). Resolving these relationships requires more detailed work, including assessment of the specific mechanisms activated by IIS. For example, in reproductives of the ant *Harpegnathos saltator*, brain produced ILPs activate MAPK in the fat body and ovaries, but not the PI3K/Akt pathway (20), while ovarian activation of PI3K/Akt signaling occurs in other ant species (102, 103). These different responses to ILPs in the ovaries could mediate divergent phenotypic outcomes.

The unresolved complexities in IIS continue when looking among members of the worker caste in social insects. Honey bee workers show dietary and physiological changes corresponding to adult age-related behavioral shifts ("age polyethism"), including a massive loss of lipid stores in the fat body associated with the transition from nursing to foraging behaviors (107). As the fat body shrinks during aging, increased ILP production leads to increased JH and behavioral changes (97, 108–111). However, while older workers have higher whole-body IIS activity compared to younger workers, they have higher brain IIS (112) but lower fat body IIS (113). IIS activity differences could also correspond to tissue-specific divergence in downstream pathways. For example, a brain biomarker for honey bee foraging behavior is a extracellular signal-regulated kinase (ERK), a member of the MAPK pathway (114), which has been associated with learning and memory processes in the context of food acquisition (67). In contrast, in the fat body, IRS (the PI3K/Akt pathway) is activated in nurse bees who consume an amino acid rich diet compared to foragers; decreased IRS/IIS signaling results in precocious foraging (113). Thus, two different IIS downstream pathways in two different tissues both contribute to the same phenotypic outcome. Other honey bee species, the wasp *Polistes metricus*, and the ant *Temnothorax longispinosus* show similar age- and tissue-related patterns (88, 115–117), while the bumble bee *Bombus terrestris*, stingless bee *Tetragonisca angustula*, and ant *Solenopsis invicta* show the opposite, at least in terms of age patterns (118–120). The mechanistic implications of these complexities remain unclear.

Notably, many studies in eusocial insects use gene expression data exclusively to implicate IIS in caste differences. These data do

not necessarily reflect circulating ILP levels or the quantity of stored ILPs that could be released to activate IIS. More work examining protein interactions and phosphorylation downstream of ILP receptor binding is necessary to validate and interpret the role of IIS in the context of behavioral differences between queens and workers or among workers.

Wing length and weapon size polyphenisms

Juvenile nutrition and IIS activity are involved in the development of discrete adult polyphenisms in wing length in some hemipterans and weapon size in some coleopterans. When food quality is low, some hemipterans produce long-winged morphs that disperse at a cost to fecundity (121). As hemimetabolous insects, the switch between morphs can happen until the last nymphal instar, allowing for rapid response to environmental conditions (121). IIS patterns and wing morph expression are similar across several species: in soapberry bugs (*Jadera haematoloma*), linden bugs (*Pyrrhocoris apterus*), and pea aphids (*Acyrthosiphon pisum*), high quality food or low population densities lead to elevated IIS activity (inferred by pathway manipulation and gene expression data) and the development of wingless morphs (30, 51, 122, 123). However, in the brown planthopper (*Nilaparvata lugens*), this pattern is generally reversed (31, 124). Downstream mechanisms could include GSK3, which is associated with wing deformities (125, 126). Wing tissues are particularly sensitive to ILPs and variation in IIS does not affect allometry or growth in other tissues (121). This tissue specificity extends beyond species with conspicuous polyphenisms, e.g., *D. melanogaster* and the tobacco hornworm *Manduca sexta*. Thus, developmental nutrition may have other subtle effects on adult flight, dispersal phenotypes, and reproductive capacity across diverse species (19, 127).

In *Scarabaeidae* beetles, exaggerated male weapons like horns are common. Males with high-quality larval nutrition have large weapons and engage in male-male fighting over mates, while males with poor nutrition have small or no weapons and rely on sneaker tactics (10). As in the wing example, other tissues are unaffected by variation in ILPs. When nutrition is high-quality, ILPs drive weapon tissue proliferation through InR activation (128, 129). Without these signals, the transcription factor FOXO stops cell proliferation and the development of weapon structures (130). There is some interesting variation in how IIS acts in different beetle species. In the rhinoceros beetle *Trypoxylus dichotomus*, InR knockdown results in greatly diminished horns (130). In contrast, InR knockdown has no effect on horn growth in the dung beetle *Onthophagus nigriventris*, but FOXO knockdown suppresses growth in both the horns and genitalia (10, 29, 128, 131). IIS has also been implicated in more subtle variation in flight and fighting capabilities in bark beetles (*Dendroctonus ponderosae*) and crickets (*Gryllus assimilis* and *Gryllus firmus*5, 132–134), suggesting it may play a more generalized role in competition-related behavior and polymorphisms.

In the dimorphic horned beetle examples, developmental IIS leads to differences in adult morphology and behavior, but it is unclear whether IIS exerts organizational effects on the brain during development, or continuously regulates adult behavioral differences. For example, variation in *doublesex* expression and serotonin levels in the adult brain predict differences in aggression across dimorphic males (135, 136). *Doublesex* is a target of IIS in developing horn tissues and serotonin impacts the body size threshold that distinguishes the horn morphs (29, 137), but it is unknown whether IIS regulates either mechanism in the adult brain. Similarly, in the pea aphid, differential ILP expression between nymphal winged and wingless individuals occurs in the thorax, but ILPs are also differentially expressed in the brain and thorax during adulthood, suggesting further phenotypic impacts (51, 138). Understanding the relationships in activity of IIS across the life stages could lead to new insights about the evolution and regulation of phenotypic plasticity. IIS appears to integrate environmental cues over the lifetime to modulate behavioral expression, and as such, it could serve as a mechanism that impacts the duration of environmental effects (139, 140).

Discussion

IIS's role in communicating nutritional state and regulating feeding behaviors has been elaborated over evolutionary time to coordinate reproductive physiology, courtship and mating behaviors, maternal provisioning behaviors, social insect caste differentiation, and the development and adult regulation of dimorphic dispersal and reproductive phenotypes.

Food choice and food-related locomotion are broadly associated with IIS, but there is substantial species-level variation in food cues, nutrients and preferences, locomotion patterns, and the ecological contexts that regulate foraging behaviors. Future studies could investigate the mechanistic bases of this species-level variation, in terms of how both internal state and external information modulate IIS and cause behavioral change. Insects present some particularly interesting and economically relevant contexts where IIS is essential to feeding behavior, including grasshopper (*Oedaleus asiaticus*) plague activity resulting from sub-optimal diets (141) or changes in feeding behavior due to crowding in armyworms (*Mythimna separata*142). Examining IIS activation, including ILP production and modes of action in the brain across diverse taxa is critical to understanding the evolution of IIS and may also highlight new tools for pest control.

Substantial gaps remain in understanding the role of IIS in coordinating activities between the brain and peripheral tissues. These mechanisms are diverse and context dependent even in well studied species like *D. melanogaster* (15). However, certain emergent patterns may be conserved. For example, in *D. melanogaster*, different ILPs are responsible for within and cross-tissue signaling. ILP number varies greatly among taxa (143), possibly reflecting the diversity of contexts requiring IIS regulation, or the tissues involved. Most species have 1 or 2 InRs that activate tissue-specific downstream targets (144) but the mechanisms that allow specificity in downstream interactions, including how limited numbers of InR receptors give rise to diverse effects from numerous peptides, are still mostly unknown (55). While the most-studied model species *D. melanogaster* has only one InR, many other species

have two, and Blattodea three, which can lead to novel relationships and interactions that should be studied further (144). For example, in the brown planthopper, InR2 directly inhibits InR1 during wing morph development, while the third Blattodea receptor is hypothesized to have a role in social termite evolution (124, 144).

Identifying the downstream pathways activated specifically by IIS is challenging as many of them can be affected by several other signaling pathways (20, 23, 145–147). This is especially problematic in non-model organisms where genetic tools and experimental approaches to manipulate ILP abundance are not well-developed. It is also important to consider the possibility that some peptides identified as insulin-like may belong to other peptide classes (e.g., IGF-like), which could suggest divergent downstream effects (20, 38, 105). Future studies could address these complexities by at least elaborating on the details of tissue-specific IIS and confirming the involvement of IIS using direct measures of ILP abundance and scaffolding or phosphorylation state of downstream targets (24).

Another compelling pattern that emerges from eusocial caste differentiation is that IIS is used to integrate cues associated with seasonal timing and other abiotic factors. For example, in the social paper wasp *Polistes metricus*, late season larvae become reproductive gynes that will overwinter and establish new nests the following year. As such, larvae are fed more and have activated IIS (148). The ant *Pogonomurmex rugosus* can only produce new queens after the original queen has hibernated, a transition caused by environmental signals like temperature that induce numerous physiological and behavioral changes in queens, including decreased metabolism and feeding. Hibernated queens have increased ILP expression, which increases the amount of vitellogenin deposited in eggs leading to new queen production (149). These provide additional examples of the ways in which IIS has been co-opted in novel contexts associated with nutrition variation.

Despite broad connections between IIS and behavior, mechanistic work outside of *D. melanogaster* remains limited. More diverse functional information could elucidate the conserved and divergent aspects of IIS among species and contexts, for example, in terms of where ILPs originate in the body (59), or how the different IIS components interact with each other (121). Our current model systems have highly derived phenotypes that may hinder attempts to form generalizable hypotheses. Broadening work in other taxa will also help explain why IIS is inconsistently used to regulate the same phenotypes across species (89). For example, some fig wasp species have winged and wingless males (150) that differ in aggression and weapon size (151). Although these phenotypes resemble the bugs and beetles discussed above, no link has been made to IIS or nutrition. Is this an independent evolutionary event with repeated co-option of the IIS pathway? Comparative investigations of the evolutionary origins of phenotypes like polyphenisms could help determine whether IIS is a "toolkit pathway" that has been repeatedly deployed over evolutionary time to give rise to similar phenotypes (120, 152). Its ubiquity among species and behaviors suggests this could be the case.

Author contributions

AW: Conceptualization, Writing – original draft, Writing – review & editing. CR: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Molecular characterization, localization, and physiological roles of ITP and ITP-L in the mosquito, *Aedes aegypti*

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The insect ion transport peptide (ITP) and its alternatively spliced variant, ITP-like peptide (ITP-L), belong to the crustacean hyperglycemic hormone family of peptides and are widely conserved among insect species. While limited, studies have characterized the ITP/ITP-L signaling system within insects, and putative functions including regulation of ion and fluid transport, ovarian maturation, and thirst/excretion have been proposed. Herein, we aimed to molecularly investigate *Itp* and *Itp-l* expression profiles in the mosquito, *Aedes aegypti*, examine peptide immunolocalization and distribution within the adult central nervous system, and elucidate physiological roles for these neuropeptides. Transcript expression profiles of both *AedaeItp* and *AedaeItp-l* revealed distinct enrichment patterns in adults, with *AedaeItp* expressed in the brain and *AedaeItp-l* expression predominantly within the abdominal ganglia. Immunohistochemical analysis within the central nervous system revealed expression of *Aedae*ITP peptide in a number of cells in the brain and in the terminal ganglion. Comparatively, *Aedae*ITP-L peptide was localized solely within the pre-terminal abdominal ganglia of the central nervous system. Interestingly, prolonged desiccation stress caused upregulation of *AedaeItp* and *AedaeItp-l* levels in adult mosquitoes, suggesting possible functional roles in water conservation and feeding-related activities. RNAi-mediated knockdown of *AedaeItp* caused an increase in urine excretion, while knockdown of both *AedaeItp* and *AedaeItp-l* reduced blood feeding and egg-laying in females as well as hindered egg viability, suggesting roles in reproductive physiology and behavior. Altogether, this study identifies *Aedae*ITP and *Aedae*ITP-L as key pleiotropic hormones, regulating various critical physiological processes in the disease vector, *A. aegypti*.

KEYWORDS

ion transport peptide, ionoregulation, feeding, reproductive biology, anti-diuretic

Introduction

Neuropeptides comprise a large and diverse class of signaling molecules that, together with their receptors, play a significant role in controlling a myriad of behavioral and physiological processes, including reproduction, feeding, development, energy homeostasis, water and ion balance, and more (1–4). The crustacean hyperglycemic hormone (CHH) family of peptides are a large neuropeptide superfamily that includes structurally-related peptides composed of 72 to more than 80 amino acids (5) containing three highly conserved intramolecular disulfide bonds (6). Functional roles for the CHH family of peptides are linked to molting, stress responses, reproduction, and homeostatic regulation of energy metabolism (7). The insect ion transport peptide (ITP) and its alternatively spliced variant, ITP-like or ITP-long (ITP-L) belong to the CHH family of peptides (8, 9) and are widely conserved among insect species, including lepidopterans, such as the silkworm *Bombyx mori* (9, 10), and the tobacco hornworm *Manduca sexta* (11). While investigations examining the roles of ITP and ITP-L are limited, studies have suggested functions of both peptides as regulators of ion and fluid transport across the ileum of the desert locust *Schistocerca gregaria* (12), ecdysis in *M. sexta* (11), ovarian maturation in the red flower beetle *Tribolium castaneum* (13), and thirst/excretion regulation and clock neuron modulation in the fruit fly *Drosophila melanogaster* (14, 15).

The insect ITP was originally identified in *S. gregaria* (9, 12, 16), where it drives chloride-dependent movement of fluid across the ileum, hence suggesting a role as an anti-diuretic hormone (17). Subsequently, Meredith et al. identified the complete amino acid sequence of *Schgr*ITP, with a 72-residue mature peptide sequence and six cysteine residues proposed to participate in disulfide bridge formation (18). The mature *Schgr*ITPL peptide is only four amino acids longer than ITP, containing a unique carboxy-terminus (19, 20). Studies have revealed that both peptides share a common N-terminal sequence, whereas the C-terminal sequences diverge significantly, thus were predicted to arise from alternative splicing (18). Due to the shared N-terminus between the two peptide precursors, earlier studies proposed that the N-terminus permits the peptides to bind to its receptor (19). ITP is a potent stimulator of ileal short circuit current, whereas ITP-L is devoid of such activity, suggesting an antagonistic role of ITP-L on the putative ITP receptors in the locust hindgut (21).

Differential tissue immunolocalization of ITP and ITP-L in *M. sexta* and *B. mori* revealed ITP expression in bilaterally-paired neurosecretory cells in the brain with projections to the retrocerebral complex, whereas ITP-L expression was seen in peripheral neurosecretory cells and neurons of the ventral ganglia (9). Further investigations confirmed ITP localization exclusively to the central nervous system, and ITP-L to the central nervous system and peripheral tissues (18, 22, 23), suggesting differential functional roles for the alternatively spliced peptides. In 2007, Dai et al. were the first to identify a conserved ITP gene (*Itp*) in the mosquito, *Aedes aegypti*, which by alternative splicing, encodes for *Aedae*ITP-L; a longer peptide isoform with an unblocked C-terminus, and *Aedae*ITP; a shorter peptide with an amidated C-terminus. To date, the expression pattern, tissue distribution, and putative physiological function of either ITP or ITP-L has not been determined in *A. aegypti*. Herein, this study set out to characterize the tissue-specific expression and localization, as well as determine functional roles of *Aedae*ITP and *Aedae*ITP-L in the *A. aegypti* mosquito. Using a combination of molecular and physiological techniques, *Aedae*ITP and *Aedae*ITP-L was characterized in the adult stage, with expression and localization of *Aedae*ITP in the brain and the terminal ganglion while *Aedae*ITP-L was detected in the pre-terminal abdominal ganglia of the ventral nerve cord. Furthermore, using RNA interference (RNAi), the current results provide strong evidence that *Aedae*ITP and *Aedae*ITP-L play essential roles in osmotic and ionic regulation, reproductive physiology and mating behavior in the *Aedes* mosquito. Overall, these findings advance our understanding of ITP and ITP-L neuropeptides in mosquitoes and provide novel research directions for elucidating neuropeptidergic signaling in the disease-vector, *A. aegypti*.

Materials and methods

Animals

Aedes aegypti eggs (Liverpool strain) were collected from an established laboratory colony as described previously (24, 25) and hatched in double-distilled water in an incubator at 26°C on a 12:12 hour light: dark cycle. Larvae were fed a solution of 2% (w/v) brewer's yeast and 2% (w/v) Argentine beef liver powder (NOW foods, Bloomingdale, IL, USA). For colony upkeep, female mosquitoes were fed sheep's blood in Alsever's solution (Cedarlane Laboratories Ltd., Burlington, ON, Canada) using an artificial feeding system (24). Adults were provided with 10% sucrose solution *ad libidum*.

Tissue/organ dissections, RNA extraction, and cDNA synthesis

One- and four-day old adult female (n=30) and male (n=40) *A. aegypti* were briefly anaesthetized with CO_2 and submerged in Dulbecco's phosphate buffered saline (DPBS; Wisent Corporation, St. Bruno, QC, Canada), and the following body segments and tissues/organs were dissected and isolated: head, thorax, midgut, Malpighian tubules (MTs), hindgut, reproductive tissues (ovaries, testes, and accessory reproductive tissues) and carcass (remaining cuticle, musculature, fat body, and abdominal ganglia). For the central nervous system expression profile, the brain, thoracic ganglia, and abdominal ganglia were collected. Whole adult RNA was obtained by collecting fourth instar larvae (n=10), early- and late-stage pupae (n=10), and one- and four-day old adult female (n=10) and male (n=11) mosquitoes. For the starvation and desiccation assay, whole adult male (n=6-7) and females (n=5-6) were isolated 24 h or 48 h post treatment. To confirm knockdown efficiency following double-stranded RNA (dsRNA) treatment, whole adult male (n=5-6) and female (n=5) were isolated four-, six-, and eight-days post injection. Whole adult and organ samples

were stored in 1x RNA protection buffer at -20°C until further processing. Samples were then thawed at room temperature and total RNA was isolated using the Monarch Total RNA Miniprep Kit following manufacturers protocol with an on-column DNase treatment to remove genomic DNA (New England Biolabs, Whitby, ON, Canada). Purified total RNA samples were subsequently aliquoted onto a Take3 micro-volume plate and quantified on a Synergy Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). To determine *AedaeItp* and *AedaeItp-l* transcript levels, cDNA was synthesized from 500 ng (developmental expression profile), 80 ng (spatial expression profile), and 250 ng (starvation assay and dsRNA injections) total RNA using the iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Mississauga, ON, Canada) following manufacturers protocol, including a ten-fold dilution of cDNA following synthesis.

RT- quantitative PCR

To measure expression profiles for *AedaeItp* and *AedaeItp-l*, transcript abundance was quantified on a StepOnePlus™ Real Time PCR system (Applied Biosystems, Carlsbad, CA, USA) using PowerUP™ SYBR® Green Master Mix (Applied Biosystems, Carlsbad, CA, USA). Cycling conditions were as follows: 1) uracil-DNA glycosylase (UDG) activation 50°C for 2 min, 2) 95°C for 2 min, and 3) 40 cycles of i) 95°C for 15 seconds and ii) 60°C for 1 minute. Gene-specific primers for *AedaeItp* and *AedaeItp-l* were designed over multiple exons (see Supplementary Table S1 for list of primers) based on a previously reported mRNA sequence (Genbank Accession Numbers: (*Itp*) AY950503 and (*Itp-l*) AY950506) (9). To ensure specificity for each individual peptide-specific transcript, reverse primers for *AedaeItp* (nucleotides 418-438) and *AedaeItp-l* (nucleotides 418-428) were designed over transcript-specific exon-exon boundaries that, in the case of *AedaeItp-l*, includes exon 3 since this exon is absent in *AedaeItp* (Supplementary Figure S1). Relative expression levels were determined using the C_T method (26) and normalized to the geometric mean of *rp49* and *rps18* reference genes, which were previously determined as optimal endogenous controls (27). Developmental expression profiles consisted of an average of 5-6 biological replicates that each included triplicate technical replicates for each target gene. Spatial expression profiles, starvation assay and dsRNA knockdown experiments consisted of 3-4 biological replicates. Primer specificity for target mRNA was assessed by conducting no reverse-transcriptase and no-template controls along with performing standard curves to calculate primer efficiencies.

Immunohistochemistry

To examine *Aedae*ITP and *Aedae*ITP-L immunoreactivity in the central nervous system, whole one- and four-day old adult male and female *A. aegypti* were collected and incubated in freshly prepared 4% paraformaldehyde (PFA) fixative overnight at 4°C. The following day, tissues/organs dissections of the central nervous system consisting of the brain, thoracic ganglia, and the abdominal ganglia, were performed in DPBS. Tissues/organs were then permeabilized by incubating on a rocker for 1 h at RT in 4% Triton X-100 (Sigma Aldrich, Oakville, ON, Canada), 10% normal sheep serum (NSS) (v/v) and 2% BSA (w/v) prepared in DPBS, followed by three 15 min washes in DPBS. After the last wash, the DPBS was removed and substituted with a 1:1000 dilution of primary antiserum solution (0.4% Triton-X- 100, 2% NSS (v/v), and 2% BSA (w/v)) in DPBS (prepared the day before use and incubated at 4°C to reduce non-specific binding) on a rocker for 96 h at 4°C. The custom primary antiserum solution was raised in rabbit against a synthetic peptide (SSFFDIECKGQFNKA) antigen corresponding to a 15-amino acid region of *Aedae*ITP and *Aedae*ITP-L (nucleotides 154-198 on common exon 2, amino acids 1-15 of the shared N-terminal sequence, Supplementary Figure S1), thus targeting both *Aedae*ITP and *Aedae*ITP-L (Biomatik, Kitchener, ON, Canada). Following incubation, tissues/organs were washed with DPBS four times on a rocker over the course of an hour and subsequently incubated overnight at 4°C with a goat anti-rabbit Alexa Fluor® 568 IgG (H+L) secondary antibody (Molecular Probes, Life Technologies, Eugene, OR, USA) diluted 1:200 in 10% NSS made up in DPBS and protected from light. The following day, tissues/organs were washed three times with DPBS for 15 min each. As a negative control, the anti-*Aedae*ITP/ITP-L primary antiserum was preincubated with 10 µM antigen (SSFFDIECKGQFNKA) overnight prior to use. Additionally, tissues/organs were also incubated with a no-primary control (0.4% Triton-X-100, 2% NSS (v/v), and 2% BSA (w/v) prepared in DPBS). Tissues/organs were mounted on cover slips with mounting media comprised of DPBS with 50% glycerol containing 4 µg/mL 4'6-diamidino-2-phenylindole dihydrochloride (DAPI) and were visualized on a Zeiss LSM 800 confocal laser microscope (Carl Zeiss, Jena, Germany) and processed with the Zeiss LSM Image Browser software or visualized on a Lumen Dynamics XCite™ 120Q Nikon fluorescence microscope (Nikon, Mississauga, ON, Canada).

Starvation and blood feeding assay

To examine the potential roles of *Aedae*ITP and *Aedae*ITP-L in mosquito feeding/starvation, adult males and females were isolated post-emergence and sorted into three treatment conditions: desiccated (no food or water provided), fed (10% sucrose *ad-libitum*), and starved (only water provided). The adults were collected after 24 h or 48 h, and mRNA transcript levels of *AedaeItp* and *AedaeItp-l* were examined by RT-qPCR (as described above). To investigate whether a protein-rich meal influences the transcript abundance of *AedaeItp* and *AedaeItp-l*, adult female mosquitoes (four-six day old) were given 20 min to blood feed on sheep's blood in Alsever's solution, and all blood fed females were subsequently isolated after 1, 6, 12 and 24 h post-bloodmeal. Blood fed females were compared to control, similarly aged (five-six day old) females that were provided sucrose *ad libitum*. Post isolation, mRNA transcript levels of *AedaeItp* and *AedaeItp-l* were examined by RT-qPCR (as described above).

Preparation and microinjection of *AedaeItp* and *AedaeItp-l* dsRNA

Gene-specific primers were designed to amplify a region of the *AedaeItp* and *AedaeItp-l* transcripts as a template for dsRNA synthesis (ensuring no overlap with RT-qPCR primers, Supplementary Table S1, Supplementary Figure S2). Similar to the gene-specific probes, ds*AedaeItp* primers were designed over common exons 1 and 2, that targets and knocks down both *AedaeItp* and *AedaeItp-l* mRNA, whereas *AedaeItp-l* primers were designed over the unique exon 3 targeting only *AedaeItp-l* transcript (Supplementary Figure S1). The *AedaeItp* and *AedaeItp-l* target regions were amplified and cloned into pGEM-T-Easy vector and subsequently subcloned into the L4440 vector, which possesses two T7 promoters, each flanking either side of the multiple cloning site. L4440 was a gift from Andrew Fire (Addgene plasmid#1654; http://n2t.net/addgene:1654; RRID : Addgene_1654). The *AedaeItp* and *AedaeItp-l* targets were screened with a M13 forward (5'-TGTAAAACGACGCCAGT-3') and L4440 reverse primer (5'-AGCGAGTCAGTCAGTGAGCGAG-3') and reamplified with a T7 primer serving as a forward and reverse primer. Double stranded RNA was synthesized by *in vitro* transcription using the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, Whitby, ON, Canada) following manufacturers recommendations. Following synthesis, the dsRNA was incubated at 75°C for 5 min for denaturation and left at RT for 15 min to allow rehybridization, followed by RNA purification using a Monarch RNA Cleanup Kit following the manufacturer's protocol (New England Biolabs, Whitby, ON, Canada). One-day old male and female adult mosquitoes were briefly anesthetized using CO_2 and injected in the thorax with 1 µg of *AedaeItp*, *AedaeItp-l* or control *Egfp* (enhanced green fluorescent protein) dsRNA dissolved in nuclease-free H_2O in a total volume of 200nL using a Nanoject III Programmable Nanoliter Injector (Drummond Scientific, Broomall, PA, USA).

In vivo urine production assay

To determine if *Aedae*ITP and/or *Aedae*ITP-L influences urine output, adult female mosquitoes were injected with 500 nL of a HEPES buffered saline (HBS), consisting of 11.9 mM HEPES, 137 mM NaCl, and 2.7 mM KCl, titrated to a pH of 7.45 and filter sterilized before use. Based on an established protocol (28), four-day old females were injected and placed into a graduated, packed-cell volume tube (MidSci, St. Louis, MO, USA) for two hours at 28°C with three mosquitoes per tube and excretion volumes were measured. Specifically, following the incubation period, mosquitoes were removed from the tube, which was then centrifuged at 16,000x*g* for 30 s to allow for the excreted volume to be measured visually under a dissecting microscope, via the graduated column at the bottom of the tube. Treatment females were either four-days old ds*Egfp*, ds*AedaeItp*, ds*AedaeItp-l* females (injected at one-day old), or non-dsRNA injected four-days old female mosquitoes that served as controls. Mosquito images were captured using a Zeiss Stemi 508 microscope with an Axiocam 208 color camera (Carl Zeiss, Jena, Germany).

Mating and egg-laying assay

A. aegypti mosquitoes were separated at the pupal stage and individually placed into a 24-well plate to allow adults to emerge. One-day old non-mated male and female adults were then isolated and injected as follows: 1) ds*AedaeItp* or ds*AedaeItp-l* knockdown females mated with virgin males, 2) ds*AedaeItp* or ds*AedaeItp-l* knockdown males mated with virgin females, and 3) ds*AedaeItp* or ds*AedaeItp-l* knockdown females mated with ds*AedaeItp* or ds*AedaeItp-l* knockdown males. For 1), ds*AedaeItp* or ds*AedaeItp-l* females were grouped with one-day old virgin males at a 1:2 ratio of female: male per insect box (BugDorm-5 insect box, MegaView Science Co. Taiwan) between 18 and 24 h after dsRNA injection. After four-five days post-mating, knockdown females were provided a bloodmeal. For 2), ds*AedaeItp* or ds*AedaeItp-l* males were mated with two-three-day old virgin females, and females were provided a bloodmeal 48 h post-mating. Lastly, for 3), ds*AedaeItp* or ds*AedaeItp-l* males and females were mated, and females were provided a bloodmeal four-five days post injection. For all blood feeding assays, females were given 20 min to blood feed, and blood fed females were subsequently isolated and weighed individually before being placed in an inverted 25 cm^2 cell culture flask (Corning) lined with filter paper containing 3 mL of distilled water (dH_2O) from larvae rearing containers to promote egg laying. Laid eggs were collected after 4 days and were semi-desiccated for 72 h and counted. Females were then removed and weighed before spermathecae were dissected and viewed under a microscope to confirm insemination. Eggs were placed in 40 mL of dH_2O with 1 mL larval food (1:1 ratio of 2% brewer's yeast and 2% liver powder), and hatched larvae (if any) were counted after 48 h.

Sperm quantification

Sperm quantification in the paired seminal vesicles (male sperm storage organs) and testes of male *A. aegypti*, along with the spermathecae (sperm storage organs) of female *A. aegypti* 4 days post dsRNA injection was performed following previously published protocols (29, 30). The seminal vesicles and testes from male along with spermathecae from female mosquitoes (9-11 mosquitoes per dsRNA mating treatment from 3-4 mating replicates) were placed in a 96-well plate with 100 µL PBS, and gently torn open using ultrafine forceps to release spermatozoa. An additional 10 µL PBS was used to rinse the forceps and the PBS with spermatozoa was mixed thoroughly using a P100 pipette. Five 1 µL droplets of the PBS/spermatozoa mixture were spotted onto a microscope slide (previously treated with poly-L-lysine to promote sperm attachment), allowed to air dry completely, and subsequently fixed with 70% ethanol. Slides were mounted using mounting media comprised of DPBS with 50% glycerol containing 4 µg/mL DAPI, and the nuclei of spermatozoa within each 1 µL

droplet was imaged under 4X magnification using an Olympus IX81 inverted microscope (Olympus Canada, Richmond Hill, ON, Canada). The nuclei of spermatozoa were counted in each 1 µL droplet, averaged across all five droplets for each animal, and multiplied by the dilution factor to determine total spermatozoa numbers within the seminal vesicle, testes, and spermatheca.

Statistical analyses

All graphs were created and statistical analyses performed using GraphPad Prism v9.1 (GraphPad Software, San Diego, CA, USA). Data was analyzed accordingly using an unpaired t-test or one-way or two-way ANOVA with the appropriate post-hoc test as indicated in each figure caption, with differences between treatments considered significant if $p<0.05$.

Results

Tissue/organ-specific expression profile of *Aedaeltp* and *Aedaeltp-l* transcripts

Post-embryonic stages and selected tissues/organs of mosquitoes were examined for *AedaeItp* and *AedaeItp-l* transcript expression and compared between males and females. Developmental expression profiling revealed enrichment of *AedaeItp* transcript abundance in adult stage mosquitoes (Figure 1A), with greatest and significant enrichment in one- and four-day old adult males compared to fourth instar larvae. In contrast, *AedaeItp-l* transcript was significantly enriched in late pupal and adult stage mosquitoes, including one-day old males and females (Figure 1B). Transcript abundance of *AedaeItp* was significantly higher compared to *AedaeItp-l* abundance in adult-stage mosquitoes, with over a three-fold higher abundance of *AedaeItp* transcript compared to *AedaeItp-l* (Figure 1C). Additionally, *AedaeItp* transcript abundance was exclusively and significantly enriched in the head (Figures 2A, C) and brain (Figures 2B, D) in both adult male and female mosquitoes. Comparatively, expression of *AedaeItp-l* transcript was significantly enriched in the carcass (Figures 2E, G) and abdominal ganglia (Figures 2F, H) in adult mosquitoes.

*Aedae*ITP- and *Aedae*ITP-L-like immunoreactivity in the central nervous system

Using whole mount immunohistochemistry, the central nervous system from one-day old male and female mosquitoes revealed *Aedae*ITP- and *Aedae*ITP-L-like immunostaining in three pairs of lateral neurosecretory cells in the medial anterior region of each brain hemisphere with axonal processes projecting anteriorly near an additional single pair of lateral neurosecretory cells (Figures 3A–E). A number of varicosities and blebs can be seen peripherally near the axonal projections, suggestive of release sites of these neuropeptides (Figures 3A–E). In abdominal ganglia 2-6,

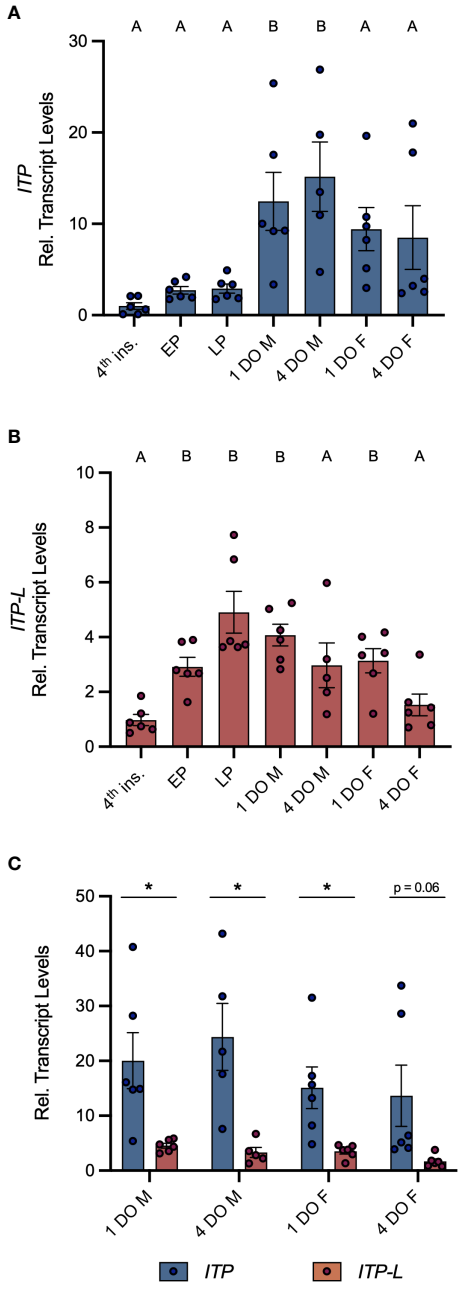

FIGURE 1
Developmental transcript expression profile of *Aedaeltp* and *Aedaeltp-l* in *A. aegypti*. Expression of *Aedaeltp* **(A)** and *Aedaeltp-l* **(B)** transcript was analyzed in post-embryonic stages of the mosquito shown relative to transcript levels in fourth instar larvae. Data labeled with different letters are significantly different from fourth instar larvae (mean ± SEM; one-way ANOVA with Dunnett's multiple comparison, $p<0.05$, n=5–6 biological replicates). **(C)** Comparison expression profile of *Aedaeltp* and *Aedaeltp-l* transcript levels in one and four day old adult male and female mosquitoes. Significant differences between *Aedaeltp* and *Aedaeltp-l* transcript levels are denoted by an asterisk (*) (mean ± SEM; unpaired t-tests, $p<0.05$, n=5–6 biological replicates). 4th ins, 4th instar larvae; EP, early pupa; LP, late pupa; M, male; F, female; DO, day old.

*Aedae*ITP- and *Aedae*ITP-L-like staining was observed in a single pair of neurosecretory cells, positioned laterally on either side of each ganglia (Figures 3F, G). The terminal ganglion, which is a

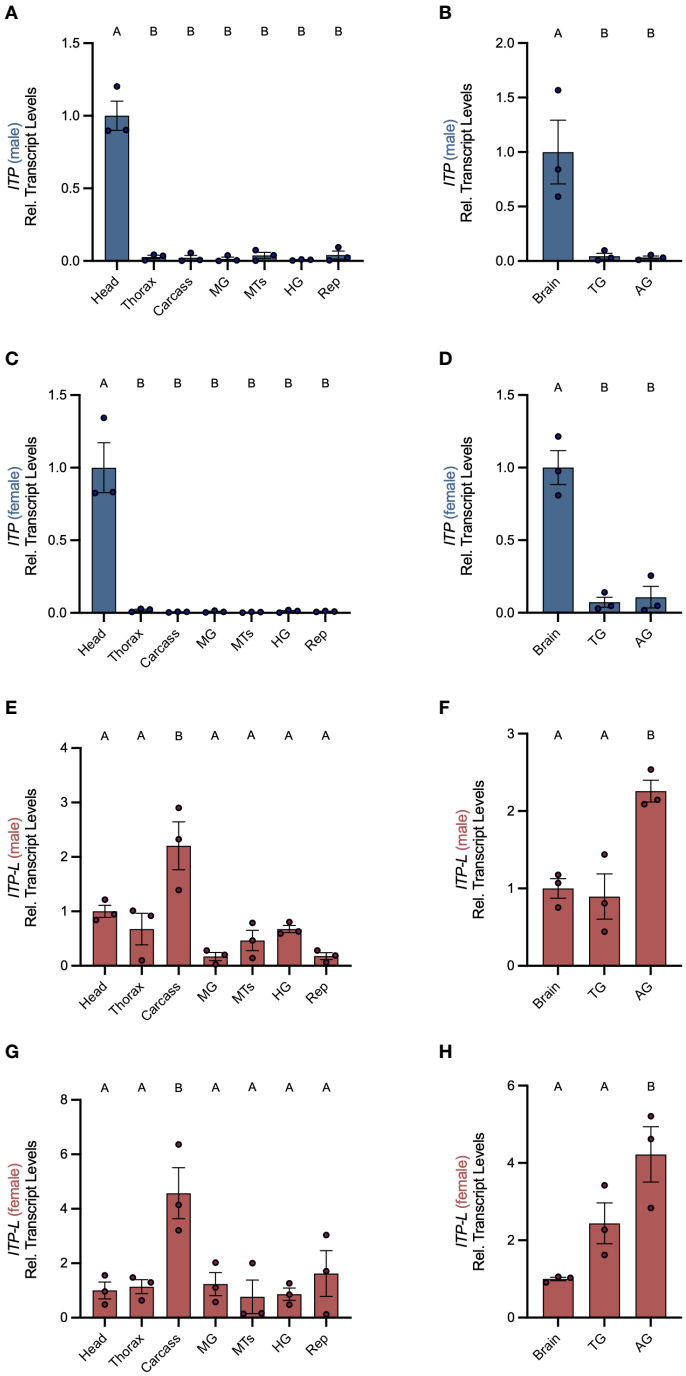

FIGURE 2
Spatial transcript expression profile of *Aedaeltp* and *Aedaeltp-l* in *A. aegypti*. Expression of *Aedaeltp* **(A–D)** and *Aedaeltp-l* **(E–H)** transcript levels were analyzed in various tissues/organs from one-day old adult males **(A, B, E, F)** and females **(C, D, G, H)** shown relative to transcript levels in the mosquito head/brain. MTs, Malpighian tubules; MG, midgut; HG, hindgut; Rep, reproductive tissues/organs; TG, thoracic ganglia; AG, abdominal ganglia. Bars labeled with different letters are significantly different from head/brain (mean ± SEM; one-way ANOVA with Dunnett's multiple comparison, $p<0.05$, n=3 biological replicates).

fusion of abdominal ganglia 7 and 8, revealed *Aedae*ITP- and *Aedae*ITP-L-like immunostaining in a single pair of neurosecretory cells located in the anterior region of the ganglion (corresponding to abdominal ganglia 7) (Figure 3H). Staining in these cells and projections were absent in control treatments where either the antiserum was preabsorbed with the ITP antigen or the omission of the primary antiserum (Supplementary Figure S1).

Roles of *Aedae*ITP and *Aedae*ITP-L in desiccation and starvation stress and blood feeding

Post emergence, one-day old adult male and female mosquitoes were placed in either a fed (provided a 10% sucrose meal), desiccated (no food or water provided), or starved (provided a

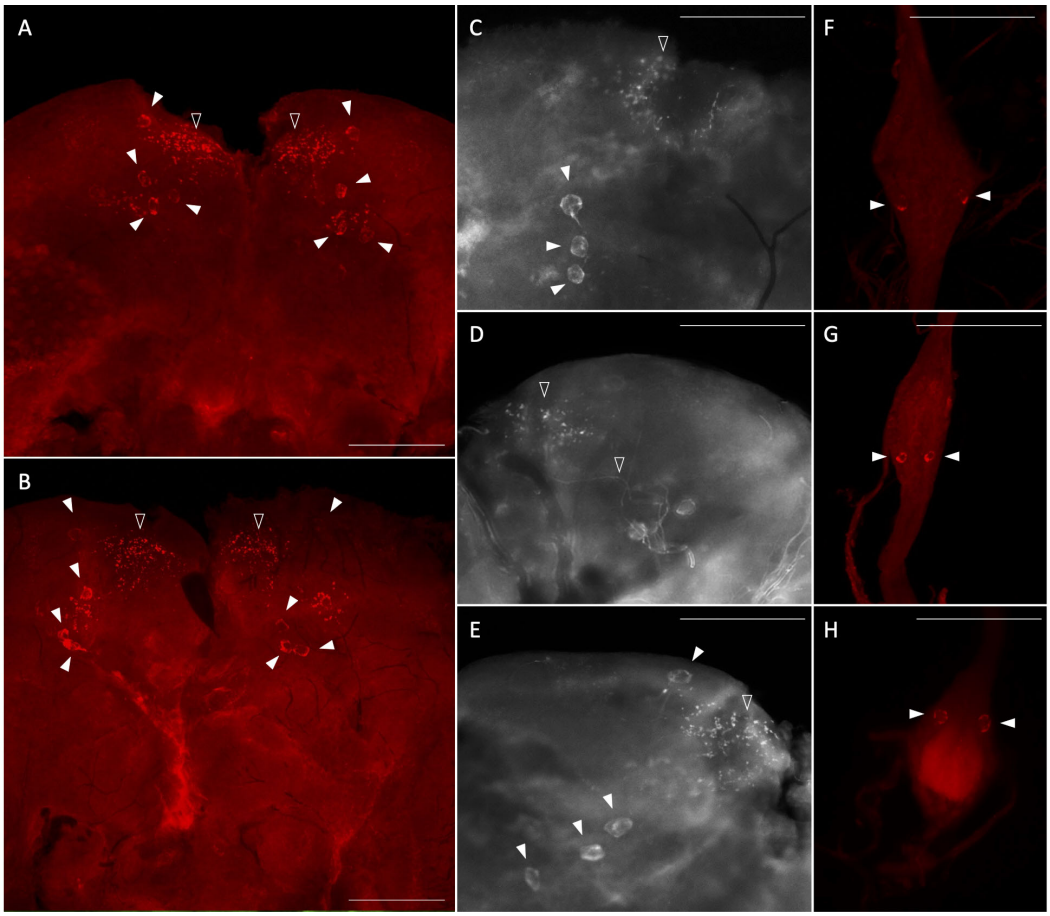

FIGURE 3
Immunolocalization of *Aedae*ITP and *Aedae*ITP-L in the central nervous system of the *A. aegypti* mosquito. *Aedae*ITP- and *Aedae*ITP-L-like immunoreactivity was examined in **(A)** male and **(B)** female brains, in four pairs of neurosecretory cells (indicated by white arrowheads), with axonal processes projecting anteriorly **(C–E)**, towards varicosities and blebs on the periphery of the brain (indicated by empty arrowheads). **(F, G)** Ventral view of abdominal ganglia showing a single pair of lateral neurosecretory cells and **(H)** an anterior pair observed in the terminal ganglion. Scale bars: **(A, B)** 200 µM; **(C–H)** 100 µM.

water meal, no sucrose) condition and isolated after 24 h or 48 h to examine a potential role of *Aedae*ITP and *Aedae*ITP-L in desiccation and starvation stress (Figure 4A). When adult male and female mosquitoes were placed in the starved or desiccated treatment for 24 h, there was no difference in mRNA abundance of either *AedaeItp* or *AedaeItp-l* compared to control fed conditions (Figures 4B, D, F, H). However, when the mosquitoes were subjected to these treatments for 48 h, there was a significant enrichment of both *AedaeItp* and *AedaeItp-l* transcript levels in the desiccated condition (ranging between ~1.75 and ~3.5-fold) compared to control fed animals (Figures 4C, E, G, I) while no change to the transcript levels in animals that were starved but provided with water. Next, to determine if *Aedae*ITP and/or *Aedae*ITP-L may play a role in relation to blood feeding, four- to six-day old adult female mosquitoes were provided a bloodmeal to examine whether a protein-rich meal influences the transcript abundance of *AedaeItp* and *AedaeItp-l* (Figure 5A). *AedaeItp* mRNA abundance did not change significantly compared to control, sucrose-fed females over any of the measured timepoints between 1 and 24 h post-blood feeding (Figure 5B) although abundance trended lower at the 1, 6 and 12 hr post-blood feeding timepoints. Similarly, *AedaeItp-l* mRNA abundance did not change significantly at any of the post blood-feeding timepoints in comparison to control, sucrose-fed females (Figure 5C).

dsRNA knockdown of *AedaeItp* and *AedaeItp-l* in adult *Aedes* mosquitoes

RNA interference of *AedaeItp* and *AedaeItp-l* expression was accomplished through dsRNA-injections of one-day old adult male and female mosquitoes (Supplementary Figure S3A). Given the conserved exons 1, 2, and 4 between *AedaeItp* and *AedaeItp-l*, the ds*AedaeItp* primers were designed over a common exon 2 resulting in the knockdown of both *AedaeItp* and *AedaeItp-l* transcripts. However, ds*AedaeItp-l* primers were designed over the unique exon 3 allowing knockdown of only the *AedaeItp-l* transcript. Relative to ds*Egfp*-injected control mosquitoes, *AedaeItp* and *AedaeItp-l* transcripts

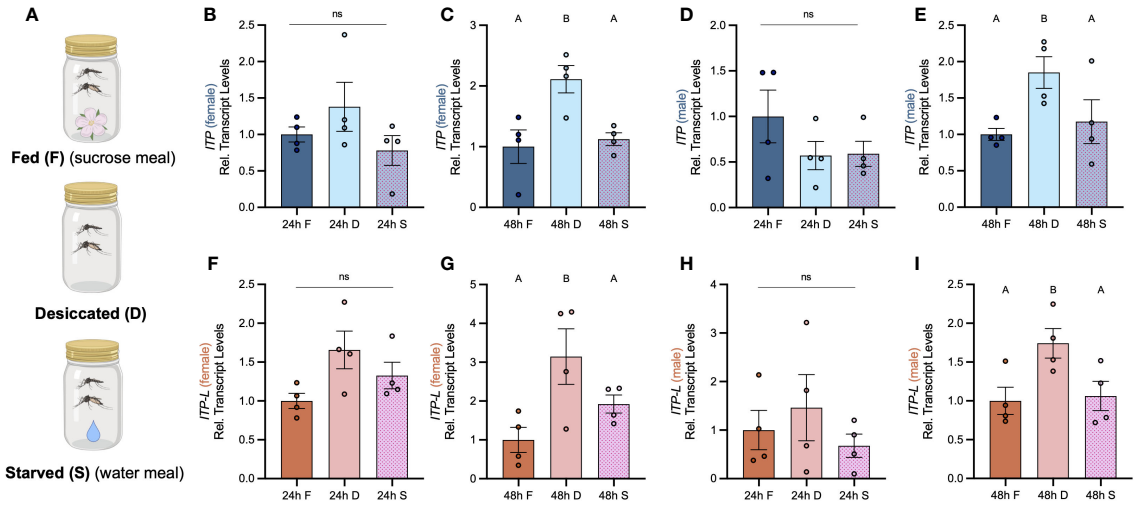

FIGURE 4
Effect of desiccation and starvation stress on transcript levels of *AedaeItp* and *AedaeItp-l* in whole body samples of adult *A. aegypti*. **(A)** Post-emergence, adult males were placed in a fed (sucrose meal provided), desiccated (no food or water provided), and starved (only water provided) condition for 24 h and 48 h, and abundance of **(B–E)** *AedaeItp* and **(F–I)** *AedaeItp-l* transcript were analyzed, shown relative to transcript levels in the control F, fed; D, desiccated; S, starved. Bars labeled with different letters are significantly different from the 24 h fed adult controls (mean ± SEM; one-way ANOVA with Bonferroni multiple comparison, $p<0.05$, $n=4$ biological replicates) and ns denotes no statistical significance.

were significantly reduced by ~75% in four-day old male (Supplementary Figure S3B) and female (Supplementary Figure S3C) mosquitoes injected with ds*AedaeItp*. Comparatively, ds*AedaeItp-l* treatment resulted in a significant decrease in *AedaeItp-l* transcript abundance by ~80% in males (Supplementary Figure S3B) and ~60% in females (Supplementary Figure S3C) in four-day old adults, whereas *AedaeItp* transcript abundance was unaffected. To confirm injection alone does not influence *AedaeItp* and *AedaeItp-l* transcript levels, four-day post-ds*Egfp* injected animals were compared to four-day old non-injected mosquitoes (Supplementary Figure S3D), with no significant changes in *AedaeItp* and *AedaeItp-l* transcript abundance in ds*Egfp*-injected males and females compared to non-injected mosquitoes. *AedaeItp* and *AedaeItp-l* transcript restored to normal levels within six and eight days post-injection in males and eight days post-injection in females (Supplementary Figures S3E–H).

dsRNA knockdown confirms *Aedae*ITP and *Aedae*ITP-L immunolocalization

To further confirm ds*AedaeItp*- and *AedaeItp-l* knockdown and differentiate between *Aedae*ITP and *Aedae*ITP-L immunolocalization, staining in the CNS was examined in mosquitoes four-days post-dsRNA injection. Wholemounts of ds*AedaeItp*-injected male and female mosquitoes showed no *Aedae*ITP- and *Aedae*ITP-L-like immunostaining in the brain (Supplementary Figures S4A, B), abdominal ganglia (Supplementary Figure S4C), and in the terminal ganglion (Supplementary Figure S4D). In contrast, ds*AedaeItp-l*-injected mosquitoes did not exhibit changes to immunostaining in the brain (Supplementary Figures S4E, F), with similar staining as described above (Figures 3A–E), and in control ds*Egfp*-injected mosquitoes (Supplementary Figures S5A, B). However, as expected,

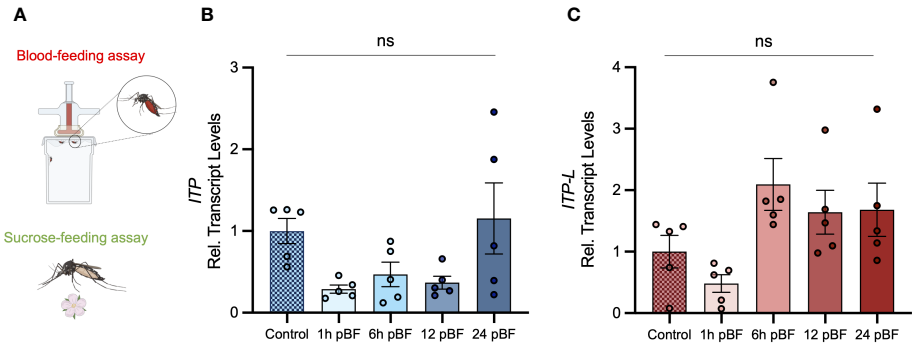

FIGURE 5
Effect of blood feeding on transcript levels of *AedaeItp* and *AedaeItp-l* in adult *A aegypti*. **(A)** Four- to six-day old adult females were blood fed and isolated at 1, 6, 12, and 24 post-blood feeding and **(B)** *AedaeItp* and **(C)** *AedaeItp-l* transcript levels were analyzed, shown relative to control, sucrose-fed females. pBF (post-blood feeding). No significance (ns) reflects comparisons with control, sucrose-fed females (mean ± SEM; one-way ANOVA with Tukey's multiple comparison, $p<0.05$, $n=5$ biological replicates).

knockdown of *AedaeItp-l* resulted in abolished staining of neurosecretory cells in the pre-terminal abdominal ganglia (Supplementary Figure S4G), and interestingly, no change to the immunostaining in the terminal ganglion (Supplementary Figure S4H), with a single pair of immunoreactive cells as described above (Figure 3H) and also observed in control ds*Egfp*-injected mosquitoes (Supplementary Figures S5C, D).

AedaeItp knockdown influences urine output in adult females

To determine if *Aedae*ITP and/or *Aedae*ITP-L influences ionoregulation and urine excretion, we volume loaded ds*AedaeItp* and ds*AedaeItp-l* injected females with saline and measured their urine output over two hours post volume loading. Four-day old HBS-injected females excreted a volume of 429.5 ± 53.49 nL of urine, which was significantly higher (~4-fold) compared to control four-day old non-HBS injected females (106.8 ± 15.39 nL) (Figure 6A). Notably, ds*Egfp* treated females excreted a similar volume of urine (391.6 ± 61.03 nL) compared to HBS-injected females. In contrast, ds*AedaeItp* mosquitoes injected with HBS secreted a significantly higher amount of urine, 972.7 ± 70.18 nL, approximately 2.5-fold higher compared to ds*Egfp*-injected females. Interestingly, no significant change in urine output was observed in ds*AedaeItp-l* females injected with HBS (604.2 ± 78.87 nL), compared to HBS loaded control (non-dsRNA injected) and ds*Egfp*-injected females. The overall effect of ds*AedaeItp* and ds*AedaeItp-l* injections on urine output was studied by examining abdomen distension immediately after and two hours post-HBS injection. Two hours post-volume loading, a less distended abdomen was observed in ds*AedaeItp*-injected females (Figures 6H, I) compared to control ds*Egfp*- and HBS-injected females (Figures 6B–G). Comparatively, a moderately distended abdomen was observed in ds*AedaeItp-l*-injected females (Figures 6J, K) two hours post saline-injection.

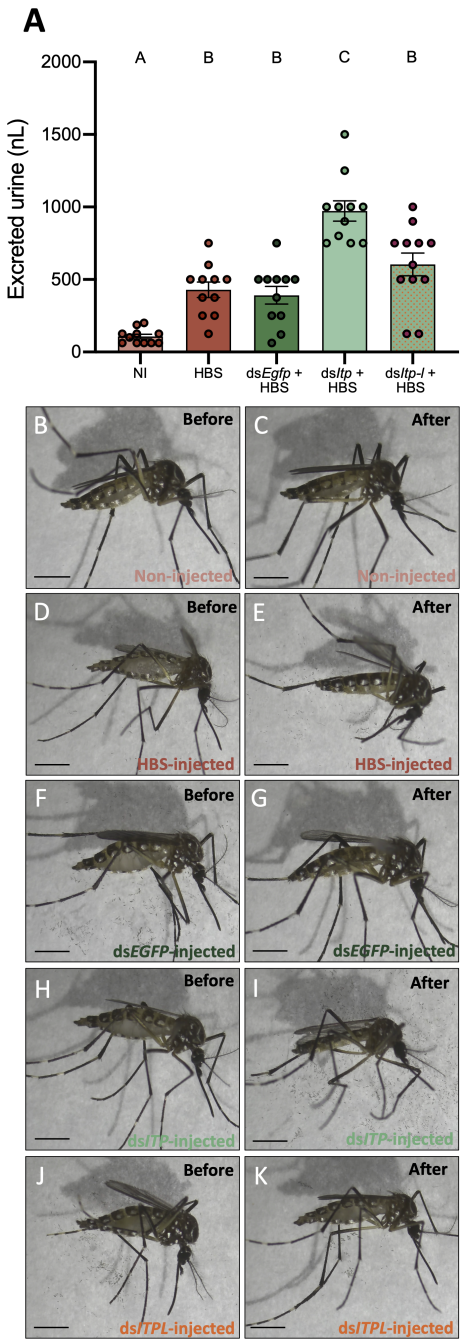

FIGURE 6
Effect of *AedaeItp* and *AedaeItp-l* knockdown on urine excretion in adult female *A. aegypti*. **(A)** Four-day old females were injected with 500 nL of a HEPES buffered saline (HBS) and allowed to excrete for two hours. Bars labeled with different letters are significantly different from each other including non-injected (NI) animals (mean ± SEM; one-way ANOVA with Bonferroni multiple comparison, $p<0.05$, n=11–12). **(B–K)** Images of female mosquitoes immediately following and two hours post-HBS injection. Scale bars: 1 mm.

AedaeItp and *AedaeItp-l* knockdown influences male and female reproductive success

To assess the roles of *Aedae*ITP and *Aedae*ITP-L in reproductive behavior and physiology, dsRNA-injected virgin mosquitoes were placed into one of the following mating combinations: 1a) ds*AedaeItp* or 1b) ds*AedaeItp-l* females mated with control, non-dsRNA males, 2a) ds*AedaeItp* or 2b) ds*AedaeItp-l* males mated with control, non-dsRNA females, and 3a) ds*AedaeItp* or 3b) ds*AedaeItp-l* females mated with ds*AedaeItp* and ds*AedaeItp-l* males. When ds*AedaeItp* females were mated with normal males, there was a significant reduction in the incidence of blood feeding (Figure 7A), reduced bloodmeal engorged by the female (Figure 7B), reduction in the number of eggs laid (Figure 7C), and an overall reduction in the percentage of larvae hatching per female (Figure 7D) was observed. More specifically, there was a ~90% reduction in preference for blood feeding by ds*AedaeItp* females and ~75% reduction in ds*AedaeItp-l* females (Figure 7A) with both treatments leading to a reduction in the bloodmeal volume imbibed (Figure 7B). Females treated with ds*AedaeItp-l* oviposited a similar number of eggs and had a comparable percentage of hatched larvae as control ds*Egfp*-females while ds*AedaeItp* caused a drastic reduction (~50%) in eggs oviposited

by females and a more dramatic impact on larval hatching success (Figures 7C, D). In males injected with either ds*AedaeItp* or ds*AedaeItp-l* and mated with normal females, this did not influence female preference for blood feeding or the volume of blood imbibed (Figures 7E, F). However, normal females mated with ds*AedaeItp* and ds*AedaeItp-l* injected males had significantly reduced number of eggs oviposited (Figure 7G), as well as a reduced percentage of viable eggs and larvae hatching per female (Figure 7H). Interestingly, ds*AedaeItp* and ds*AedaeItp-l* females mated with ds*AedaeItp* and ds*AedaeItp-l* males almost completely abolished the preference for blood feeding (Figure 7I) with the few that did blood feed imbibing a significantly lower blood volume (Figure 7J), with almost no eggs laid by the female (Figure 7K), and complete absence of larval hatching (Figure 7L). Notably, the reduced preference for blood feeding limited the number of blood fed females used for subsequent studies. No major changes were observed in the weight of blood fed females post-egg laying (4 days post-feeding) in any of the treatment regimens (Figures 7B, F, J), indicating long term volume balance is not impacted by knockdown of *AedaeItp* or *AedaeItp-l* although there was a small (but significant) increase in the weight of *AedaeItp-l* injected females (Figure 7B). Mated males and females injected with control ds*Egfp* had similar preference for blood feeding, weight of blood fed females, produced similar number of eggs and comparable larval hatching as non-injected females (Supplementary Figure S6).

AedaeItp and *AedaeItp-l* knockdown reduces spermatozoa count

In light of the results above, it supports the notion that knockdown of *AedaeItp* and *AedaeItp-l* may have a distinct role in male reproductive biology separate from their effects on females since pairings between normal females and knockdown males revealed females had normal preference for blood feeding as well as bloodmeal weight; however, oviposition rates by females and larval hatching rates were significantly reduced. Considering the overall reduced preference for blood feeding in ds*AedaeItp* and

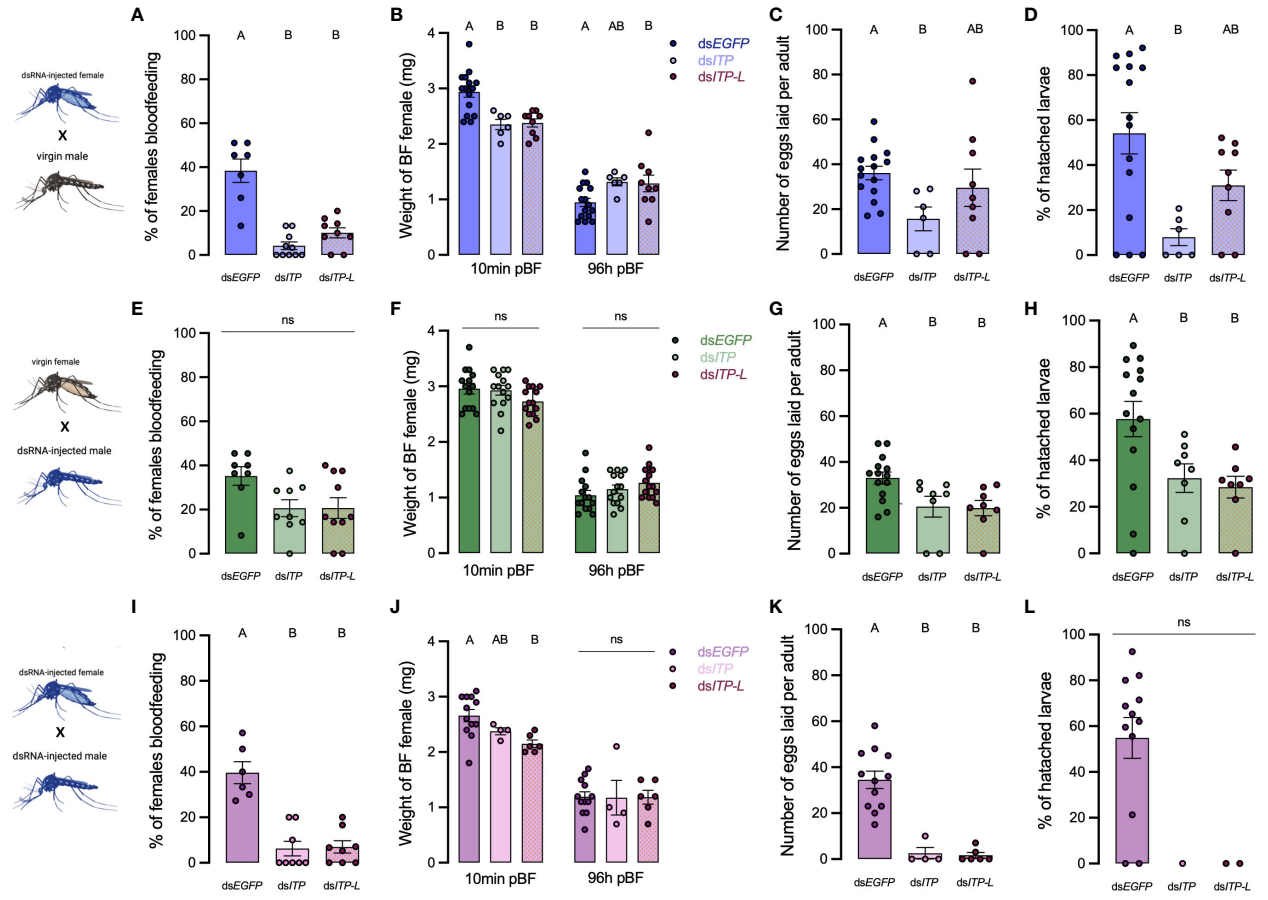

FIGURE 7
Effect of *Aedaeltp* and *Aedaeltp-l* knockdown on blood-feeding, egg laying, and larval hatching (egg viability) in adult *A. aegypti*. **(A–D)** ds*Aedaeltp* or ds*Aedaeltp-l* females mated with normal males, **(E–H)** ds*Aedaeltp* or ds*Aedaeltp-l* males mated with normal females, and **(I–L)** ds*Aedaeltp* or ds*Aedaeltp-l* females mated with ds*Aedaeltp* or ds*Aedaeltp-l* males. The effect of ds*Aedaeltp* or ds*Aedaeltp-l* knockdown was tested on **(A, E, I)** preference for blood feeding, **(B, F, J)** weight of blood fed female before and after egg collection, **(C, G, K)** number of eggs oviposited, and **(D, H, L)** percentage of larval hatching. Bars labeled with different letters are significantly different from control, ds*Egfp* injected age/time-matched adults (mean ± SEM; one-way ANOVA with Bonferroni multiple comparison, $p<0.05$, [**(A, E, I)**, n=6–11 mating replicates, each point represents individual replicate values] [**(B–D, F–H, J, K)**, n=1–15, each point represents data from individual females], (ns denotes no statistical significance).

ds*AedaeItp-l* females, we speculated that *Aedae*ITP and *Aedae*ITP-L may play an essential role in spermatozoa production and release. Consequently, sperm was collected separately from the testes and seminal vesicles of four-day old ds*AedaeItp*- and ds*AedaeItp-l*-injected males (from all three mating conditions described above) and spermathecae of four-day old ds*AedaeItp*- and *AedaeItp-l*-injected females (again, from all three mating conditions noted above), and the quantity of mature spermatozoa was compared to ds*Egfp*-injected animals (Figures 8A, B). For spermatozoa collected from the seminal vesicles, ds*AedaeItp*- and ds*AedaeItp-l*-injected females mated with normal males produced similar mature spermatozoa counts compared to control ds*Egfp* animals (Figure 8C, Supplementary Figures S7A, B, E). Interestingly, knockdown resulted in significant reductions in the number of spermatozoa in the seminal vesicle, from both ds*AedaeItp*- and ds*AedaeItp-l*-injected males mated with control females and when mated with ds*AedaeItp*- and ds*AedaeItp-l* injected females (Figure 8C, Supplementary Figures S7C, D, F, G). Similar trends were observed for the number of spermatozoa collected directly from the male testes (Figure 8C, Supplementary Figures S7H–N). Comparatively, *AedaeItp*- and *AedaeItp-l* knockdown resulted in an ~85% reduction in mature spermatozoa counts in the spermatheca in all three mating conditions (Figure 8C, Supplementary Figures S7O–U). Animals injected with ds*Egfp* resulted in similar number of spermatozoa in the testes, seminal vesicles, and spermathecae compared to non-injected animals (Supplementary Figure S8).

Discussion

ITP and ITP-L belong to the CHH family of neuropeptides and have been functionally characterized in many insect species (6, 7, 11, 13, 15, 22, 23, 31). However, while several studies have examined ITP signaling pathways in insects, ITP/ITP-L receptors have generally not been identified and characterized thus far, except for in the domestic silk moth *B. mori* (6). In a recent study using *Drosophila melanogaster* as a conserved tumor model, isoform F of ion transport peptide (ITPF) was found to be secreted by gut tumor cells and acts as an antidiuretic hormone targeting the tachykinin receptor (TkR99D) in Malpighian tubules leading to compromised renal function that results in the accumulation of excess fluid (32). This latest report is an intriguing considering tachykinins have been reported as diuretic factors in a number of insects (see 33) including recently in *D. melanogaster* (34).

In the present study, the *A. aegypti* ITP and ITP-L peptides have been localized, confirming the distribution of these peptides in neurosecretory cells and processes within the mosquito central nervous system. Additionally, prospective physiological functions

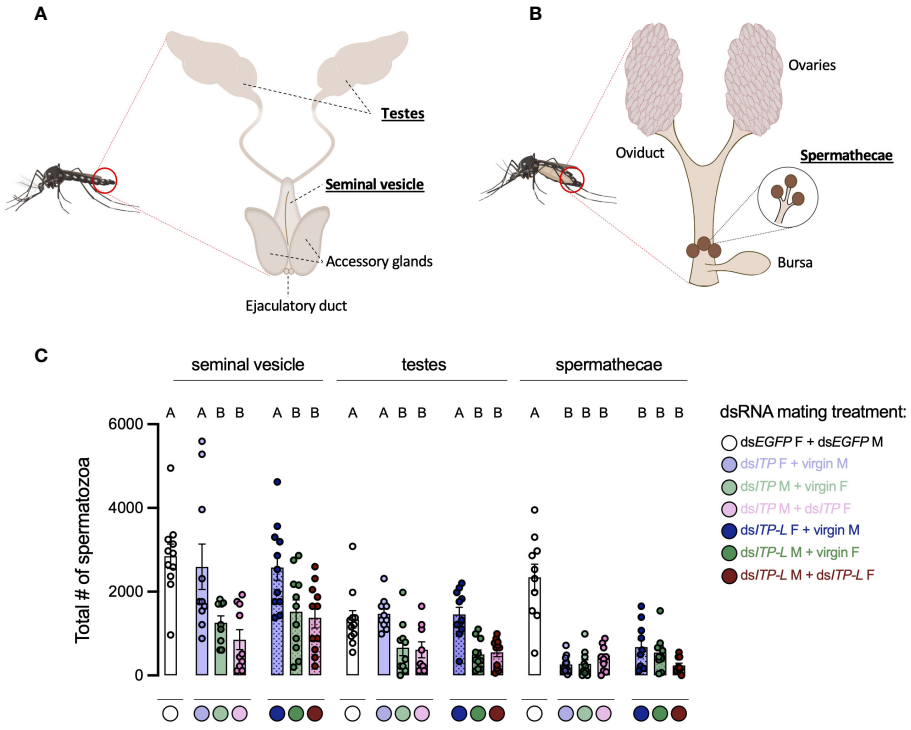

FIGURE 8
Total number of spermatozoa in the male testes and seminal vesicle along with female spermathecae of adult *A. aegypti* following RNAi (dsRNA)-mediated knockdown of *Aedaeltp* or *Aedaeltp-l*. Schematic diagram showing the **(A)** male and **(B)** female reproductive system of the adult mosquito. **(C)** Total spermatozoa number within the male testes and seminal vesicle, and female spermathecae of adults four-days after ds*Aedaeltp* or ds*Aedaeltp-l* injection. M, male; F, female. Bars labeled with different letters are significantly different from the organ-specific (seminal vesicle, testes and spermathecae) number of spermatozoa in control, ds*Egfp* injected adults (mean ± SEM; one-way ANOVA with Bonferroni multiple comparison, $p<0.05$, n=9–11, each point represents individual replicate values).

have been investigated for *Aedae*ITP and *Aedae*ITP-L including roles in feeding, urine output, and reproductive success of adult male and female mosquitoes. This is the first report that examines the distribution, localization, and physiological function of the ITP/ITP-L signaling system in *A. aegypti* mosquitoes.

Distribution pattern of *Aedae*ITP and *Aedae*ITP-L in the CNS

Expression profiles of transcripts encoding *A. aegypti* ITP and ITP-L were measured to reveal potential functional or sex-specific roles for these peptides. Examination of the developmental and tissue-specific expression profile revealed enrichment of *AedaeItp* and *AedaeItp-l* in both one- and four-day old male and females, with significant enrichment of *AedaeItp* in the brain and *AedaeItp-l* in the abdominal ganglia. The expression and distribution of ITP and ITP-L in the central and peripheral nervous system has been examined in numerous insects (9, 18, 22, 23). While *Itp* mRNA expression has been detected only in the nervous system, evidence has suggested *Itp-l* expression in peripheral tissues as well. In *T. castaneum*, *Itp-l* transcript expression was found to be highly expressed in the midgut (13), and in the Malpighian tubules and hindgut in *S. gregaria* (18).

Previous studies in *M. sexta* revealed that *Mas*ITP and *Mas*ITPL are differentially expressed in mainly nonoverlapping populations of central and peripheral neurons, which includes neuronal projections from the CNS (9). RT-PCR, immunohistochemistry, and *in situ* hybridization studies indicated expression of *Mas*ITP exclusively in the brain where it was localized to two neuron types; in type Ia_2 neurosecretory cells, with axonal projections to the retrocerebral complex (35–38) and in small neurons adjacent to type Ia_2 cells, established as interneurons since their projections remain within the protocerebrum (9). Thus, in *M. sexta*, it was suggested that ITP is released as a neurohormone from type Ia_2 cells into the hemolymph, whereas ITP produced in the small interneurons may serve transmitter or modulatory functions in the brain (9). Similarly in *S. gregaria*, ITP is believed to be synthesized in neurosecretory cells of the pars intercerebralis of the brain where it is then transported for storage and eventual release in the corpora cardiaca (18). The existence of ITP-L transcripts was first reported in *S. gregaria* (39), while the mature peptide was identified by Dai et al. (9) demonstrating ITP-L transcript and peptide distribution in the central and peripheral nervous system of insects, including *B. mori*, *M. sexta*, and the grasshopper, *Schistocerca americana*. Relatively weak ITP-L immunoreactivity was observed in the brain type Ia_2 cells but was found to be completely absent in axons and terminals within the retrocerebral complex (9). ITP-L peptides are abundant in the ventral ganglia, flight muscle, MTs, and ileal tissues, indicating a possible distinctive function from ITP (17, 18).

Through RNAi-mediated knockdown, we confirmed *Aedae*ITP immunoreactivity in at least four pairs of neurosecretory cells in the anterior region of the protocerebrum and in a single pair of lateral neurosecretory cells in the terminal ganglion. In contrast, *Aedae*ITP-L immunoreactivity was observed in one pair of lateral neurosecretory cells in each abdominal ganglia of the ventral nerve cord. In general, expression patterns of *AedaeItp* and *AedaeItp-l* were similar to those described in *M. sexta*, *T. castaneum*, *B. mori*, and *D. melanogaster* (9, 13, 20, 22). In *T. castaneum*, *Itp* expression was in five pairs of brain cells on the dorsal side of the protocerebral hemispheres and in a pair of cells in the abdominal terminal ganglion (13). Similarly, ITP expression was observed in four pairs of brain cells in *D. melanogaster*. While still inconclusive, previous immunohistochemical studies in other insects allude to protocerebral cells with projections to the corpora cardiaca and allata, and the cells in the terminal abdominal ganglion may have projections to the hindgut (22) and possibly to reproductive organs (13), which provide insight on novel functions for the ITP/ITP-L signaling system. Given the immunoreactivity of *Aedae*ITP in the terminal ganglion, this suggests potential iono-regulatory roles in the hindgut, acting possibly as an anti-diuretic hormone to increase water or ion reabsorption, similar to activity seen in the desert locust (12, 16).

Roles of ITP and ITP-L in feeding and urine excretion

The challenges of osmotic and ionic regulation vary between distinct environmental conditions that the adult mosquito might encounter. In desiccating environments, insects must safeguard water balance and reduce the rate of water loss (40). The current findings reveal an increase in both *AedaeItp* and *AedaeItp-l* transcript levels after 48 hours of combined desiccation and starvation stress in both adult male and female mosquitoes, which was not observed in animals undergoing starvation stress alone. These findings corroborate data reported in *Drosophila*, where ITP was linked as a natural component of desiccation and osmotic stress responses, since both stressors triggered an increase in *Itp* expression, while ITP knockdown reduced survival under desiccation and osmotic stress (15). *Droso*ITP plays roles in hunger, thirst, and excretion in *Drosophila* suggesting that ITP-regulated changes to physiology and behavior represent critical insect responses to cope with reduction in body water (15).

The discovery of the first anti-diuretic hormone mediating its effects on the insect hindgut was described by Audsley et al. (12) when ITP was purified from the corpora cardiaca of the locust, *S. gregaria*. A conserved *Itp* gene was later uncovered in the genome of the mosquito, *A. aegypti*, raising the prospect for a similar role in maintaining iono- and osmo-regulation (9). *A. aegypti* mosquitoes are reliant on an efficient excretory system comprised of the MTs and hindgut (1), functioning to counter disturbances to their hemolymph. The MTs, which are functional analogs of vertebrate kidneys, are responsible for the formation of primary urine (1), driven by the V-type H^+-ATPase (41) that permits transport of Na^+ and K^+ cations across the membrane (42) via a putative H^+/cation exchanger (43). The MTs are regulated by various diuretic and anti-diuretic hormones, which in *A. aegypti* includes the biogenic amine 5-hydroxytryptamine (5HT) (44, 45), DH_{31} (46), DH_{44} (47, 48), kinin-like peptides (49, 50) and CAPA that inhibits the activity of select diuretic hormones (25, 51, 52). The primary urine then enters

the hindgut, where it is further modified through secretory and reabsorptive processes. Here, we show that *Itp* knockdown (but not *Itp-l* knockdown) leads to increased excretion of urine, supporting a possible anti-diuretic role for *Aedae*ITP. Thus, it suggests that *Aedae*ITP promotes water reabsorption in the hindgut similar to mechanisms described in *S. gregaria* where *Schgr*ITP was found to stimulate chloride-dependent water reabsorption in the ileum, promoting an increase in Na^+, K^+ and Cl^- transport (12, 16). Interestingly, while *Schgr*ITP promotes a reabsorptive role on ileal tissue, ITP-L did not display any stimulatory effect, instead inhibiting the stimulatory effect of synthetic ITP (21). A key step to understanding the ITP and ITP-L actions in the *Aedes* mosquito is to identify the as yet unknown *A. aegypti* ITP receptor. The first presumed receptors for ITP and ITPL were characterized in the silkworm *Bombyx mori* (6). Specifically, Nagai et al. (6) identified three *B. mori* orphan GPCRs as receptors for ITP and ITP-L, that all responded to recombinant ITP, with elevating levels of intracellular cGMP upon receptor binding (6), which support the suggested ITP's mode of action on ileal ion transport involving this second messenger in *S. gregaria* (53). In the locust, *Schgr*ITP is proposed to bind to two different receptors, a G-protein coupled receptor and a membrane bound guanylate cyclase, on the ileal basolateral membrane, increasing both cyclic GMP (cGMP) and cyclic AMP (cAMP) levels, to regulate ion and fluid transport (53). cGMP stimulates Cl^- reabsorption and H^+ secretion across the ileum, whereas cAMP stimulates Na^+, K^+, and Cl^- reabsorption (53). To further understand how these second messengers facilitate the physiological actions of *Schgr*ITP on the ileum will require the endogenous ITP receptor(s) to be characterized.

Role of ITP and ITP-L in reproductive behavior and success

Female *A. aegypti* are day-biting mosquitoes, taking a single or multiple bloodmeals to obtain vitamins, proteins, minerals and other nutrients for egg development (42). Transcript levels of *AedaeItp-l* and *AedaeItp* levels remained unchanged over the time points we examined post blood feeding. Nonetheless, to elucidate whether ITP and ITP-L signaling might be involved in mosquito reproductive biology, RNAi was utilized to knockdown expression of *Itp* and *Itp-l* in adult *A. aegypti.* Overall, *AedaeItp* and *AedaeItp-l* knockdown female mosquitoes had a lower preference for blood feeding, laid fewer eggs, and had significantly reduced larval hatching. In *T. castaneum*, ITP and ITP-L are required throughout all life stages and are essential for reproduction and offspring survival (13). Knockdown of both ITP and ITP-L resulted in dramatic decreases in egg numbers and in survival of eggs, with reduced ovaries that lack mature ovarioles in the ITPL knockdown females (13). In contrast, ITP knockdown females had fully developed ovaries, however showed reduced oviposition rates and offspring survival. These developmental defects in *T. castaneum* were suggested to be due to hormonal imbalance in ovarian development, or indirectly caused by mating deficiencies, preventing exposure to male ejaculatory products essential for completion of ovarian development (13).

Considering the finding that *AedaeItp* and *Itp-l* knockdown mosquitoes resulted in fewer eggs laid by females mated with knockdown males, we predicted that transfer of sperm or sperm storage may be targeted. The regulation and entry of sperm into, protection within, and release from the storage organs (seminal vesicle in males, and spermathecae in females) requires both male and female-derived molecules (54–56). In male mosquitoes, spermatogenesis occurs in the paired testes, allowing for mature sperm cells, spermatozoa, to be synthesized (24, 29) and transported to the seminal vesicles via the vas deferens (57–59). During mating, male *A. aegypti* deposit sperm from the seminal vesicles into the female reproductive tract initially in the bursa and is later transferred into the spermathecae for long-term storage (60, 61). Herein, the results revealed lower spermatozoa counts in testes and seminal vesicles of males with *Itp* and *Itp-l* knockdown. Thus, this indicates that *Aedae*ITP/ITP-L knockdown reduces the incidence of blood feeding by females, and additionally, knockdown reduces the number of spermatozoa in male seminal vesicles and in the spermathecae of females, which ultimately results in fewer eggs laid and reduced larval hatching. It remains to be investigated exactly what role *Aedae*ITP and ITP-L play in male and female reproduction or in blood feeding behavior. However, given the already established roles of these neuropeptides in *T. castaneum* reproduction (13), it is possible that *Aedae*ITP and ITP-L are similarly involved in regulation of male or female reproductive biology or mating behavior, influencing successful mating and transfer of spermatozoa into the female *A. aegypti*. Indeed ITP and ITP-L are multifunctional neuropeptides involved in metabolism, regulation of water and ion homeostasis, cuticle expansion and melanization, and reproduction (13, 15, 20, 23). The role in reproduction has been supported by ITPL expression in the *B. mori* male reproductive system with innervations of the accessory glands, the seminal vesicles, and the ejaculatory ducts (62), all organs critical for successful mating and reproduction. ITPL expression has also been found in the seminal fluid of the brown plant hopper, *Nilaparvata lugens* (23), which is transferred to females during mating. Future research examining *Aedae*ITP and ITP-L signaling can provide greater insight on the actions of these neuropeptides in mosquito reproductive biology.

ITP and ITP-L peptides are highly homologous to the CHH peptides, which has been linked to molting, energy metabolism, immune defense, reproduction, and homeostatic regulation of osmotic and other stress responses (7, 63). In locusts, ITP stimulates fluid reabsorption and Cl^-, Na^+, and K^+ transport, while inhibiting secretion of H^+ in the ileum (16, 18). In *Drosophila*, ITP plays an essential role in development and locomotion (14, 15), and water homeostasis by protecting the fly from water loss by increasing thirst, reducing excretion rate, and promoting ingestion of water (15). Studies have also established functions of ITP during ecdysis in *M. sexta* (11) and wing expansion in *N. lugens* (23), while ITP-L has been linked to ovarian maturation in *T. castaneum* (13) and produced as a seminal fluid protein in *N. lugens* (23). Differences in the primary structure and cellular distribution patterns of ITP and ITP-L peptides suggest they may serve different biological functions. In conclusion, the current results expand our understanding of ITP and ITP-L in insects,

providing evidence of differential expression, insight into their cell-specific distribution, and revealing novel independent physiological roles for these neuropeptides in the *A. aegypti* mosquito. Importantly, these findings also contribute towards our understanding of *A. aegypti* reproductive biology, which is of medical importance given their propensity of feeding on human hosts and role as a vector of several viruses. As such, given these neuropeptides appear to hold pleiotropic actions related to successful mating and reproduction, further insights into ITP and ITP-L signaling could contribute towards development of novel strategies for decreasing the fitness of these vectors, that may improve control of these anthropophilic mosquitoes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://borealisdata.ca/dataverse/PaluzziLab, N/A.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

FS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. J-PP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/finsc.2024.1374325/full#supplementary-material

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Protein localization of aquaporins in the adult female disease vector mosquito, *Aedes aegypti*

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The female *Aedes aegypti* mosquito is a vector for several arboviral diseases, due to their blood feeding behavior and their association with urban communities. While ion transport in *Ae. aegypti* has been studied, much less is known about mechanisms of water transport. Rapid water and ion excretion occurs in the adult female mosquito post blood meal and involves a set of organs including the midgut, Malpighian tubules (MTs), and hindgut. The MTs are responsible for the formation of primary urine and are considered the most important site for active transport of ions. Within the cells of the MTs, along with various ion transporters, there are aquaporin water channels that aid in the transport of water across the tubule cell membrane. Six aquaporin genes have been molecularly identified in *Ae. aegypti* (AQP1–6) and found to be responsible for the transport of water and in some cases, small solutes such as glycerol. In this study, we used immunohistochemistry to localize AaAQP1, 2, 4, 5, and 6 in the adult female *Ae. aegypti*, in non-blood fed and post blood feeding (0.5 and 24hr) conditions. We further examined the main water transporting aquaporin, AaAQP1, using western blotting to determine protein abundance changes in isolated MTs pre- and post-blood feeding. Using fluorescence *in situ* hybridization, *aqp1* mRNA was found exclusively in the principal cells of female MTs. Finally, we used immunogold staining with transmission electron microscopy to determine subcellular localization of AaAQP1 in the Malpighian tubules under non-blood fed conditions. Interestingly, AaAQP1 was found to be predominantly in the principal cells of the MTs, dispersed throughout the brush border; however, there was also evidence of some AaAQP1 localization in the stellate cells of the MTs.

KEYWORDS

water, osmoregulation, transport, protein expression, blood feeding

Introduction

The mosquito, *Aedes aegypti* is responsible for the spread of deadly arboviral diseases such as Zika virus (1), Chikungunya (2), yellow fever (3), and dengue fever (4). Adult *Ae.*

aegypti are terrestrial and feed on plant nectar for essential nutrients. Females also obtain a blood meal from vertebrate hosts to utilize the protein for egg maturation (5). Upon the initiation of blood feeding, female mosquitoes intake a large quantity of water and ions, which must be dealt with quickly. Rapid excretion of water and ions, such as Na^+ and K^+, has been shown to begin before a female *Ae. aegypti* has finished taking a blood meal (6, 7). Insects, including *Ae. aegypti*, have specialized organs that are responsible for osmoregulation and rapid urine excretion, namely the Malpighian tubules (MTs) and the hindgut. The MTs are comprised of two main epithelial cell types; the more abundant principal cells and the intercalated stellate cells that together are responsible for the production of primary urine, which is driven by an apical V-type H^+-ATPase (VA) (8). Specifically, the VA maintains a proton gradient that is needed for secondary active transport of ions such as Na^+ and K^+ into the tubule lumen, in exchange for protons through cation proton antiporters expressed on the apical membrane of both cell types (9–11). In addition to the MTs, the posterior region of the hindgut (ie. rectum) is responsible for reabsorption of water and ions before final waste excretion (12). The excretion of ions has been studied extensively, however there is much less known about the movement of water by insect excretory organs, including the MTs.

Aquaporins are transmembrane proteins that form selective channels for water and some solutes such as glycerol and trehalose (13), that make it possible for the transcellular flow of water to occur. In *A. aegypti*, there have been six aquaporin genes identified [AaAQP1–6] (13–15). In this study, the AQP nomenclature used follows that of the Hansen lab (Table 1) (16). Functional characterization in a heterologous system revealed AaAQP 1, 2, and 5 allow significant water permeability (13) as does AaAQP6 (17), while AaAQP 4 and 5 have been identified as entomoglyceroporins, able to transport some solutes across the cell membrane (13). Sequencing (14), heterologous expression data (13), and gene replacement data (18) suggest that AaAQP4 and AaAQP5 have a different amino acid composition resulting in a larger pore diameter, allowing the transport of solutes like glycerol, urea, erythritol, adonitol, mannitol, and trehalose in addition to water. AaAQP5 has a comparatively high permeability to water, similar to AaAQP1, which is significantly higher than AaAQP4 that is a poor water transporter (13).

AaAQP1, 2, 4, 5, and 6 mRNA has been identified in the MTs of non-blood fed females (16). A blood meal in female *Ae. aegypti* increases mRNA levels of AaAQP1, 4, and 5 in the MTs, specifically between 3–48hr post-blood meal (13, 16). Previous work done in larval *Ae. aegypti* established the localization of AaAQPs throughout the alimentary canal with abundance of AaAQP1, 4, and 5 shown in the MTs (19), as well as in adult *Ae. aegypti* where AaAQP1 was localized to the tracheolar cells (20). To date, there has been no comprehensive characterization of AQPs in the adult *Ae. aegypti* mosquito; particularly at the protein level. In addition, identifying mechanisms by which AQPs in *Ae. aegypti* are regulated is important for understanding how a blood meal in female *Ae. aegypti* may affect localization, abundance and ultimately the function of AaAQPs. In *Ae. aegypti*, control and modification of MT function involves circulating hormones, including neuropeptides (21). The rate of fluid secretion by MTs increases with application of the neuropeptide, diuretic hormone 31 (DH_{31}) and decreases with application of the anti-diuretic hormone, CAPA (21). The actions of DH_{31} and CAPA are mediated by intracellular signaling that involves assembly and disassembly of the VA in the apical membrane of principal cells (22). Through the control of fluid secretion by the MTs, AaAQP regulation is also possible, however there have been limited studies on invertebrate AQP regulation. It has been proposed that AQPs can be phosphorylated during periods of stress, in addition to the possibility that they are packaged into membrane vesicles on demand, for example during diuresis (23). The goal of this study was to provide a deeper understanding of the localization of AQPs in the adult female *Ae. aegypti* and to gain better insight on AaAQP1 expression and regulation before and after blood feeding by female mosquitoes.

Materials and methods

Mosquito rearing

Aedes aegypti eggs (Liverpool) were gathered from a long-standing colony reared at York University in Toronto, Ontario Canada. Filter paper was placed in small cups filled with ddH_2O, where adult females were able to lay their eggs at the surface of the water. Females were fed twice weekly with sheep's blood in Alsever's solution (Cedarlane Laboratories, Burlington, Ontario Canada)

TABLE 1 Sizes and functional characteristics of aquaporins examined in the current study found in *Aedes aegypti* along with their orthologs in the African malaria vector mosquito, *Anopheles gambiae*, and the fruit fly, *Drosophila melanogaster*.

Name in *Aedes aegypti*	Accession Number	Length	Putative AQP Function	*Anopheles gambiae* Homolog	*Drosophila melanogaster* Homolog
AaAQP1	XP_001656931	249	Water-selective AQP	XP_319584	DRIP – CG9023
AaAQP2	XP_001649747	264	Water-selective AQP	XP_319585	PRIP – CG7777
AaAQP4	XP_001650168	292	Entomoglyceroporin	XP_554502	Eglp2 – CG5398
AaAQP5	XP_001650169	249	Entomoglyceroporin	XP_318238	Eglp4 – CG4019
AaAQP6	XP_001648046	261	Water-selective AQP	XP_309823	CG12251

Table data, including accession numbers, was compiled based on phylogenetic data described previously (16).

using an artificial feeding method (24). Egg strips were then air dried and hatched as necessary, in 1L dechlorinated water baths. Larvae were fed with a 1:1 liver powder-yeast mixture dissolved in ddH_2O. As the larvae pupated, the pupae were collected in small 10mL containers and placed in mosquito cages. Each mosquito cage was provided with a 10% sucrose-soaked cotton ball to allow the mosquitoes to feed on a simulated nectar meal *ad libitum*. Male and female pupae were combined in each mosquito cage. For non-blood fed conditions, ~10–15 female *Ae. aegypti* were isolated from mosquito cages at ~10–12 days post-emergence and their Malpighian tubules (MTs) were dissected out in physiological saline (25). Treatment conditions for female *Ae. aegypti* included 0.5hr and 24hr post blood meal (PBM), where ~10–15 females were blood fed at ~10–12 days old as described above. The females were allowed to feed for ~15min and then the time was initiated for each post-blood fed treatment with females that engorged on blood identifiable by their red abdomen. The rearing and treatments protocols were then kept consistent for each biological replicate.

Immunohistochemistry

Immunohistochemistry was completed for whole body (WB) adult female *Ae. aegypti*, to localize the different AaAQPs. Immunohistochemistry was also completed for isolated adult female MTs from *Ae. aegypti*, localizing AaAQP1. For each treatment, NBF, 0.5hr PBM, 24hr PBM in WB and isolated MT sections, 4-5 individual mosquitoes (biological replicates) were studied. For each individual mosquito, 5–6 technical replicates were completed. Procedures were completed following previously published protocols (26–28). All tissues were fixed in Bouin's fixative and dehydrated in a series of ethanol and xylene. Paraffin embedded tissues were then sectioned using an Epredia HM325 manual microtome (Epredia, Kalamazoo, Michigan United States) and placed on Fisherbrand™ ColorFrost™ Plus Adhesion Microscope slides. Samples were processed such that tissue sections from non-blood fed (NBF), 0.5hr PBM, and 24hr PBM mosquitoes were placed on the same slide. The slides were processed in a multi-day procedure, with stepwise washes in 1xPBS. WB tissue sections were probed with one of the following; Anti-AaAQP1 affinity-purified primary antibody (1:1000 rabbit polyclonal antibody against CFFKVRKGDEESYDF, Genscript, NJ, USA) (27), Anti-AaAQP2 affinity purified primary antibody (1:50 rabbit polyclonal antibody against CNGLGNTGLKENVQD, Genscript, NJ, USA) (29), Anti-AaAQP4 affinity purified primary antibody (1:500 rabbit polyclonal antibody against PAEQAPSDVGKSNQS, Genscript, NJ, USA) (27), Anti-AaAQP5 affinity purified primary antibody (1:1000 rabbit polyclonal antibody against FRREVPEPEYNRELT, Genscript, NJ, USA) (27), or Anti-AaAQP6 affinity purified primary antibody (1:50 rabbit polyclonal antibody against CSFRNMFLADKAKAE, Genscript, NJ, USA). WB tissue sections were also probed with a mouse monoclonal anti-a5 antibody for Na+/K+-ATPase (NKA) (Douglas Fambrough, Developmental Studies Hybridoma Bank, IA, USA, 1:10 dilution) as a membrane marker. Malpighian tubule tissue sections were probed with the same AaAQP1 antibody as previously listed, as well as a guinea pig anti-V_1 antibody for V-type H^+-ATPase (VA) (Ab 353-2, gifted by H. Wieczorek, Osnabruck, Germany, 1:5000 dilution) as a membrane marker, to specifically distinguish the apical membrane of the MTs. For secondary antibodies, a goat anti-rabbit AlexaFluor 594 (Jackson Immunoresearch) antibody was used (1:400 dilution) to visualize all of the AaAQPs, a goat anti-mouse secondary antibody conjugated to Cy2 (Jackson Immunoresearch) was used (1:500 dilution) to visualize NKA, and a goat anti-guinea pig AlexaFluor 488 (Jackson Immunoresearch) antibody (1:500 dilution) was used for VA. For control slides, the primary antibody was omitted to confirm an absence of staining where only secondary antibody was added. Similar controls were completed for all AaAQP primary antibodies used. Aside from omission of the primary antibody, control and experimental slides were treated identically. All samples were mounted on slides with ProLong® Gold antifade reagent with DAPI (Life Technologies, Burlington, Ontario Canada). Slides were viewed and images captured using an Olympus IX81 fluorescent microscope (Olympus Canada, Richmond Hill, Ontario Canada) in combination with CellSense® 1.12 Digital Imaging software (Olympus Canada). VA staining (AlexaFluor 488) and NKA staining (Cy2) were viewed using the Brightline GFP filter set and AaAQP staining (AlexaFluor 594) was viewed using the Brightline TRITC filter set (Olympus Canada). Exposure and gain settings were first determined by viewing sections from NBF mosquitoes and then the identical acquisition settings were used to view and capture images of the 0.5hr PBM and 24hr PBM mosquito sections all on the same slide. This same procedure was repeated for each slide of processed samples containing NBF and PBM mosquito sections. Identical acquisition settings were also used on control slides, to confirm that in the absence of primary antibody, no staining of AaAQPs was observed.

Probe synthesis and fluorescence in-situ hybridization

Investigation into the identification and localization of *aqp1* mRNA began with *de novo* sequencing of the gene in *Ae. aegypti*. The originally reported transcript of *aqp1* by Pietrantonio et al. (20) was used as a template for our work, however it was discovered that annotations in the reference genome have reported different predicted transcripts variants of the *aqp1* gene, which ultimately yield different C-termini of the AaAQP1 protein. Through standard PCR and rapid amplification of cDNA ends (RACE), we confirmed the *aqp1* gene sequence (GenBank Accession: PP003259), which matches the originally reported sequence by Pietrantonio et al. (20) (see Supplementary Materials and Methods and Supplementary Results). We also used heterologous expression in human embryonic kidney (HEK293T) cells to verify our custom antibody against AaAQP1 specifically detects this water channel and the immunoblot results demonstrate a band size that matches that observed in protein extracted from MTs (Supplementary Figure S2).

To synthesize a template suitable for fluorescence *in situ* hybridization (FISH) with DIG-labelled RNA probes, *aqp1* FISH forward and reverse primers (Supplementary Table S1) were used to amplify a 583bp *aqp1* fragment. Primers were designed using the

Primer3 plugin in Geneious® 8.1.8 (Biomatters Ltd., Auckland, New Zealand). The *aqp1* gene was then amplified using bacterial cloning, by ligating the product into the pGEM T-Easy cloning vector (Promega, Madison, Wisconsin, USA) and the previously described standard protocol for cloning (12) was followed to amplify the specific portion of the *aqp1* gene. During colony screening, *aqp1* FISH primers with added T7 promoter sequences were used to yield *aqp1* gene fragments with added T7 sequences for RNA probe synthesis. Confirmation of fragment size was done by running an agarose gel and colonies yielding PCR products with correct size were chosen for overnight inoculation and then plasmid DNA was isolated by standard column-based plasmid mini-prep (Bio Basic Inc., Markham, Ontario, Canada), according to the protocol associated with the kit. Products were diluted 1:100 and ran on an agarose gel to confirm product size and band intensity. Then several replicate PCR reactions were completed to generate a large volume of both the anti-sense and sense DNA template products used for preparing RNA probes for experimental and control preparations, respectively. Products were pooled and purified using the Monarch PCR and DNA Cleanup Kit (5µg). The DNA template concentrations were determined using the Synergy Multi-Mode Microplate Reader (BioTek, Winooski, USA). To synthesize the digoxigenin (DIG) labeled RNA probes, the purified DNA products (either anti-sense or sense template) were added to a PCR tube with reaction buffer, a T7 polymerase, and DIG RNA Labeling Mix (Roche Applied Science, Mannheim, Germany), following the T7 RNA Polymerase Kit (New England BioLabs, Ontario, Canada). The tubes were incubated overnight at 37°C and the following day, the probe products were diluted 1:10 and were treated with a DNAse I to remove the DNA template. Products were run on a RNase-free non-denaturing agarose gel to confirm RNA probe size and band intensity.

To prepare tissue samples for FISH, we followed a protocol previously described (30). First, MTs were dissected from 3–4 day old adult female *Ae. aegypti* and transferred into a microcentrifuge tube containing 200µL of sterile PBS. The PBS was then replaced with freshly prepared 4% paraformaldehyde (PFA) and placed on a rotator for 1hr at room temperature to fix the tissues. The PFA was removed and tissues were washed five times with sterile PBT (sterile PBS + 0.1% Tween-20). Tissues were then quenched with 1% H_2O_2 for 20 min at room temperature to quench endogenous peroxidase activity which would otherwise result in elevated non-specific background fluorescence. Following this, tissue samples underwent permeabilization using 4% Triton X (960µl PBT + 40µL Triton X-100) with tubes set on a rocker at room temperature for 1hr. Tissues were then washed three times with PBT and followed by a second fixation with 4% PFA, with tissues rotating at room temperature for 20 min. The MTs were then rinsed with 1:1 PBT : Hybridization solution (Hyb) (50% formamide, 5x SSC, 100ug/mL heparin, 100ug/mL sonicated salmon sperm DNA and 0.1% Tween-20), followed by a single wash with 100% Hyb. All subsequent incubations above or below room temperature were carried out on a thermocycler. Pre-Hyb solution was made during this time by aliquoting 250µL per sample of Hyb solution into PCR tubes which were then placed at 100°C for 5 min, followed by a 5 min incubation on ice. After removal of 100% Hyb from the sample tubes, pre-Hyb solution was added to the sample and tissues were incubated at 56°C for 1hr. During this time, 4ng/µL of anti-sense (experimental) or sense (control) probe was prepared in pre-Hyb solution and then applied to the tissues overnight at 50°C. Solutions for the following day were prepared at this time, including 100% Hyb, 3:1 Hyb : PBT, 1:1 Hyb : PBT, 1:3 Hyb : PBT, and 100% PBT, all incubated overnight at 50°C.

The next day, tissues were washed in the following 50°C pre-warmed solutions; twice with 100% Hyb, once with 3:1 Hyb : PBT, once with 1:1 Hyb : PBT, once with 1:3 Hyb : PBT, and once with 100% PBT. Then, blocking of tissues to reduce non-specific staining was done with 1% blocking solution, PBTB (0.1g Molecular Probes block reagent; Invitrogen, Carlsbad, USA + 9.5mL PBT), rotating for 1hr at room temperature. The tissues were then incubated for 1.5hr with 1:200 mouse anti-DIG biotin-conjugated antibody (Jackson Immuno Research, West Grove, USA) diluted in PBTB, rotating at room temperature. From this step and onward, tissue samples were protected from light exposure. Tissues were then washed with PBTB four times, for 15 min each, rotating at room temperature. The tissue samples were then incubated with 1:50 HRP-streptavidin in PBTB for 1hr, rotating at room temperature to bind with the biotin-conjugated anti-DIG primary antibody. Tissues were once again washed with PBTB four times, for 15 min each, rotating at room temperature. Following manufacturer instructions, the samples were then incubated in 100µL diluted tyramide solution (Life Technologies, Eugene, USA) for 5 min at room temperature and then immediately followed by the addition of 100µL of stop solution (Life Technologies, Eugene, USA). The solution was then removed, and ten PBS washes were completed, following by an overnight incubation with PBS, rotating at room temperature and protected from light. Tissue samples were mounted on slides the following day using in-house mounting media (1:1 PBS:glycerol containing 4µg/mL DAPI) and were imaged with the EVOS FL Auto Live-Cell Imaging System (Life Technologies, Burlington, ON). The fluorescence *in situ* hybridization experiments were completed in at least four biological replicates that each included experimental (anti-sense probe) and control (sense probe) preparations. Image acquisition settings were identical for all sample preparations including experimental samples treated with anti-sense probes and control samples treated with sense-probes.

Transmission electron microscopy and immunogold staining

Transmission electron microscopy (TEM) techniques and imaging was carried out by Dr. Ali Darbandi at the Nanoscale Biomedical Imaging Facility at The Hospital for Sick Children Research Institute – Peter Gilgan Centre for Research and Learning. The MTs from non-blood fed adult female *Ae. aegypti* were dissected in physiological saline (25) and fixed in 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1M sodium cacodylate for 2hr at room temperature. The MTs were then rinsed in buffer and dehydrated in a graded ethanol series (50%, 70%, 90%, and 100%) for 20 min each at 4°C. Following this, two 1:1 ethanol/LR white acrylic resin changes were made for 30 min each and then

tissue samples were embedded in LR white resin where blocks were left to cure overnight at 60°C. MT sections of 70nm thickness were cut on a Leica EM UC7 ultramicrotome (Ontario, Canada) and the sections were stained with uranyl acetate and lead citrate. The grids were imaged using an electron microscope at the Nanoscale Biomedical Imaging Facility at The Hospital for Sick Children Research Institute – Peter Gilgan Centre for Research and Learning.

pt?>For immunogold labeling, MT samples were prepared on TEM grids as previously described by Schwartzbach and Osafune (31), completed at the Peter Giligan Centre for Research and Learning, Toronto CA. Then, each individual grid was placed on a 100µl droplet of 0.01M sodium citrate at 95°C for 15 min, followed by a brief 5 min cooling period and two washes with 0.15M glycine for 15 min and 1xPBS for 5 min. The grids were placed on individual 100µl droplets of antibody dilution buffer (ADB) blocking for 30 min at room temperature, followed by three washes with 1xPBS, 1% BSA, and 0.05% Tween®20 (Bio-Rad) for 5 min each and a 2hr wash in 1x PBS, 10% BSA, and 0.05% Tween®20 (Bio-Rad). The tissues were probed with 1:5 anti-AaAQP1 affinity purified rabbit polyclonal antibody in 1x PBS, 10% BSA, and 0.05% Tween®20 (Bio-Rad) overnight at 4°C. The grids were washed five times in 1x PBS with 1% BSA for 5 min each, followed by a 1hr incubation in a colloidal gold AffiniPure goat anti-rabbit secondary antibody conjugated with 18nm gold particles (Jackson Immunoresearch) in 1% BSA and 0.05% Tween®20 (1:10) (Bio-Rad). For control samples, they were treated identical to the experimental grids previous to this step. However, for control grids the primary anti-AaAQP1 antibody was omitted, to show its specificity relative to the treated samples. All tissue samples (control and experimental) were then treated with 2% glutaraldehyde in PBS for 5 min, before a series of washes with ddH_2O and the grids were stained with uranyl acetate and lead citrate. Imaging was completed at the same facility using the electron microscope.

Gel electrophoresis and western blotting

Gel electrophoresis and western blotting was completed on protein samples isolated from adult female *Ae. aegypti* MTs. For each biological replicate (n=5–6), MTs were collected from 75 individual female mosquitoes under physiological saline (25), from NBF, 0.5hr PBM, and 24hr PBM groups. Protein processing for all samples was done using the Mem-PER Plus Membrane Protein Extraction kit (Thermo Fisher Scientific, Burlington, Ontario Canada) as recently described (32). First, the cytosolic fraction of the MT protein was separated by adding 60µl of permeabilization buffer from the kit to each sample tube, with 1:200 protease inhibitor cocktail (Thermo Fisher Scientific, Burlington, Ontario Canada). The tubes were set to mix on a rotator at 4°C for 10min, before centrifugation at 16,000g for 15min at 4°C. The supernatant was collected as the cytosolic fraction. The remaining pellet was then re-suspended in 60µl of solubilization buffer with 1:200 protease inhibitor cocktail (as above) and set to incubate at 4°C for 30min. The samples were then centrifuged at 16,000g for 15min at 4°C and the supernatant was collected as the membrane fraction. Protein concentrations were measured using a Bradford assay, relative to bovine serum albumin (BSA) standards.

The MTs samples were prepared for SDS-PAGE, where MT protein was combined with 50mM Tris buffer and 6x loading buffer [225mmol L^{-1} Tris-HCl, pH 6.8, 3.5% SDS, 35% glycerol, 12.5% ß-mercaptoethanol, and 0.01% bromophenol blue]. The samples were then heated for 5 min at 100°C, placed back on ice briefly, and centrifuged at 10,000g for 1 min. For each replicate, 5µg of protein were loaded in 12% SDS-PAGE gels. Following gel electrophoresis at 120V, a wet transfer was completed where MT protein was transferred onto a polyvinyl difluoride membrane at 90V for 2hr on ice in 1x transfer buffer (0.225g Tris, 1.05g glycine, 20% methanol in 1L of ddH_2O). Following the transfer, the membranes were placed in 5% milk blocking buffer (5g skim milk powder, 100mL 1x Tris-buffered saline [TBS-T; 0.12g Tris, 0.9g NaCl, 0.1mL Tween®20 (Bio-Rad), 0.1mL NP-40 (Sigma-Aldrich) in 1L of ddH_2O]) at room temperature for 1hr on a rocker. The membranes were then probed with 1:1000 specific anti-AaAQP1 affinity-purified primary antibody (1.282 µg/mL) (27) overnight at 4°C and then washed in 1x TBS-T the following day before incubation in horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody at 1:5000 in blocking buffer for 1hr at room temperature. After a second series of washes with 1x TBS-T, 2mL of prepared chemiluminescent Clarity™ Western ECL substrate kit (BioRad, Hercules, California, United States) was applied to each membrane for 5 mins. The membranes were viewed using a Chemi-Doc MP Imaging System (BioRad, Hercules, California, United States) and protein bands were normalized against Coomassie staining of total protein. The protein bands were analyzed using ImageJ 1.53a Software (USA) to quantify protein abundance, normalized to total protein.

Statistics

All data was analyzed using Prism® 9 software (GraphPad Software Inc., California, USA). Normalized protein abundance values were plotted as mean values ± standard error of the mean (SEM). For *Ae. aegypti* western blot graphs, data was normalized to non-blood fed control groups. An unpaired t-test was completed for each data set to determine if there were significant changes in AaAQP abundance values. The ROUT outlier test was used to determine if there were outlier values, which were removed accordingly.

Results

Localization of AaAQPs in adult female *Ae. aegypti*

AaAQP1, 2, 4, 5, and 6 were localized in whole body (WB) tissue sections of adult female *Ae. aegypti* mosquitoes with Na^+/K^+-ATPase (NKA) used as a membrane marker.

AaAQP1

AaAQP1 immunoreactivity was primarily detected in the Malpighian tubules (MTs) and the fat body (FB), under non-blood fed (NBF) conditions (Figure 1A). AaAQP1 immunoreactive staining in the MTs appears aggregated in some areas but, for the most part, is evenly distributed at the apical side of the cell, in the NBF conditions (Figure 1A). At 0.5hr post blood meal (PBM), staining of AaAQP1 appeared uniform at the apical membrane of the MTs, with stronger staining intensity compared to NBF mosquitoes (Figure 1B). At 24hr PBM, immunoreactive staining of AaAQP1 is similar to the 0.5hr PBM in the MTs (Figure 1C). AaAQP1 staining in the fat body appeared to decrease in intensity at 0.5hr PBM in comparison to the NBF group (Figure 1B). However, at 24hr PBM, staining intensity of AaAQP1 in the fat body appeared more intense in comparison to the 0.5hr PBM group, but less intense compared to the NBF condition (Figure 1C). Interestingly, prominent AaAQP1 immunoreactive staining was localized to the ovaries in both the 0.5hr and 24hr PBM groups (Figures 1B, C). Minimal immunoreactivity of AaAQP1 was detected in midgut sections visible in the 0.5hr PBM group (Figure 1B). Furthermore, AaAQP1 immunoreactivity was found in the apical membrane of the hindgut in the 0.5hr PBM group and as well as on the apical side of the cells in the rectal pads in the 24hr PBM group, showing intense staining (Figures 1B, B', C).

AaAQP2

Immunoreactivity for AaAQP2 was detected in the MTs, showing dispersed and apical membrane staining in the NBF group (Figure 1D). In both the 0.5hr and 24hr PBM groups, the overall staining intensity of AaAQP2 immunoreactivity remains the same but staining appears uniformly at the apical membrane, in comparison to the NBF group (Figure 1E). Immunoreactivity of AaAQP2 appeared intense in the fat body under NBF conditions, with a reduction in intensity in the 0.5hr PBM group, and a partial recovery of staining intensity in the 24hr PBM group (Figures 1D–F). Further, AaAQP2 immunoreactivity was found in the ovaries of the

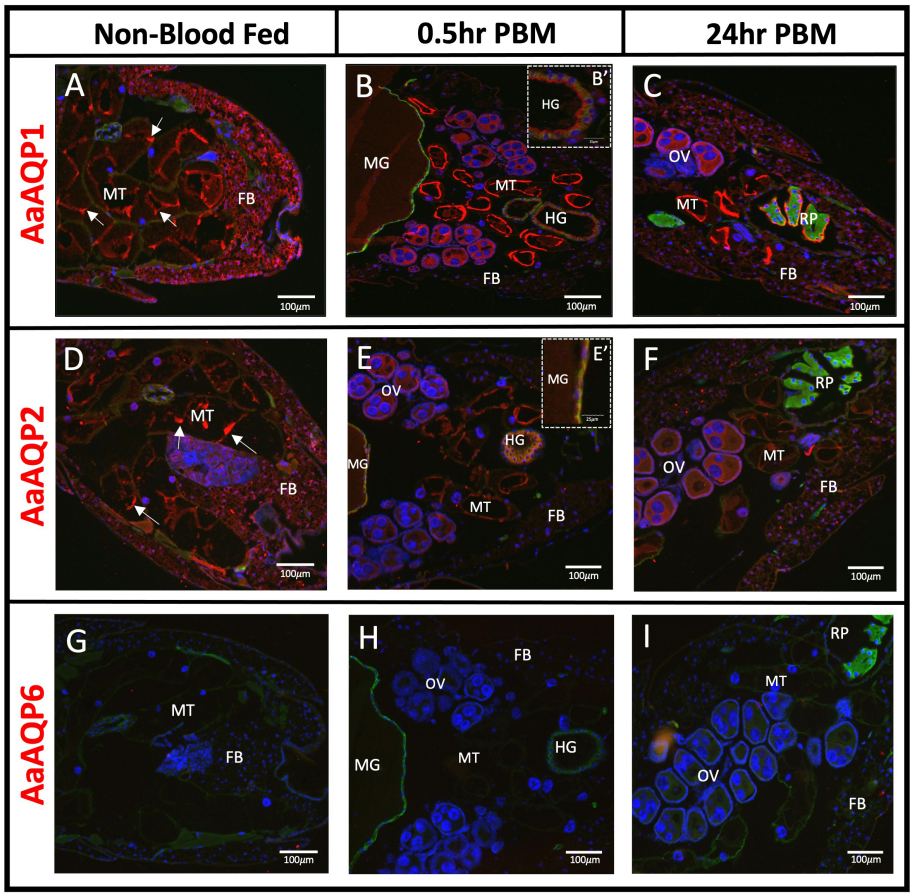

FIGURE 1
Localization of AaAQP1, 2, and 6 in whole body adult female *Ae. aegypti*. Immunohistochemical localization of water selective AaAQPs (red staining) in female *Ae. aegypti* mosquitoes (~10–12 days old). Each AaAQP (red staining) was localized under non-blood fed conditions, 0.5hr post blood meal conditions, and 24hr post blood meal conditions. Whole body sections of the abdominal segment immunolocalizing AaAQP1 in **(A–C)**, AaAQP2 in **(D–F)**, and AaAQP6 in **(G–I)**. In **(B)**, an inset image **(B')** shows AaAQP1 membrane localization in the HG. In **(E)**, an inset image **(E')** shows AaAQP2 membrane localization in the MG. All images were taken at 10x magnification (n=3). The Na^+/K^+-ATPase (NKA) was used as a membrane marker (green staining) and nuclei were stained with DAPI (blue). MT, Malpighian tubules; FB, fat body; OV, ovaries; HG, hindgut; MG, midgut; RP, rectal pads. White arrows indicate aggregated staining of AaAQP in the MTs.

blood fed groups. Finally, AaAQP2 immunoreactive staining was also present in the midgut and hindgut tissue in the 0.5hr PBM, where it was co-localized with the NKA membrane marker appearing at the basolateral membrane (Figure 1E, E').

AaAQP6

Mosquito abdominal sections were void of any AaAQP6 immunoreactive staining in NBF and PBM conditions (Figures 1G–I).

AaAQP4

Immunoreactivity for AaAQP4 was detected in the MTs, under all three conditions (Figures 2A–C). Staining appeared at the apical membrane of the MTs, with similar intensity seen in the NBF and 0.5hr PBM groups, but an increase in staining intensity was observed in the MTs in the 24hr PBM group (Figure 2C). Immunoreactivity for AaAQP4 was also found in the fat body, appearing intense in the NBF group, reduced in the 0.5hr PBM group, and partially recovered in the 24hr PBM group (Figures 2A–C). AaAQP4 was also found in the hindgut of 0.5hr and 24hr PBM tissue sections, with relatively low immunofluorescence (Figures 2B, C). Additionally, minimal immunoreactivity was observed in the ovaries at 0.5hr and 24hr PBM (Figures 2B, C).

AaAQP5

Immunoreactivity for AaAQP5 in NBF mosquitoes appeared relatively low and uniformly distributed at both the apical and basolateral membranes of the MTs (Figure 2D). AaAQP5 immunoreactive staining was found mainly in the fat body in NBF conditions; however, staining was absent in the fat body in the 0.5hr PBM but was localized to the epidermis of the cuticle (Figure 2E). At 0.5hr PBM, there was some localized staining seen in the ovaries (Figure 2E). At 24hr PBM, AaAQP5 immunoreactive staining in the fat body returned (Figure 2F) with no additional staining observed in the midgut, ovaries and hindgut.

AaAQP1 is expressed by principal cells in the MTs of adult female *Ae. aegypti*

Sections of isolated MTs confirmed that AaAQP1 staining is present on the apical membrane of the epithelia (Figures 3A–I), where co-localization with the V-type H^+-ATPase (VA) can be observed in the 0.5hr PBM mosquito tubules (Figure 3E). Importantly, *in situ* hybridization using an anti-sense probe demonstrated that *aqp1* mRNA in adult female *Ae. aegypti* MTs was associated with the larger and more abundant principal cells. In the proximal tubule, *aqp1* transcript was localized in the cytosol around the nuclei of principal cells (Figure 4A) while *aqp1*

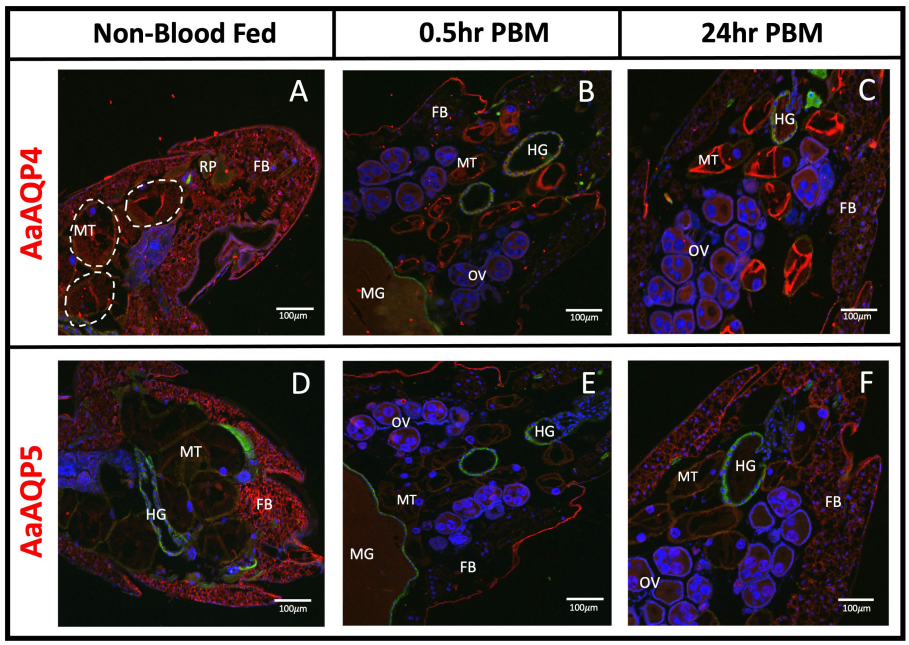

FIGURE 2
Localization of AaAQP4 and 5 in whole body adult female *Ae. aegypti*. Immunohistochemical localization of entomoglyceroporin AaAQPs (red staining) in female *Ae. aegypti* mosquitoes (~10–12 days old). AaAQP4 and 5 (red staining) were localized under non-blood fed conditions, 0.5hr post blood meal conditions, and 24hr post blood meal conditions. Whole body sections of the abdominal segment immunolocalizing AaAQP4 in **(A–C)** with basolateral membrane of MTs encircled in dashed line and AaAQP5 in **(D–F)**. All images were taken at 10x magnification (n=3). The Na^+/K^+-ATPase (NKA) was used as a membrane marker (green staining) and nuclei were stained with DAPI (blue). MT, Malpighian tubules; FB, fat body; OV, ovaries; HG, hindgut; MG, midgut; RP, rectal pads. White arrows indicate aggregated staining of AaAQP in the MTs.

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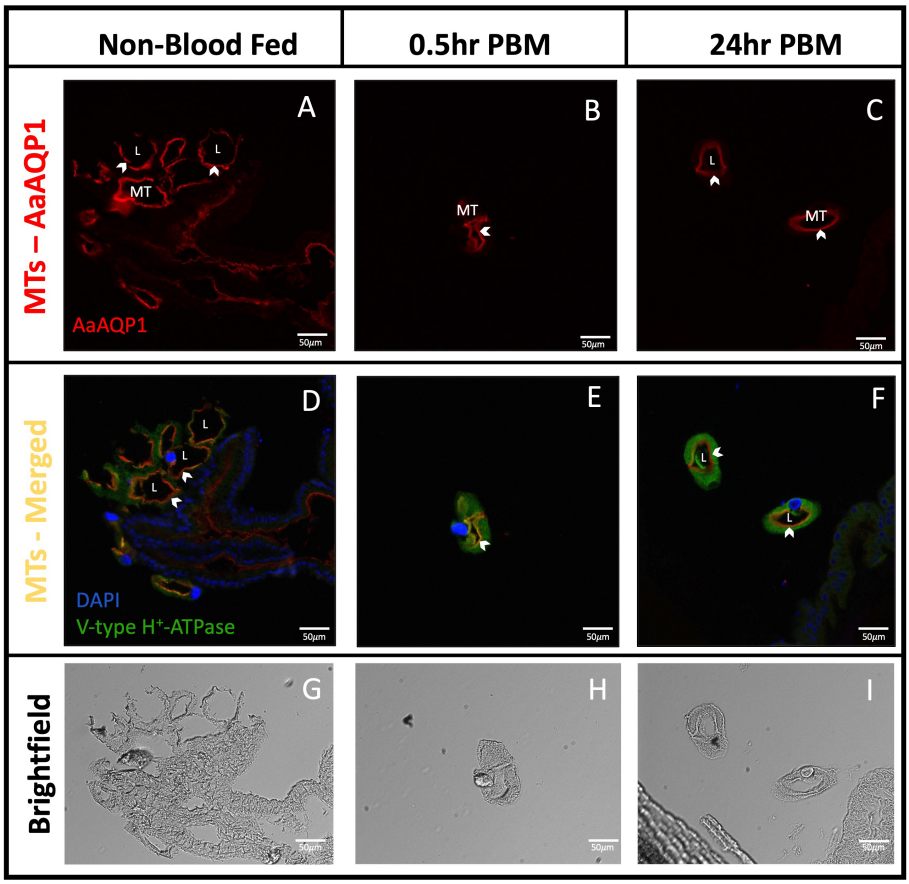

FIGURE 3
Localization of AaAQP1 in Malpighian tubules of adult female *Ae. aegypti*. Immunohistochemical localization of AaAQP1 (red staining) in isolated Malpighian tubules (MTs) of female *Ae. aegypti* (~10–12 days old) MTs were isolated from non-blood fed mosquitoes, and mosquitoes that were fed blood either 0.5hr or 24hr prior. **(A–C)** shows localization of AaAQP1 at the apical membrane of the MTs. White arrows indicate the apical membrane of the MTs. **(D–F)** shows localization of AaAQP1 at the apical membrane of the MTs, merged with staining observed for V-type H^+-ATPase (VA), which was used as an apical membrane marker (green staining). Co-localization of AaAQP1 with VA appears yellow/orange in colour. **(G–I)** shows brightfield images of MT sections, which was used to identify apical and basolateral membranes. All images were taken at 20x magnification (n=4) and nuclei were stained with DAPI (blue).

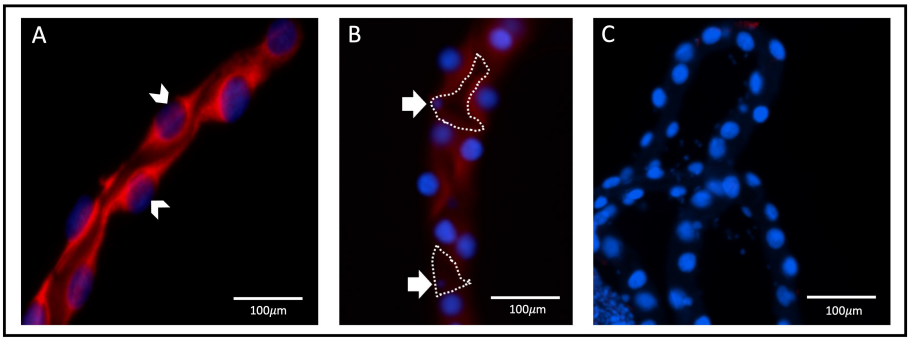

FIGURE 4
aqp1 localization with fluorescence *in situ* hybridization in Malpighian tubules. Localization of *aqp1* mRNA (red staining) in the Malpighian tubules (MTs) of female *Ae. aegypti* mosquitoes (~3–4 days old), using fluorescence *in situ* hybridization. Tubules were dissected from non-blood fed adult female mosquitoes (n=3). **(A)** *aqp1* localization in the distal tubule, found associated with the principal cell nuclei, indicated by the white arrow. **(B)** *aqp1* localization in the proximal tubule, found associated with the principal cells. Stellate cell nuclei indicated by the white arrow, with cell structure outlined in dashed lines, showing an absence of staining in encircled region. **(C)** MTs where an absence of *aqp1* staining is seen, after incubation and treatment with the sense (control) probe.

transcript staining was absent in stellate cells (Figure 4B). In the distal portion of the adult female tubule, very strong *aqp1* transcript levels were observed with consistent and exclusive staining to the principal cells of the MTs (Figures 4A, B). No staining was observed in MTs treated with the sense probe (Figure 4C), confirming the localization of *aqp1* transcript detected with the anti-sense probe (Figures 4A, B).

Sub-cellular localization of AaAQP1 in MTs of adult female *Ae. aegypti*

Immunogold labeling and transmission electron microscopy were used to localize AaAQP1 at the sub-cellular level in MTs of adult female mosquitoes. In NBF MTs, immunogold particles were predominantly localized to the principal cells (PCs) and appear scattered throughout the apical region in the brush border (Figures 5A, B). Additionally, some immunogold particles were also detected in reduced brush border of the less abundant stellate cells (SCs) (Figure 5C). Control grids, where the primary antibody was omitted, showed a complete absence of gold particles, relative to the presence of gold particles in the sections treated with both primary and secondary antibodies (Supplementary Figure S1).

Quantitative assessment of AaAQP1 protein expression in MTs of adult female *Ae. aegypti*

A ~25kDa band representing the putative monomer for AaAQP1 was observed in western blots of protein homogenates from MTs of adult female *Ae. aegypti* probed with custom anti-AaAQP1 antibody (Figure 6). Blood feeding did not result in any changes in AaAQP1 protein abundance in the MTs. There were no significant differences between AaAQP1 levels in MTs of the 0.5hr PBM and 24hr PBM groups, normalized to the NBF control. Therefore, there were no differences found in AaAQP1 protein in post blood fed female MTs relative to the NBF control. Heterologous expression of the AaAQP1 protein in human embryonic kidney cells yielded a protein of identical size, ~25kDa, confirming the specificity of the custom antibody utilized in this study (Supplementary Figure S2).

Discussion

In this study, we immunolocalized the aquaporins AaAQP1, 2, 4, 5, and 6 in organs of the adult female mosquito, *Ae. aegypti*, before and after a blood meal. Furthermore, a primary aim of this study was to better understand the function of the primary water channel, AaAQP1, in the adult female *Ae. aegypti* mosquito. For this reason, we focused more extensively on AaAQP1 expression at both the protein and transcript levels in the Malpighian tubules since earlier studies have shown its mRNA is enriched in this organ (16).

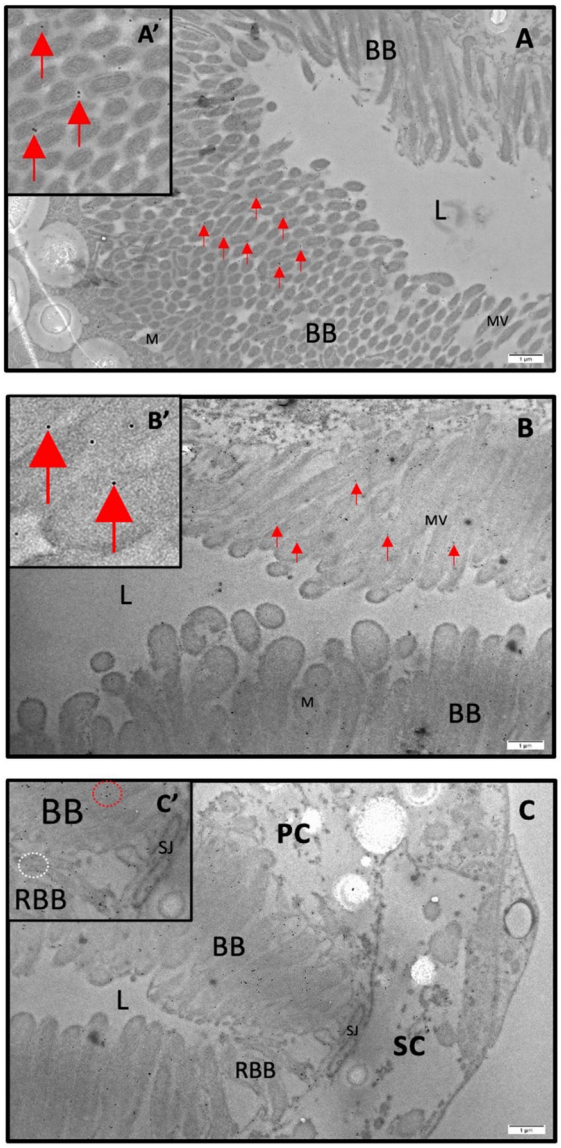

FIGURE 5
Immunogold staining of AaQP1 in female Malpighian tubules of *Ae. aegypti*. Subcellular localization of AaAQP1 in adult female *Ae. aegypti* (~10–12 days old) Malpighian tubules, using transmission electron microscopy (TEM) with immunogold staining (n=3). AaAQP1 staining is represented by the presence of 18nm gold particles, indicated by the red arrows. Inset images magnify individual gold particles. **(A, B)** Cross sections of non-blood fed mosquito Malpighian tubules, showing the apical membrane of the principal cells. Gold particles appear dispersed through the membrane fraction specifically within the brush border, with no pattern of gold particle collection. **(C)** Cross section of non-blood fed mosquito Malpighian tubules showing predominant gold particle presence in principal cells; however, there is some presence of gold particles also within the reduced brush border of the stellate cells. BB, brush boarder; L, lumen; M, mitochondria; MV, microvilli; RBB, reduced brush boarder.

AaAQP localization in the fat body

The transcript expression of AaAQPs in the fat body was previously reported where relatively low levels of *aqp1–6* mRNA were found (16, 33). Our data demonstrated immunolocalization of

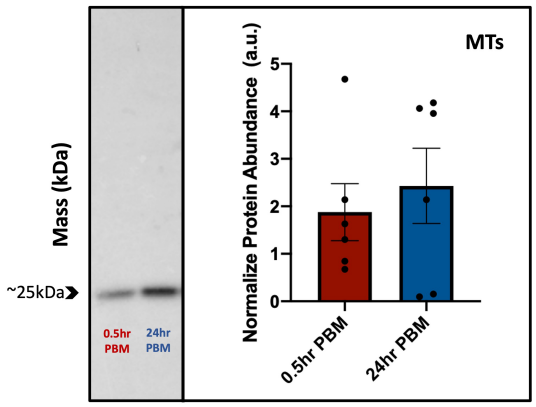

FIGURE 6
Protein abundance of AaAQP1 in female *Ae. aegypti* Malpighian tubules post blood meal. Normalized protein abundance of AaAQP1 in female *Ae. aegypti* Malpighian tubule tissue samples, in 0.5hr post-blood meal and 24hr post-blood meal groups. Values were normalized to the AaAQP1 immunoreactive band detected in Malpighian tubule protein samples from non-blood fed control mosquitoes. Malpighian tubules were isolated from ~75 individuals, for each biological replicate (n=5–6). A ~25kDa band (putative monomer) was detected for AaAQP1, and an unpaired t-test showed no significant changes in the protein abundance between the two blood fed treatment groups (p>0.05).

AaAQP1 (Figures 1A–C), 2 (Figures 1D–F), 4 (Figures 2A–C) and 5 (Figure 2D–F) in the fat body, but did not observe AaAQP6 immunoreactive staining in this tissue (Figure 1G–I). The intensity of AaAQP1, 2, 4, and 5 immunoreactive staining in the fat body decreased at 0.5hr PBM compared to NBF suggesting that blood feeding leads to lowered expression of these aquaporins in the fat body. Using transcriptomic analysis, Price at al. (33) detected three of the six mosquito aquaporins expressed in the fat body, including AaAQP1, 4, and 5 (Table 1). Notably, Price et al. reported an apparent decrease in the transcript abundance of AaAQP4 and AaAQP5 in the fat body 24 hours after a blood meal (33). Our results are consistent with this data and furthermore suggest that there is an abrupt decrease in AaAQP4 and AaAQP5 protein expression after a blood meal (Figure 2). It has been hypothesized that a decrease in overall transporters PBM in *Ae. aegypti*, including AaAQP4 and 5, may be accounted for by the increase in transporters present in yolk proteins PBM, during embryogenesis (33). On the other hand, AaAQP1 transcript was not detected in NBF fat body although low levels were detectable in this tissue at 24hr PBM (33), which is consistent with our observations of the partial recovery of AaAQP1 immunoreactive staining at 24hr PBM (Figure 1C). In addition, the expression of an AaAQP2 ortholog, AgAQP1 in the malaria vector, *Anopheles gambiae*, found a significant increase in the fat body, 48hr PBM (34). A similar trend in the expression of AaAQP2 is apparent in the fat body where staining intensity is largely diminished at 0.5hr PBM followed by partial recovery at 24hr PBM (Figures 1D–F). These findings suggest that blood feeding may lead to a temporary short-term reduction (~0.5hr PBM) in AaAQP1 and AaAQP2 expression in the fat body of mosquitoes but, the expression of these two aquaporins partially recovers within a day or two of blood feeding.

It is possible that AaAQP1 and 2 proteins are more immediately relevant for the MTs or HG, during post-prandial diuresis and early-stage blood meal digestion when the animal is dealing with a large load of water associated with the 0.5hr PBM female mosquitoes. During late-stage blood meal digestion, such as 24hr PBM, increased AaAQP1 and 2 staining in the fat body might relate to roles in water transport during egg maturation.

The fat body is an important multifunctional organ for female mosquitoes participating in nutrient storage, metabolic homeostasis, and production of yolk precursor proteins for egg production (35). In the previtellogenic female mosquito that has not blood fed, the fat body stores nutrients, and the trophocytes (i.e. fat body cells) contain numerous, relatively large lipid droplets which are synthesized at the endoplasmic reticulum (33, 36, 37). The fat body trophocytes are activated by a blood meal, shifting their function into yolk precursor protein (YPP) factories, which includes vitellogenin and lipophorin that are secreted into the haemolymph where they are then taken up by developing oocytes in the ovaries (33, 36, 37). During this time of YPP synthesis, the size of lipid droplets fluctuates, but by 24hrs PBM, they are reduced in size and by 48hrs PBM, they have recovered to pre-blood meal sizes (37). Furthermore, by 36hrs PBM, the fat body trophocytes revert back to a nutrient storage organ (36). Abundant immunoreactive staining of AaAQP4 and 5 correlates to the nutrient storage stage of the fat body trophocytes where lipid synthesis and storage are likely occurring at higher rates. When trophocytes are activated to synthesize YPPs for secretion into the haemolymph, AaAQP4 and 5 immunoreactive staining is low. Since AaAQP4 and 5 are entomoglyceroporins which have been shown to transport glycerol in a heterologous system, their function in the trophocytes may be to facilitate glycerol transport for the synthesis of lipids when the main function of the fat body is nutrient storage (13). AaAQP1 and 2 are the orthologs of *Drosophila* DRIP and PRIP, respectively and both have been shown to exhibit a preference for water transport (13, 16). The intensity of AaAQP1 and AaAQP2 immunoreactive staining suggests that similar to the entomoglyceroporins, their most abundant expression in the fat body coincides with nutrient storage and metabolic function of the trophocytes. Since lipid metabolism requires water, these aquaporins may play a critical role in regulating water content of the trophocytes as they synthesize fats. Comparatively, diminished AaAQP1 and 2 immunoreactive staining coincides with when trophocytes are synthesizing YPPs and secreting these proteins and stored fats into the haemolymph, a time when water regulation may not be as vital. AaAQP1 and 2 immunoreactive staining recovers as the fat body reverts back to nutrient storage and lipid synthesis.

AaAQP localization in the ovaries

The ovaries are the site for oocyte production and maturation. In *Ae. aegypti*, follicles in the ovaries containing oocytes are kept in a previtellogenic state until a blood meal is imbibed (38). Upon blood feeding the ovaries take up proteins and fats from the haemolymph, originating mainly from the fat body, to produce

mature oocytes (37, 39). In general, oocyte maturation is also accompanied by water uptake in follicles and the AaAQP2 ortholog of *Bombyx mori* was implicated in this process (40). In particular, (40) showed that the highest expression of the AaAQP2 ortholog in *Bombyx mori* was during vitellogenesis, aiding in water transport and hydration of oocytes. Furthermore, the localization of an AaAQP1 ortholog in *B. mori* oocytes suggests that this AQP may function to prevent dehydration, as its highest expression is found during late-stage oogenesis known as choriogenesis, when the vitelline envelope is developed to protect the egg from water loss, which occurs ~2 days pre-eclosion (40). In the current study, AaAQP1 (DRIP ortholog) and AaAQP2 (PRIP ortholog) immunoreactive staining was identified in the ovaries of PBM mosquitoes (Figure 1), which is consistent with earlier reported transcript expression of these AQPs in the ovaries (13). Additionally, it was found that AgAQP1A, the ovarian-specific AaAQP2 ortholog in *Anopheles gambiae*, was found to be abundant in non-blood fed female ovaries, with significant increases in abundance 24–48hr post blood meal (41). Earlier, (34) examined AgAQP1 abundance *in vivo* and found that expression was significantly increased 48hr post blood feeding. Together, this suggests that both AaAQP1 and AaAQP2 may participate in water uptake during oocyte maturation or prevention of dehydration of oocytes in *Ae. aegypti*. Further studies to pinpoint the exact localization of these AQPs in the ovaries of *Ae. aegypti* is needed. Additionally, the current study found an entomoglyceroporin ortholog to the *D. melanogaster* Eglp1, known as AaAQP4, was immunolocalized in the ovaries after blood feeding (Figure 2). Relatively low levels of AaAQP4 transcript has been shown to be present in adult female *Ae. aegypti* ovaries, pre- and post-blood meal (16). A role for AaAQP4 in the ovaries is still unclear and requires further investigation.

AaAQP localization in the midgut

The posterior midgut of the female mosquito stores and digests the blood meal and is the gut region where fluid and nutrients are absorbed into the haemolymph (42, 43). Immunoreactivity for AaAQP2 was found on the haemolymph-facing basolateral surface of the posterior midgut in 0.5hr PBM mosquitoes (Figure 1E, E'). Since the large volume load imbibed with a blood meal is absorbed into the haemolymph and subsequently secreted by the Malpighian tubules, basolaterally localized AaAQP2 may participate in transporting water across the midgut epithelium into the haemolymph during this time. Similarly, it was found that the PRIP ortholog, BcAQP2 in the insect *Bactericera cockerelli* was found to be highly expressed in the gut of the animal, likely responsible for transport of water post-food digestion (44). It has also been recently found that in *Ae. aegypti*, *aqp2* mRNA is significantly downregulated 3hr PBM (45). In addition, some immunoreactivity of AaAQP1 was found on the lumen-facing apical surface of the posterior midgut in 0.5hr PBM mosquitoes. It is possible that AaAQP1, a water-specific aquaporin, is important in the midgut epithelial tissue post blood feeding, to aid in removal of water from the midgut. Furthermore, various DRIP orthologs have been identified in the midgut of insects such as in larval midge *Belgica antarctica* (46), the rice striped stem borer, *Chilo suppressalis*, with confirmation of expression in whole adult female animals (47), 5th-instar *Rhodnius prolixus* posterior midgut samples (48), and in the columnar midgut cells of the silkworm *Bombyx mori* (49).

AaAQP localization in the hindgut and rectal pads

The hindgut of adult female *Ae. aegypti* contains an elongated anterior segment, known as the ileum, and a more posterior region called the rectum, which contains six luminal rectal pads. Both segments have been implicated in ion and water absorption through the localization of major ion-transporting pumps such as Na^+/K^+-ATPase and V-type H^+-ATPase (50). After a blood meal, alterations to the basolateral membrane of rectal pads suggest an increase in absorptive activity (51). Not surprisingly, transcript expression of AaAQP1 and AaAQP2 was detected in the ileum and rectum of female mosquitoes (13) and we detected immunoreactivity of both in the hindgut (Figure 1). Specifically, AaAQP1 was detected on the apical membrane of the ileum and in the apical membrane of the rectal pads (Figure 1) where it could play a role in absorbing water from the gut contents prior to excretion. In particular, the strong immunoreactive staining on the apical membrane of rectal pads 24hr PBM fits with the implied increased absorptive activities during this time (23, 52). AaAQP2 was detected in the ileum at 0.5hr PBM but, we could not verify if AaAQP2 was in the rectal pads because we did not have sections of rectal pads for NBF or 0.5hr PBM. Notably, AaAQP2 immunoreactivity was not observed in the rectal pads at 24hr PBM (Figure 1F), which is consistent with the very lower transcript abundance in the rectum reported earlier (13). Taken together, these findings indicate that AaAQP2 may also aid in water absorption shortly after the mosquito takes a blood meal.

AaAQP localization in the Malpighian tubules

The Malpighian tubules (MTs) in insects are responsible for the production of ion-rich primary urine through the secretion of ions and water from the haemolymph. Using immunohistochemistry, we localized AaAQP1 (Figures 1A–C), 2 (Figures 1D–F), 4 (Figures 2A–C), and 5 (Figures 2D–F) in the MTs of adult female *Ae. aegypti* while AaAQP6 immunoreactivity was not detected in this organ (Figures 1G–I). We detected AaAQP2 immunoreactivity at the apical membrane of the MTs which manifested as discrete aggregated staining in the NBF group and more evenly distributed staining around the luminal circumference of the MT in post-blood fed mosquitoes (Figures 1E, F). There is a possibility that the aggregated staining is indicative of AaAQP2 expression in the small stellate cells of the MTs in NBF mosquitoes, although the evenly distributed staining observed after blood feeding is more akin to expression in the principal cells. AgAQP1, the AaAQP2

ortholog in *Anopheles gambiae*, was localized to the proximal principal cells as well as the distal stellate cells, in the MTs of NBF mosquitoes (34, 41). In *Drosophila melanogaster* MTs the expression of the ortholog of AaAQP2 (PRIP) is enriched in the stellate cells but is also expressed by principal cells (53, 54). The transition from aggregated staining to an even distribution of AaAQP2 at the apical membrane of the MTs after blood feeding suggests that expression of AaAQP2 in the MTs may become associated with the stellate cells as well as the principal cells, to deal with an increased demand for water transport through the tubule epithelium. This may be necessary because principal cells are the more abundant cell type in the MTs of *Ae. aegypti* making up the majority of the tubule, whereas the relatively small volume that stellate cells make up may not be able to handle the large volume of fluid secretion that occurs after blood feeding (55).

Entomoglyceroporin immunoreactivity was detected in the principal cells of the Malpighian tubules. AaAQP4 immunoreactivity was localized to the apical side while AaAQP5 immunoreactivity appeared at the basolateral membrane (Figure 2). Two entomoglyceroporin orthologs are also expressed in the principal cells of *Drosophila* MTs (53). In *Aedes albopictus* AQP4 transcript abundance is decreased with blood feeding in the Malpighian tubules while there is a short-lived increase in AQP5 transcript abundance (56). Conflicting observations have been reported in *Ae. aegypti* with both a short-lived increase in AaAQP4 transcript or a more sustained increase (13, 16). On the other hand, a sustained increase in AaAQP5 transcript was reported and later confirmed (13, 16). It appears that the increase in AaAQP4 mRNA may result in a greater protein abundance since we detected an increased intensity of AaAQP4 immunoreactive staining at 24hr PBM. We did not detect any changes in the intensity of AaAQP5 immunoreactive staining after blood feeding, which might suggest that the brief increase in *aqp5* mRNA seen by Esquivel et al. in (56), is required to maintain the baseline AaAQP5 protein levels. AaAQP5 was shown to be an efficient water transporter in a heterologous system and its knockdown increases adult mosquito survival under desiccation stress and reduces fluid secretion by MTs in larvae (13, 19).

Previous studies using an antibody generated against *Cicadella viridis* AQPcic reported that AQP1 expression is confined to the tracheolar cells of *Ae. aegypti* female MTs (20, 57); however, the antigen region which this antibody was generated against shares relatively low identity (4/15 residues, ~27%) with the *Ae. aegypti* AaAQP1. However, in the present study, using a custom AaAQP1 affinity-purified antibody, immunoreactivity was detected at the apical membrane of the MTs, showing aggregated staining under NBF conditions followed by distinct continuous staining along the circumference of the apical membrane of the MTs in PBM female mosquitoes (Figures 1A–C). AaAQP1 antibody specificity was confirmed by *de novo* confirmation of the full *aqp1* coding sequence with rapid amplification of cDNA ends (RACE), followed by the heterologous expression of AaAQP1 in HEK293T cells (see Supplementary Results). Through western blotting using our custom AaAQP1 antibody, expression of AaAQP1 in HEK293T cells revealed a single ~25kDa band, representing the putative AaAQP1 monomer (Supplementary Figure S2). Using FISH, the *aqp1* transcript was localized to the principal cells in NBF female MTs. Furthermore, subcellular localization of AaAQP1 in NBF female MTs using immunogold labeling found AaAQP1 dispersed within the principal cells, specifically to the extensive brush boarder which is thinner in stellate cells (Figure 5). In both *Ae. aegypti* and *Anopheles gambiae*, transcript abundance of AQP1 is significantly higher in blood fed versus sugar fed mosquito MTs suggesting that AaAQP1 is important in voiding the excess water load imbibed with a blood meal (13, 58), which is supported by reduced diuresis in AaAQP1 knockdown mosquitoes (16). Our findings show that staining of AaAQP1 in the MTs appears uniformly at the apical membrane after a blood meal but no changes in immunoreactive staining intensity were observed. TEM with immunogold labeling confirmed that AaAQP1 is predominantly localized to the principal cells of the MTs, although some presence was detected in the smaller and less abundant stellate cells. Through western blotting, we confirmed that the protein abundance of AaAQP1 in MTs is unchanged after blood feeding in comparison to non-blood fed mosquitoes (Figure 6). This raises the possibility that activity of AaAQP1 is regulated by translocation to the membrane and/or through post translational modifications rather than expression. A recent study by Kandel and colleagues examined AQP regulation in *Ae. aegypti* by phosphorylation and concluded that it is more likely that AQPs are regulated by membrane translocation during the post-blood feeding period in adult female mosquitoes (23).

In conclusion, this study established a comprehensive localization of AaAQP1, 2, 4, 5, and 6 in adult female *Ae. aegypti*, during NBF, 0.5hr PBM, and 24hr PBM conditions. In particular, AaAQP1 (DRIP ortholog) was localized to the MTs, fat body, ovaries, and hindgut pre- and post-blood feeding. It was also found that AaAQP1 is specifically localized to the principal cells of the MTs. Furthermore, AaAQP2 (PRIP ortholog) was localized to the MTs, fat body, ovaries, midgut, and hindgut. The entomoglyceroporin AaAQP4 (Eglp1 ortholog) was found primarily in the MTs, with immunoreactivity also observed in the fat body, ovaries, and hindgut. The entomoglyceroporin AaAQP5 (Eglp2 ortholog) was found primarily in the fat body, with minimal immunoreactivity also found in the MTs and ovaries. AaAQP6 immunoreactivity was found to be absent throughout the abdominal tissue sections, which aligns with earlier studies that demonstrated enrichment of this aquaporin within the foregut of the alimentary canal (16) that was not investigated herein. Further studies on the role of each AaAQP in the various organs of female *Ae. aegypti* will provide a better understanding of the mechanisms by which water and other solutes are transported within mosquitoes.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors/.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

BP: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing. JP-P: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing. AD: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

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Supplementary material

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Voltage-gated ion channels as novel regulators of epithelial ion transport in the osmoregulatory organs of insects

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Voltage-gated ion channels (VGICs) respond to changes in membrane potential (V_m) and typically exhibit fast kinetic properties. They play an important role in signal detection and propagation in excitable tissues. In contrast, the role of VGICs in non-excitable tissues like epithelia is less studied and less clear. Studies in epithelia of vertebrates and invertebrates demonstrate wide expression of VGICs in epithelia of animals. Recently, VGICs have emerged as regulators of ion transport in the Malpighian tubules (MTs) and other osmoregulatory organs of insects. This mini-review aims to concisely summarize which VGICs have been implicated in the regulation of ion transport in the osmoregulatory epithelia of insects to date, and highlight select groups for further study. We have also speculated on the roles VGICs may potentially play in regulating processes connected directly to ion transport in insects (e.g., acid-base balance, desiccation, thermal tolerance). This review is not meant to be exhaustive but should rather serve as a thought-provoking collection of select existing highlights on VGICs, and to emphasize how understudied this mechanism of ion transport regulation is in insect epithelia.

KEYWORDS

Malpighian tubules, salt and water balance, voltage-gated ion channels, ion transport, excretion

1 Introduction

Insects are the most diverse group of animals with approximately 1 million species that span all geographical, aquatic, and terrestrial environments. In order to overcome their physiological disadvantage of having a large surface-to-volume ratio, insects have evolved and conserved a diverse range of systemic osmoregulatory functions to maintain internal homeostatic conditions (1, 2) that protect against unfavorable environmental challenges (3). Many insects face frequent and rapid salt-and-water imbalance due to their environmental ion availability changes, feeding

habits, alterations in acid-base balance, desiccation, changes in environmental temperatures, and even buoyancy.

For instance, a blood-feeding mosquito must get rid of extra water ingested with a blood meal rapidly to address the post-prandial salt-and-water imbalance. A caterpillar eating ~5 times its own body weight in food daily must excrete gargantuan amounts of metabolic wastes and plant-based xenobiotics and be able to adjust ion transport within minutes when necessary. To add insult to injury, the only way for a caterpillar to digest plant-based food and tap into the nutritional potential of plants is to raise midgut pH to ~11 to dissociate plant proteins from tannins (4). Some insects (e.g., beetles) possess a sophisticated "desiccation tolerant" physiological adaptation to cope with intermittent or evaporative water loss by accumulating and storing water in the haemolymph (2, 5, 6). Many insects face rapid and drastic changes in the temperature of their environment. Chill-susceptible insects experience chill injury or chill coma with exposure to unfavorable thermal conditions when key osmoregulatory and active ion transport mechanisms decline, disturb membrane polarization and ion balance, and negatively impact energy budgets as temperatures decrease (1, 7, 8). *Chaoborus* midge larvae control buoyancy by manipulating pH levels using ion-transporting endothelia adjacent to the air-sac's pH-sensitive protein resilin, which encourages the passive diffusion of gases across the endothelium that covers air-sac (9). All of these processes involve epithelia/endothelia that may benefit from employing fast ion transport-regulating mechanisms like the one discussed in this review.

Epithelial tissues simultaneously serve as: (i) a barrier between internal and external environment, and (ii) as a conduit for selective exchange of ions, nutrients, and wastes. Integral membrane proteins, like ion pumps, channels, aquaporins, and transporter are embedded in the membrane and form complexes that regulate V_m (10, 11) and provide a transmembrane pathway for ions and fluid (12).

Ion channels can be activated by temperature, osmolarity, ligand binding, mechanical change in the cell membrane, pH, neuroendocrine signals, and V_m (13–15) and display a spectrum of ion selectivity (Na^+, K^+, Cl, and Ca^{2+}) (16). For the purpose of this review, voltage-gated ion channels (VGICs) are broadly defined as channels belonging to the voltage-gated ion channel superfamily regardless of whether they're actually activated by changes in V_m, which is considered on a case-by-case basis. The primary function of VGICs is to generate action potentials in excitable tissues in response to changes in the cell V_m (17–23). Since the development of patch-clamp electrophysiology, protein purification and molecular and biochemical techniques, >250 types of ion channels have been identified (24, 25).

Interestingly, expression of many Ca_V, K_V, Na_V, and non-selective cation permeable VGICs has been reported *specifically in epithelia* of animals ranging from early divergent Placozoans to vertebrates (e.g., 26) (See Table 1). The genesis of the study of how

TABLE 1 Voltage-gated ion channels (VGICs) expressed in non-insect epithelia with potential roles they may play noted (where available).

Animal clade	Species	Tissue/Organ	Differential Factor/ Proposed Role	VGICs	Ref.
Placozoa	*Trichoplax adhaerens*	dorsal epithelium	–	**Ca_V1**	(27)
		outer edge of dorsal epithelium	–	**Ca_V2**	(28)
Asteroidea	*Patiria pectinifera*	coelomic epithelium	–	**Ca_V3, HVCN1, TRPA, TRPM**	(29)
Mollusca, bivalves	*Tridacna squamosa*	gill/ctenidium	light exposure	**Ca_V1**	(30)
	Crassostrea gigas	mantle epithelium	salinity, exposure to dilute seawater	**Ca_V3**	(31)
Teleosts, eels	*Anguilla japonica*	gill epithelia	environmental salinity (freshwater vs seawater)	**PVC - SCN3B, Ca_V1, TRPA1**, H_VCN1; MRC - Ca_Va2d3	(32)
	Anguilla anguilla	swim bladder epithelium	metabolic activity (rest vs. exercise)	**Ca_V1**, Ca_V2, Ca_V3, $Ca_Va_2d_2$, $Ca_V\beta$, CLCN, HCN, HVCN1, KCNA, KCNB, KCNC, KCND, KCNF, KCNG, KCNH, KCNMA1, KCNN, KCNQ, KCNT, KCNV, Na_V, TRPC, TRPM	(33)
Amphibia	*Rana esculenta*	Basolateral in distal convoluted tubule and intercalated cells of collecting duct in kidney nephron	K^+ secretion/reabsorption	**KCNQ1**	(34)
Chondrichthyes	*Squalus acanthias*	Rectal gland	function unclear	**KCNQ1**	(35)

(Continued)

TABLE 1 Continued

Animal clade	Species	Tissue/Organ	Differential Factor/ Proposed Role	VGICs	Ref.
Mammals	*Homo sapiens*	HK-2 kidney epithelial cells	cytokine TGF-β1 stimulation	**HCN, KCNA**, KCNH, KCNMA1, KCNQ, KCNS, TRPA, TRPC, TRPM, TRPV	(36)
		Intestinal and gastric, skin, lung, liver, and kidney epithelia	–	**Ca_V1**	(37)
		Vascular endothelium	K_V channels contribute to K^+ transport	**Irk/Kir, BK, TRPC**	(38)
		lung epithelia	H^+ secretion	**H_V1**	(39)
		adrenal gland, lung	function unknown	**$K_V4.3$/erg**	(40)
	Mus musculus	distal lung epithelium	development	**$Ca_V\beta$, CLCN**, KCNA, KCNH, TRPM	(41)
		collecting duct epithelial cells	–	**$Ca_V\beta$, CLCN, KCNQ, KCNS, TRPM, TRPV**	(42)
		Gastric, thyroid, intestinal and choroid plexus	K^+ transport	**KCNQ channels**	(43)
		kidney nephron	K^+ reabsorption and recycling	**KCNQ1**	(44)
		kidney, stomach, exocrine pancreas	K^+ secretion and recycling, in maintaining the resting potential, and in regulating Cl^- secretion and/or Na^+ absorption	**KCNQ1**	(45)
		kidney and colon epithelium	K^+ secretion	**BK**	(12)
		primary cilia in renal epithelia	osmotic stress response	**TRPM3**	(46)
	Canis lupus familiaris	cultured kidney epithelia	osmolality stress	**SCN1B; $Ca_V2.3$, 3.1**; KCNQ4, KCNC4, HCN2, TRPV1,2; TRPM6	(47)
			salt stress	**SCN1B; $Ca_V3.1$**; KCNQ4; KCNC3; HCN2; TRPC1; TRPV1,2	
	Didelphis virginiana	opossum kidney (OK) cells	maturation	**SCN9A**; CACNA1C; CATSPER2; CATSPER3; KCNAB2; KCNB2; KCNQ2; KCNH4; HVCN1; TRPM7; TRPM8; TRPC3; PKD2,1L2; TRPV4; HCN2	(48)
			orbital shear stress	**SCN9A; CACNA1C; CATSPER2**; CATSPER3; KCNAB2; KCNB2; KCNQ2; KCNH4; HVCN1; TRPM7; TRPM8; TRPC3; PKD2, PKD1L2; TRPV4; HCN2	
	Oryctolagus cuniculus and *Ratus norvegicus*	kidney and colon	ion transport and stabilization of the resting membrane potential	**$K_V1.3$/Shaker**	(10)
	Ratus norvegicus	pancreas, intestine, airway, kidney	provides the driving force for Cl^- transport across basolateral membrane of pancreas, airway, and intestinal epithelia	**KCNQ1**	(49)

VGICs in red decrease in mRNA abundance, while VGICs in purple increase in mRNA abundance (where available).

VGICs regulate ion transport in insect epithelia traces itself to a recent collection of studies that investigate the topic in the Malpighian tubules (MTs) of lepidopterans. Analogous to the mammalian kidney, the MTs of insects, together with the hindgut, serve as the primary osmoregulatory and excretory organs carefully balancing the uptake and recycling of vital ions and fluid while efficiently excreting ingested xenobiotics/toxins, and metabolic wastes (50).

One phenomenon is common to all insect MTs – they must secrete ions into their lumen to osmotically draw water in, and drive excretion and osmoregulation (see Figure 1) (51). Evolving approximately 350 million years ago, the highly conserved two-cell-type structure of insect MT epithelium, comprised of stellate cells (SC) and principal cells (PCs), employ physiologically distinctive cell-specific transepithelial cation and anion transporting mechanisms (2). Although, the MTs of *Drosophila melanogaster* have been used as the insect model of study for human renal pathologies (52, 53), there are substantial differences in complexity of the insect MTs between different clades of insects (54), highlighting the need for clade-specific investigation of ion transport mechanisms in osmoregulatory epithelia (see Figure 1A). For instance, how PCs and SCs transport ions does not translate between insect clades, and in many cases blind ends of MTs are either closely associated with the hindgut or are embedded into a specialized structure (15, 55, 56).

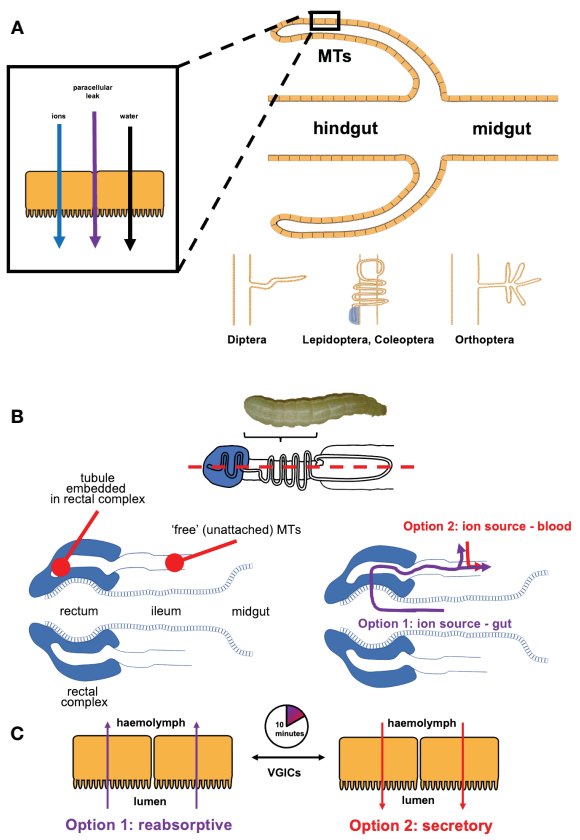

FIGURE 1
(A) Malpighian tubules (MTs) are blind outpouchings between the midgut and the hindgut of an insect - they secrete fluid by secreting ions into the lumen, and allowing water to follow by osmosis, with a paracellular junction leak component. Structure and functional adaptation depends on the insect clade. **(B)** In the larval lepidopterans, the distal end of each tubule is embedded into the rectal complex (in longitudinal section, dashed blue line, top panel). Each embedded tubule connects to the 'free' (unattached) region, suspended in the hemolymph and closely juxtaposed to the gut. Larvae have two options as a source of ions and water for secreting fluid in their MTs. The reabsorptive option (in purple) sources ions and water from the gut by way of the blind embedded tubules when the caterpillars are well fed. Some ions reabsorbed from the gut are transferred into the hemolymph across the unattached MTs. The secretory option (in red) sources ions and water from the larva's hemolymph, when dietary ions and/or water are unavailable or are in short supply (e.g., postprandially or during molting). **(C)** The switchover between reabsorption and secretion takes as little as 10 min.

In lepidopteran larvae, the distal end of MT is embedded into a specialized structure, the rectal complex (see Figure 1B). This allows the larvae to extract ions and water from their diet that they use to secrete fluid in the MTs. The remainder of the tubule (not embedded into the rectal complex) reabsorbs ions. However, when the gut is empty (e.g., during moulting or cessation of feeding), ions and water cannot be procured from the gut. A downstream region called the distal ileac plexus rapidly switches from ion reabsorption to ion secretion to enable osmotic secretion of fluid into the MTs, and to ensure that MTs function is not interrupted. This process is regulated in part by VGICs, which likely contribute to how rapidly (~10 min) the switch takes place in the MTs (Figure 1C). Given profound differences in the structure and function of MTs in insect clades, this begged the question of whether VGICs may be present in the MTs of other insects? Perhaps, even in other osmoregulatory epithelia of insects?

In addition to the MTs and the hindgut, many insects employ epithelia/endothelia that either play a direct role in osmoregulation (e.g., anal papillae of mosquitoes (e.g., 57, 58), collophore epithelia of springtails (e.g., 59)), or employ ion transport that is tied to other functions - e.g., gas bladder endothelia that regulate buoyancy in midge larvae (e.g., 9), blood-brain barrier endothelia that maintain the ion content of neuron-bathing fluid (e.g., 60). Caution must be used, however in the study of whether VGICs regulate ion transport in many of these organs, as excitable tissues layers (nerves, muscles) may confound results obtained in whole-organ studies.

Lastly, MTs epithelia play important roles in the immune response, oxidative stress, and response to xenobiotics and toxins (61). A speedy response to any of these using fast-acting VGICs would surely be of benefit to insects.

2 Voltage-gated Ca^{2+} channels

Ca_V channels are selectively permeable to Ca^{2+} and open in response to membrane depolarization (62, 63). In the osmoregulatory epithelia of insects, Ca_V1 has been detected in the MTs of *Drosophila melanogaster*, and implicated in intracellular signaling, directional Ca^{2+} transport and the regulation of diuresis (64). Likewise, Ca_V1 was shown regulate epithelial ion transport in the MTs of larval *Trichoplusia ni* (65), and larval and adult *Aedes aegypti* (58). Additionally, the same study has localized Ca_V1 in hindgut epithelium of the blood-fed adults of *Aedes aegypti*, where it may play a role in the regulation of post-prandial diuresis. The

function of other Ca_V channels in insect epithelia remains unclear. In most animals studied to date, Ca_V channels demonstrate conserved biophysical properties across clades, and Ca_V2 has different properties from Ca_V1 and Ca_V3 (66). The use of isoform-specific inhibitors may help discern the roles of other Ca_V3 isoforms in the MT of insects. Typical V_m of epithelial cells in MTs varies depending on the insect clade and osmoregulatory status of the animal (e.g., post-prandial, ion-rich diet). The use of high- and low-voltage activated Ca_V channels may provide an additional link between the regulation of ion transport and Ca^{2+} signaling. Ca^{2+} as a second messenger whose role is to maintain Ca^{2+} stores and concentration, a powerful activator of potassium transport mechanisms (14).

In addition, as Ca^{2+} is a general second messenger, there is potential for Ca_V channels to be involved in the regulation of epithelial processes other than ion transport, such as acid-base balance, desiccation and thermal tolerance, buoyancy control, and regulation of blood-brain barrier.

3 Voltage-gated K^+ channels

K_V channels belong to a diverse superfamily of proteins (67, 68) distinguished by their K^+ selectivity (21), and their role in K^+ transport, recycling, and intracellular signaling (69–71). Within the K^+ channel superfamily, K_V channels and at least 9 of their subfamilies are widely expressed in the membranes of excitable and non-excitable tissues (10, 72). K_V channels can also be activated by Na^+ or Ca^{2+}, can be inwardly rectifying or delayed rectifying, and display fast or slow kinetics of opening and closing (68, 73–76). K_V channels (K_V1-K_V4) named Shaker/$K_V1.3$, Shab/K_V2, Shaw/Kv3.1, and Shal/K_V4, based on *Drosophila* studies, vary in biophysical properties (77–82).

Inward-rectifying potassium (Kir) channels received their name from the fact that the inward flow of K^+ into the cell at any given voltage is greater than the outflow at same voltage but opposite in polarity (83). Kir channels play a role in K^+ secretion into MTs of the yellow fever mosquito *Aedes aegypti*, the fruit fly *Drosophila melanogaster*, and larval lepidopteran *Trichoplusia ni* (84). In *Drosophila*, three Kir homologues irk1, irk2 and irk3 are involved in PC-based K^+ secretion (85, 86). In the MTs of yellow fever mosquito *Aedes aegypti*, transepithelial secretion of K^+ is regulated by Kir1 located in the SC's basolateral membrane and by Kir2B in the basolateral membrane of PC's (87, 88). Together Kir1 and Kir2B are credited with conducting 66% of total transepithelial K^+ secretion in *Aedes* MTs. Additionally, Kir1 has been shown to play a role in K^+ secretion in the basolateral membrane of principal cells of a larval lepidopteran *T. ni* (55). Notably, there are pronounced differences in cellular localization of Kir isoforms in the MTs of different insects but have been detected in the MTs of larval lepidopterans using transcriptomic approaches (56, 89).

KCNQ/$K_V7.1$ are VGICs found in excitable and non-excitable cells that are sensitive to extracellular [K^+], providing constant repolarizing force that controls V_m (21). Interestingly, the membrane protein Potassium Voltage-gated Channel Subfamily E Regulatory Unit 3 (KCNE3) regulates the function of K^+ channel KCNQ1 channel by co-assembling with its α subunits preventing channel closure and converting it into a voltage-independent channel (90), while remaining constitutively active and open (43) at a negative voltage that would typically close the channel (91, 92). In insect epithelia, KCNQ1 was found to be enriched specifically in Drosophila MTs, although its function in the renal tissue has not been investigated to date (93). Recently, several studies detected KCNQ channels in MTs of larval lepidopteran *T. ni* (89, 94). KCNQ1 has also been detected in the MTs of larval dipteran *Ae. aegypti* where this channel may contribute to K^+ secretion in the MTs of brackish water (BW) larvae, helping the larvae rid themselves of extra K^+ and preventing K^+ loading with BW exposure. KCNQ1 was also found in anal papillae of larval *Ae. aegypti*, where it may play a role in uptake of environmental K^+ by the AP of larvae in freshwater (FW), aiding larvae in retention of hemolymph K^+ in the face of diffusional K^+ loss to FW (58).

In addition to being directly involved in ion transport in osmoregulatory epithelia, K_V channels may be involved in response to extracellular hyperkalemia observed in chill-injured insects (e.g., 95). Low levels of K^+ are essential for the proper function of the brain in insects (96), and potential use of K_V channels by the blood-brain barrier endothelia (especially in K^+-feeding herbivores) may be advantageous for rapid rebalancing of K^+ between body compartments.

4 Voltage-gated Na^+ channels

The ability to rapidly regulate natriuresis may be advantageous to certain insects – e.g., the female mosquito can rapidly increase diuresis to efficiently remove excess Na^+ post ingestion of a blood meal (97). In blood-feeders, the electrochemical potential of Na^+ is what drives Na^+-rich fluid secretion (98). Transcripts encoding Na_V channels like Para and Nalcn have been detected in the MTs of larval lepidopterans *T. ni* (89, 94) and *Helicoverpa armigera* (99) as well as that both MTs and anal papillae of larval dipteran *Ae. aegypti* (58). Previous pharmacological studies suggested that apical Na^+ channels regulated Na^+ uptake in anal papillae (57, 100) and Na^+ channels present in the MTs of *Ae. aegypti* transport Na^+ from haemolymph to lumen via voltage gradients created by V-type H^+ ATPase (88, 101, 102). A recent study demonstrated that Nalcn is present in the water-facing membrane of anal papillae in the larval *Ae. aegypti* (58). Although a significant number of neuropeptide toxins specific to Na_V channels have been identified among vertebrates, insect Na_V channels are remarkably different, which may prove mechanistic study of their function in insect epithelia rather challenging (103).

Na^+ balance is especially important to plant-feeding insects as all excitable tissues require Na^+ for producing action potentials, but it can be quite low in the diet, necessitating bizarre behaviors like puddling in adult moths aimed at supplementation of low dietary Na^+ with that acquired from mud puddles of vertebrate urine. Thus, in addition potentially contributing to epithelial Na^+ transport Na_V channels could be used by blood-brain endothelia to quickly rebalance Na^+ between body compartments.

5 Cation-selective VGICs

5.1 Transient receptor potential channels

TRP channels are cation-permeable (Na^+, K^+, Ca^{2+}) voltage-dependent channels (104). TRP channels are activated through a range of gating mechanisms including V_m depolarization (105). TRPA channels carry out functions in many excitable and non-excitable tissues like those found in *Drosophila* gut epithelia (106, 107). Several families of TRP channels have been reported in the osmoregulatory epithelia of in insects, including insect-specific Painless and Pyrexia TRPA channels. TRPA channels have been detected in MTs of larval *T. ni* (89), *Pieris rapae* (108) and *Bactrocera dorsalis* (109). TRPA1, TRP/Painless and TRP/Pyrexia are more permeable to K^+ than to Na^+, with well-established roles in nociception and thermotaxis in excitable tissues of insects (110–112). Six TRPA channels have been detected in MTs and AP of larval *Ae. aegypti* and mRNA abundance of every channel was altered as a result of BW exposure in MTs or anal papillae (58). TRP channels may provide an additional link between epithelial V_m, ion transport, Ca^{2+} signaling, and activation of other VGICs in insect MTs.

In addition to playing a role in the regulation of epithelial ion transport, TRP channels like Painless may serve as peripheral thermal sensors since they have been shown to respond to increased temperatures *in vitro* (i.e., regardless of the context of which tissue they're expressed in) (113). Attuning the function of osmoregulatory epithelia to changes in environmental temperature may be particularly advantageous for these small ectothermic animals.

5.2 Hyperpolarization-activated cyclic nucleotide-gated channels

Whereas most VGICs open in response to depolarization, a stand-out group of VGICs is activated by hyperpolarization - the HCN channels, which are permeable to both Na^+ and K^+, and are additionally activated by cyclic nucleotides, where the latter overrides dependence on V_m changes (114). In osmoregulatory epithelia of insects, the HCN channels have been detected in the MTs of larval lepidopteran *T. ni* (57, 58, 88, 89, 94) and when HCN1 channels are blocked in MTs, ion transport switches direction from K^+ secretion to K^+ reabsorption (89). Cyclic nucleotides are known to alter fluid secretion in the MTs of larval and adult insects (e.g., 115–117). HCN channels can provide an additional link between direct activation of ion transport and second messenger-based hormone action.

6 Select unstudied VGICs detected in osmoregulatory epithelia of multiple insects

Transcriptomic studies on osmoregulatory epithelia of lepidopterans and dipterans uncovered the presence of many more VGICs (e.g., H_V, Cl_V, BK, KCNA, KCNC, KCNS, TRP M, TRP V channels), expression of which has not been confirmed using direct molecular, protein-based, or pharmacological approaches to date (26, 58, 65, 89, 94). Whether these VGICs play a role in the regulation of ion transport in insect epithelia remains unexplored. H_V and Cl_V channels may play a role in acid-base balance regulation since acid H^+ transport can contribute to acid equivalents, and Cl^- is often transported by epithelia in exchange for HCO_3^- – to the best of our knowledge, these have not been examined in osmoregulatory epithelia of insects to date. TRP channels may serve as peripheral sensors of allelochemicals and xenobiotics in herbivores as many TRPs are activated by noxious plant-based chemicals, as well as peripheral temperature sensors (see above). Many TRP channels are volume-sensing and mechanosensitive (118), both of which would offer insect epithelia an additional mechanism for fine-tuning their ion transport rapidly.

7 Discussion

7.1 Current gaps in research – what remains to be explored

7.1.1 Are VGICs connected with mechanosensation in epithelia?

The role of VGICs in excitable tissues of animals has been well established (37). In insect epithelia however, VGICs seem to play an integral role in the regulation of ion transport and of V_m yet studies on this topic remain largely scarce.

A likely advantage of the presence of VGICs in animal epithelia is the ability to quickly respond to changing ion concentrations. In the MTs of insects, this may be relayed via mechanosensation of fluid flow and hydrostatic pressure, which may result from alternating bouts of diuresis and anti-diuresis, changing epithelial cell volume by applying pressure to the cells against the basal lamina that encases the MT epithelia. Mechanosensitive ion channels found in insect epithelia (e.g., 58, 89, 94) can detect mechanical changes in the epithelial cell membrane caused by changes in fluid flow, hydrostatic pressure and cell volume, and open resulting in changes in V_m and setting off intracellular second messengers (cAMP, Ca^{2+}) cascades (119). VGICs have been shown to respond transcriptionally to mechanical stress at least in some epithelia and can be used to amplify this signal (see Table 1). An instance of this has been described in intestinal epithelia, where TRPM5 channel triggers membrane depolarization in response to nutrient levels and opening of Ca_V channels amplifies the Ca^{2+} signal.

7.1.2 How are VGICs activated in insect epithelia?

Mechanisms of VGIC activation in insect epithelia remain largely unexplored. Peptide toxins extracted from metazoan venoms that target the specific subtypes of animal VGICs may prove to be a useful pharmacological tool to gaining a better understanding of insect VGICs (103). Do VGICs respond directly to the changes in epithelial cell V_m resulting from altered intracellular and extracellular ion concentrations? Are VGICs

activated by other upstream mechanisms (e.g., mechanosensitive and/or ligand-gated ion channels)? When luminal fluid flow decreases, cell volume may increase, activating mechanosensation and recruiting additional directional ion transport. MTs of insects have recently been shown to react to changes in hydrostatic pressure (120). Ligand-gated ion channels have similarly been detected in the MTs of multiple insect clades (e.g., 121, 122) and epithelial V_m can change in response to stimulation of neurotransmitters and endocrine ligands. VGIC can also be used to amplify activation of ligand-gated ion channels.

7.1.3 Do VGICs remain voltage-gated in epithelia?

Voltage sensitivity varies between the different types of VGICs with K_V, Na_V, and Ca_V displaying high sensitivity, activating and opening following membrane depolarization (43), and TRP channels demonstrate comparatively low voltage sensitivity (16). It is widely accepted that the biophysical properties of VGICs, although conserved in the same channel across many taxa, may be modified by channel subunit assembly for VGICs that are made up of multiple subunits. For instance, K_V α subunit complexes can also co-assemble with β subunits of K_V channels creating isoforms with different biophysical properties (19, 21, 22, 72, 90, 123).

8 Conclusions

It has been decades since the first VGIC was reported in animal epithelia. Recent studies in osmoregulatory epithelia of insects point to an abundance of VGICs, several of which have already been demonstrated to regulate ion transport. Further study of the roles specific VGICs play in the regulation of ion transport in insect epithelia may prove a fruitful ground for basic knowledge needed for instance to design better targeted integrated pest management strategies.

Important questions remain about the role of VGICs in the osmoregulatory epithelia of insects. Do VGICs demonstrate the same ion selectivity in epithelia as they do in excitable tissues? Do VGICs directly contribute to directional ion transport? Do VGICs establish the driving force for ion transport? Do VGICs establish/set V_m in insect epithelia? Do VGICs assemblages in insect epithelia differ based on external salinity, dietary ion availability, and ions used to drive diuresis in MTs (e.g., K^+-eating herbivores vs Na^+-eating omnivores/carnivores/puddlers)? Are VGICs used to regulate processes connected to the osmoregulatory function in insects – e.g., acid-base balance, desiccation, thermal tolerance, buoyancy control, regulation of blood-brain barrier?

Author contributions

JD: Conceptualization, Writing – original draft, Writing – review & editing. DK: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Consuming royal jelly alters several phenotypes associated with overwintering dormancy in mosquitoes

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Introduction: Females of the Northern house mosquito, *Culex pipiens*, enter an overwintering dormancy, or diapause, in response to short day lengths and low environmental temperatures that is characterized by small egg follicles and high starvation resistance. During diapause, *Culex pipiens* Major Royal Jelly Protein 1 ortholog (CpMRJP1) is upregulated in females of *Cx. pipiens*. This protein is highly abundant in royal jelly, a substance produced by honey bees (*Apis mellifera*), that is fed to future queens throughout larval development and induces the queen phenotype (e.g., high reproductive activity and longer lifespan). However, the role of CpMRJP1 in *Cx. pipiens* is unknown.

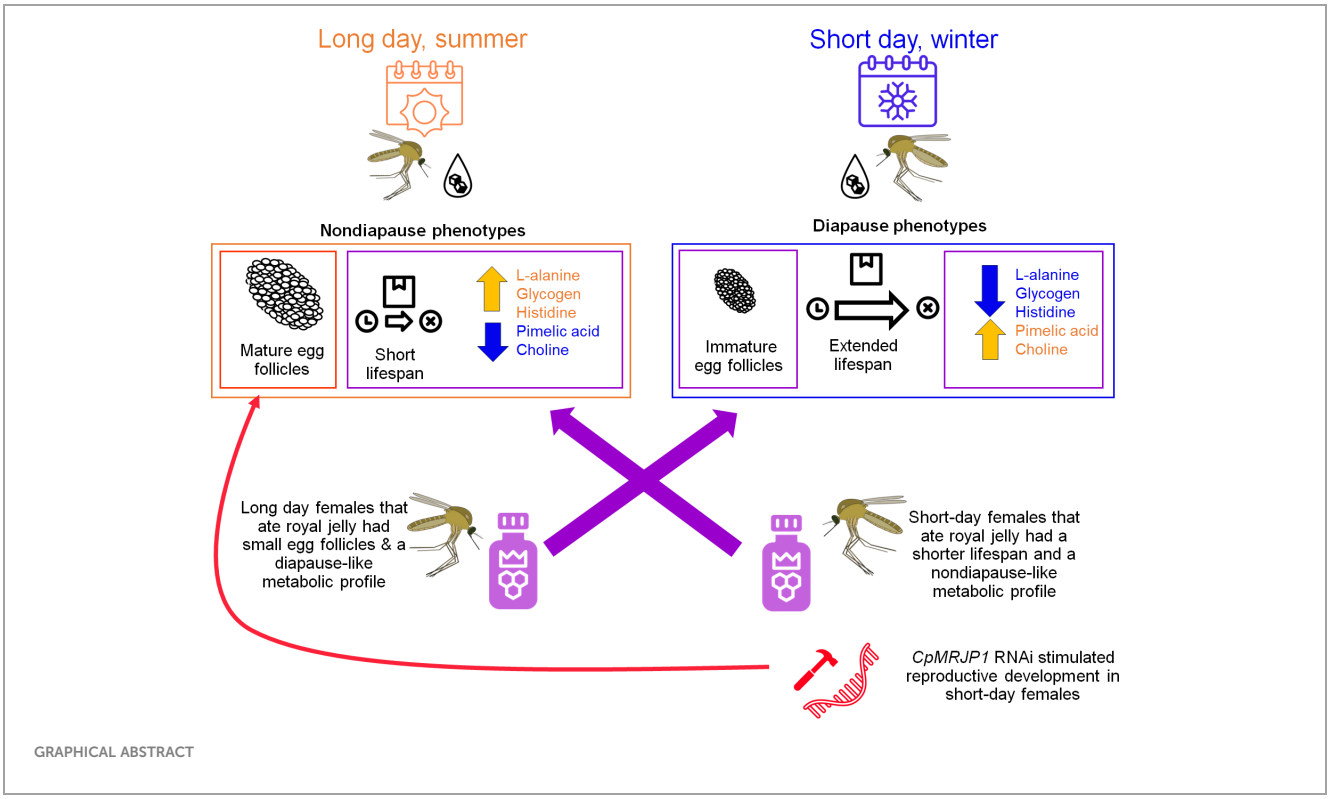
Methods: We first conducted a phylogenetic analysis to determine how the sequence of CpMRJP1 compares with other species. We then investigated how supplementing the diets of both diapausing and nondiapausing females of *Cx. pipiens* with royal jelly affects egg follicle length, fat content, protein content, starvation resistance, and metabolic profile.

Results: We found that feeding royal jelly to females reared in long-day, diapause-averting conditions significantly reduced the egg follicle lengths and switched their metabolic profiles to be similar to diapausing females. In contrast, feeding royal jelly to females reared in short-day, diapause-inducing conditions significantly reduced lifespan and switched their metabolic profile to be similar nondiapausing mosquitoes. Moreover, RNAi directed against *CpMRJPl* significantly increased egg follicle length of short-day reared females, suggesting that these females averted diapause.

Discussion: Taken together, our data show that consuming royal jelly reverses several key seasonal phenotypes of *Cx. pipiens* and that these responses are likely mediated in part by CpMRJP1.

KEYWORDS

reproductive diapause, major royal jelly protein 1 (MRJP1), metabolomics, NMR spectroscopy, machine learning, qRT-PCR, RNA interference (RNAi)



1 Introduction

Females of the Northern house mosquito, *Culex pipiens*, enter diapause in response to short daylengths and low environmental temperatures that act as harbingers of the approaching winter (1). Diapause allows mosquitoes to survive unfavorable winter conditions (2, 3), and it involves a unique suite of behavioral, hormonal and metabolic changes (4–6). In *Cx. pipiens*, exposure to short days as eggs, larvae, pupae and early adults causes adult females to enter an adult reproductive diapause (7). Diapause in females of *Cx. pipiens* is characterized by reproductive arrest, resulting in a decrease in the size of egg follicles as females divert energy away from reproduction (8). Adult mosquitoes that enter diapause feed on sugar-rich plant nectar, causing an increase in whole-body fat content (5, 9). Several genes that regulate metabolism are differentially expressed between diapausing and nondiapausing female mosquitoes; specifically, Robich and Denlinger (5) found that a gene associated with lipid accumulation, *fatty acid synthase*, was upregulated in diapausing females of *Cx. pipiens*, while two genes that encode enzymes related to digesting a blood meal, *trypsin* and *chymotrypsin-like* proteins, were down-regulated. Although many of the genes involved in generating diapause phenotypes have been well-characterized, it is still unclear what genes and proteins regulate diapause and initiate largescale metabolic and behavioral changes. However, insulin signaling and the Forkhead Transcription Factor, FOXO, have been implicated in regulating diapause responses in *Cx. pipiens* and several other insect species [reviewed in (10, 11)].

Royal jelly is produced by worker honey bees (*Apis mellifera*), and is a rich source of amino acids, lipids, vitamins, and other nutrients (12). Specifically, Nagai and Inoue (13) found that royal jelly consists of water (50–60%), proteins (18%), carbohydrates (15%), lipids (3–6%) as well as smaller amounts of water-soluble vitamin B and related components including vitamin B_1, vitamin B_2, vitamin B_6, biotin, acetylcholine, pantothenic acid, inositol, and nicotinic acid. Worker bees and drones feed on royal jelly for the first 3 and 5 days of larval life respectively, after which they feed on beebread (a type of fermented pollen) and honey. However, future queen bees consume royal jelly exclusively, and this induces female larvae to follow the queen developmental trajectory that is characterized by high reproductive outputs and a longer lifespan (14, 15). One protein in royal jelly, referred to as Major Royal Jelly Protein 1 (AmMRJP1), produces strong antibacterial activity and is the most abundant glycoprotein within royal jelly, constituting over 50% of total protein content (16, 17). Surprisingly, the gene encoding the ortholog of AmMRJP1 in *Cx. pipiens* (*CpMRJP1*) is upregulated by FOXO and is more abundantly expressed in whole bodies of diapausing females of *Cx. pipiens* (18). The tissue specificity of *CpMRJP1* transcription and translation as well as the function of CpMRJP1 in diapausing mosquitoes has not been characterized. However, the upregulation of *CpMRJP1* in diapausing mosquitoes is surprising because these females are not reproductively active but do live substantially longer than nondiapausing females, especially in the absence of food (8).

Although it is unclear how increasing the abundance of AmMRJP1 or its orthologs impacts animals, several studies demonstrate that consuming royal jelly affects the physiology of invertebrates and mammals, including humans. Integrating royal jelly into human diets can improve reproductive health, combat neurodegenerative disorders, slow aging, and promote wound-healing (19). In rats, proteins in royal jelly can also function as

antioxidants, protecting the testes of males against oxidative stress (20). Additionally, supplementing the diets of male rams with royal jelly increases sperm motility and viability (21). Similarly, royal jelly positively impacts fertility as well as semen quality and quantity in male rabbits (22). Introducing royal jelly into the diet of *Drosophila melanogaster* (L.) extends adult lifespan in males and females, possibly by increasing antioxidant activity, and stimulates feeding behavior and fecundity in females (23). Royal jelly also extends adult lifespan in the nematode *Caenorhabditis elegans* (Maupas) (24), suggesting that royal jelly may promote longevity across a wide range of invertebrates. Moreover, Fischman et al. (25) demonstrate that consuming royal jelly enhances the likelihood that alfalfa leaf cutting bees will enter diapause. While several studies have examined the role of royal jelly in animals, little is known about how consuming royal jelly might influence the seasonal responses and metabolic profile of mosquitoes, and to what extent these changes are induced by MRJP1.

Although metabolomic studies to uncover the differences between diapausing and nondiapausing *Cx. pipiens* have not been completed, previous research has examined metabolic differences between diapasuing and nondiapausing Asian tiger mosquitoes, *Aedes albopictus* (Skuse), flesh flies *Sarcophaga crassipalpis* (Macquart), and parasitic wasps, *Nasonia vitripennis* (Walker) (26–28). Not surprisingly, metabolomic studies demonstrate that diapausing mosquitoes, flesh flies, and parasitic wasps upregulate metabolites that act as cryoprotectants (26–28). In diapausing *N. vitripennis* the abundance of members of the glycolysis pathway are more abundant, reflecting an overall perturbation of the metabolic pathways in diapause (28). In *Ae. albopictus*, the monoamine neurohormones dopamine and octopamine, as well as phosphocholine and oleoyl glycine were more abundant in nondiapausing eggs compared to diapausing eggs (26). One objective of this study is to identify specific metabolites that are differentially abundant between diapausing and nondiapausing *Cx. pipiens* and how consuming diets that include royal jelly influences the overall metabolome of long and short-day reared mosquitoes.

Females of *Cx. pipiens* (L.) transmit pathogens that cause St. Louis encephalitis (29), West Nile virus (30), and canine heartworm (31) that infect millions of humans and animals each year (32). Female mosquitoes transmit these pathogens when they take a bloodmeal from a human or animal host (33). However, disease transmission is not equally distributed across time (34), in large part because diapausing mosquitoes no longer ingest blood (6) and, as a result, no longer transmit diseases (34). Therefore, uncovering molecular regulators of diapause in mosquitoes and other blood-feeding arthropods may offer novel opportunities to control these disease vectors and thereby improve human and animal health.

We first conducted phylogenetic analyses to determine how the sequence of *CpMRJP1* compared to that in other insects. To characterize how consuming royal jelly affects seasonal phenotypes in mosquitoes, we measured the abundance of *CpMRJP1* mRNA transcripts, reproductive development, lifespan, overall fat and protein content, as well as the metabolic profile of long and short-day reared females of *Cx. pipiens* that had consumed sugar water only (control) and those that consumed diets that included royal jelly. We also assessed which, if any, of the physiological impacts of royal jelly on seasonal responses were mediated by CpMRJP1 by using RNA interference (RNAi) to knock down this transcript in mosquitoes reared in long-day, diapause-averting and short day, diapause-inducing conditions. We hypothesized that mosquitoes that consumed diets including royal jelly would enter a diapause-like state regardless of environmental conditions, characterized by small egg follicles and increased longevity. Accordingly, we hypothesized that long and short-day reared mosquitoes that consumed royal jelly, and therefore had higher levels of AmMRJP1 within their guts, would exhibit a metabolic profile that was similar to that of diapausing mosquitoes that consumed sugar water. In contrast, we hypothesized that knocking down *CpMRJP1* with RNAi would prevent mosquitoes reared in short-day, diapause-inducing conditions from entering diapause and would decrease longevity.

2 Materials and methods

2.1 Sequence alignment and phylogenetic analyses

The amino acid sequence of *Culex quinquefasciatus* MRJP1 (CPIJ008700) was extracted from Vectorbase (https://vectorbase.org/vectorbase/app/) and fed into NCBI blastp search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the potential evolutionary origin of this protein. Amino acid sequences from the top dipteran matches were extracted and used for comparison and determination of phylogenetic relationships among dipteran species. As Drapeau et al. (14) indicate that *MRJP1* is closely-related to the *yellow* gene in insects, the amino acid sequences of *yellow* from *Culex quinquefasiatus*, extracted from Vectorbase, and *yellow* and its associated paralogs in *Drosophila melanogaster*, extracted from FlyBase, were used for the phylogenetic analysis. The following amino acid sequences were used to make the phylogenetic tree: *MRJP1* from *Cx. quinquefasciatus* (XP_001850268.2), *MRJP1* from *Apis mellifera* (NP_001011579.1), *MRJP1* from *Aedes aegypti* (XP_021693794.1), *MRJP1-like* from *Sabethes cyaneus* (XP_053682299.1), *MRJP1-like isoform X2* from *Anopheles funestus* (XP_049285312.1), *yellow* from *Cx. quinquefasiatus* (CQUJHB005397), *yellow* from *D. melanogaster* (FBgn0004034), *yellow-like* from *Ae. albopictus* (XP_019534039.2), *yellow-like* from *Anopheles maculipalpis* (XP_050069861.1), *yellow-like* from *An. merus* (XP_041787038.1), *yellow-like* from *An. nili* (XP_053679995.1), *yellow-like isoform X1* from *An. funestus* (XP_049285311.1), *yellow-like* from *An. moucheti* (XP_052891110.1), *yellow-like* from *An. marshallii* (XP_053669181.1), *yellow-like* from *An. stephensi* (XP_035901855.1), *yellow-like* from *An. cruzii* (XP_052868860.1), *yellow-like* from *An. aquasalis* (XP_050087457.1). The following paralogs from *D. melanogaster* were also included: *yellow-b* (FBgn0032601), *yellow-c* (FBgn0041713), *yellow-d* (FBgn0041712), *yellow-d2* (FBgn0034856), *yellow-e* (FBgn0041711), *yellow-e2* (FBgn0038151), *yellow-e3* (FBgn0038150), *yellow-f* (FBgn0041710), *yellow-f2* (FBgn0038105), *yellow-g* (FBgn0041709), *yellow-g2* (FBgn0035328), *yellow-h* (FBgn0039896).

Sequence alignment was performed using Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) and phylogenetic

analyses were conducted using the maximum likelihood method in MEGA 11 software, with bootstrap values calculated from 500 trees (35). To characterize the protein composition and domains, we conducted an InterProScan search (https://www.ebi.ac.uk/interpro/search/sequence/).

2.2 Mosquito rearing

A colony of *Culex pipiens* established in June 2013 from Columbus, Ohio (Buckeye strain) was used in this experiment. From the time that they were first instar larvae and until the adults were collected for analyses, mosquitoes were reared at 18°C and exposed to either long-day, diapause-averting conditions (photoperiod of Light: Dark 16:8 hr) or short-day, diapause-inducing conditions (photoperiod of L:D 8:16 hr). Larvae were reared in plastic containers filled with reverse osmosis water and were fed a diet of ground fish food (Tetramin Tropical Fish Flakes) according to the procedure described by Meuti et al. (36). To optimize our metabolomic procedure and allow us to acquire spectra from single mosquitoes, we conducted a preliminary experiment where mosquito pupae were transferred to cages that contained sugar water only. For all subsequent experiments, pupae from both the long and short-day photoperiods were randomly and equally divided into two cages, one that contained sugar water only (control) and one that contained 2 g of Royal Jelly (Starkish) that was dissolved in 1.5 mL of 10% sucrose solution (experimental treatment). Adult mosquitoes consumed their prescribed dietary treatments *ad libitum* (4 treatments total; n≅150 adults/treatment). One week after peak adult emergence, mosquitoes were euthanized and collected for experimental analyses.

2.3 Measuring the abundance of *MRJP1* mRNA

Quantitative real time PCR (qRT-PCR) was used to assess how the abundance of *CpMRJP1* was affected by supplementing the diet of adult mosquitoes with royal jelly. The procedure for qRT-PCR was based on (37). We designed primers for *CpMRJP1* (CPIJ008700-RA) using Primer3 (Forward: TGAACGATCGTC TGCTGTTC; Reverse: TCCTCCCACATGGTATCGTT; (38). A standard curve verified that the primers met the MIQE guidelines (39). RNA was isolated from female mosquitoes (n=5 females/biological replicate; 5 biological replicates/rearing condition and feeding treatment) one week following adult emergence using TRIzol according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using the SuperScript III kit (Invitrogen), following the manufacturer's instructions. All qRT-PCR reactions were done in triplicate on a 96-well plate using a CFX Connect qRT-PCR machine (BioRad). Each well contained a 10 µL reaction, consisting of 5 µL of iTaq Universal SybrGreen Supermix (BioRad), 400 nM of forward and reverse primers for either *CpMRJP1* or our reference gene (*Ribosomal Protein 19; RpL19;* 39), 3.2 µL of molecular grade water, and 1 µL of cDNA. qRT-PCR reactions were completed through an initial denaturation at 94°C for 2 min, followed by 40 cycles at 94°C for 15 sec and 60°C for 1 min. A melt curve was run after each qRT-PCR reaction to ensure that only a single PCR product was produced. The abundance of *CpMRJP1* transcripts was normalized to the abundance of *RpL19* using the $2^{-\Delta CT}$ method as previously described (40). We ran a model to ensure that the abundance of *RpL19* mRNA did not differ significantly among dietary treatments ($p = 0.778$) and therefore was a suitable reference gene for this study.

2.4 Assessing diapause status

To assess the diapause status of long and short day-reared females that had consumed sugar water (control) or diets that included royal jelly, we used two common markers of diapause: egg follicle length and fat content. One-week after adult emergence, we randomly euthanized 20 female mosquitoes from each dietary and rearing treatment and dissected their ovaries and egg follicles in a 0.9% saline solution (NaCl). The length of 10 egg follicles/female were measured at 200 times magnification using an inverted microscope (Nikon; n = 20 females/treatment). We also randomly selected eight, one-week-old females from the same cohorts and dietary treatments and measured the fat content in each female mosquito using a Vanillin lipid assay (41) that was modified to allow us to measure samples using a plate reader (42). The data were normalized by dividing the measured lipid content by the lean mass of the whole-body mosquito (lean mass = µg mosquito wet mass - µg of lipid).

2.5 Measuring protein content

As royal jelly is protein-rich, we also wanted to determine whether supplementing the diet with royal jelly affected the whole-body protein content of female mosquitoes. The protein content within eight, randomly selected individual female mosquitoes from the same cohort and dietary treatments as the lipid and egg follicle treatments was measured using a Bradford Assay kit (BioRad) following the manufacturer's instructions (43). In brief, each female mosquito was weighed and then homogenized in 200 µL of a 10% ethanol solution. Samples were added in triplicate to a 96-well plate, and 250 µL of Quick Start Bradford 1X Dye Reagent (BioRad) was added to each well. The absorbance of each sample was measured using a FLUOStar Omega Microplate Reader. Measurements were normalized by dividing the protein content by the wet mass of each female mosquito (44).

2.6 Evaluating longevity in the absence of food

In the field, diapausing females are able to survive for 3–6 months in without access to food (45). Therefore, we wanted to determine how dietary conditions affected the lifespan of female mosquitoes in the absence of food. Long and short-day reared

mosquito pupae were placed in cages with access to sugar water or royal jelly (4 treatments total). One week after peak adult emergence, all male mosquitoes as well as the royal jelly or sugar-water food sources were removed from each cage. The remaining females (n = 70 – 75 females/treatment) were counted and allowed constant access to water. Every week thereafter we counted and removed dead females from the cage until no female mosquitoes remained.

2.7 Performing metabolomic analyses

The experimental protocol for all metabolomic analyses employed tissue extraction followed by Nuclear Magnetic Resonance (NMR) analysis and was based on the procedure described in Wu et al. (46). One mosquito was weighed and placed in a 2 mL microtube with approximately 750 mg of ceramic beads. For our initial experiment, 3 short-day reared, diapausing and 3 long-day reared, nondiapausing mosquitoes that had consumed sugar water only were used. For our second experiment, 10 independent female mosquitoes from each photoperiodic and dietary treatment, and from the same cohort as the egg follicle, fat content, and protein measurement experiments were randomly selected (n = 40 total). Mosquito samples were homogenized in 400 µL cold methanol and 85 µL water, and the homogenate was transferred to a separate tube without beads. Next, 400 µL chloroform and 200 µL water were added to the homogenate, which was then vortexed and centrifuged (2,000 rcf for 5 min at 4°C). The aqueous (methanol) layer was isolated and collected in a new 1.5 mL microtube before being dried in an evaporator. Deuterium oxide (heavy water), trimethylsilylpropanoic acid (TSP), and boric acid were added to the evaporated extracts and vortexed. The pH of the samples was manually adjusted to 7.4 and then transferred to 5 mm NMR tubes.

The metabolites in each mosquito sample were measured using NMR spectroscopy following the procedure detailed in (47). 1D ^{1}H NOESY spectra were obtained for the aqueous extracts. In addition, one ^{1}H-^{13}C HSQC spectrum of a pooled sample was acquired for the four separate dietary and photoperiodic conditions according to (48). Avance III HD 800 and Ascend 850 MHz spectrometers, each with an inverse cryoprobe and z-gradients (Bruker BioSpin, Billerica, MA), were utilized to obtain NMR measurements, and resulting NMR spectra were analyzed as described in Newell et al. (47) and (49). Topspin 3.6.1 and AMIX 3.9.15 software (Bruker BioSpin, Billerica, MA) were used for preprocessing. The untargeted approach of spectral binning was chosen, where each spectrum is divided into small sections, or bins, which are subsequently analyzed. It should be noted that this approach provides broad coverage across various classes of metabolites, as compared to a targeted approach where only a predefined subset of metabolites of interest is quantified. In the initial experiment to optimize the protocol, 1D NMR spectra were binned with a bin width of 0.005 ppm. In contrast, for the second experiment that evaluated the effects of consuming diets that included royal jelly, the bin width was lowered to 0.003 ppm because these measurements were taken using a higher-frequency instrument (850MHz). Spectra were binned using the R package mrbin [Version 1.5.0; (50)}. Signals that showed large inter-spectra chemical shift differences were manually added to broader bins. Noise signals were automatically removed, and data was scaled using PQN (probabilistic quotient normalization) to correct for differences in sample mass and extraction efficacies. Each bin was then scaled to unit variance. Principal Component Analysis (PCA) models were generated to visualize metabolic data and screen for outliers regarding data quality. Signals of interest were identified using public databases and identifications were validated using the acquired HSQC spectrum and measurements of pure samples.

2.8 Assessing the effect of knocking down *MRP1* with RNAi

RNA interference (RNAi) was used to knock down *CpMRJP1* mRNA to evaluate how this protein affects the diapause status of female mosquitoes. The procedure for RNA interference was based on the protocol detailed in (37). Double-stranded RNA (dsRNA) specific to *CpMRJP1* and *Beta-galactosidase* from *E. coli* (*β-gal*; control) were synthesized using the Promega T7 RNAi Express Kit according to the manufacturer's instructions. We selected *β-gal* dsRNA as a control because mosquitoes do not consume lactose and hence genes encoding *β-gal* proteins are absent from their genomes, and because we have previously used *β-gal* dsRNA as a control in RNAi experiments (37, 40). We designed primers to synthesize a 230 bp fragment of *CpMRJP1* (CPIJ008700-RA) in *Cx. pipiens* using Primer3 (Forward: CACCGCCAAACCGAACAAAT; Reverse: TGAGCAGCCCAAAGTACAGG; 37), which served as the template to create dsRNA. On the day of adult emergence, 3 µg of either *β-gal* or *CpMRJP1* dsRNA was injected into the thorax of long and short day-reared mosquitoes. Following injection, females were placed into small plastic containers (4.62 x 6.75 x 7.19 inches) where they consumed 10% sucrose solution *ad libitium*. To confirm gene knockdown, RNA was isolated using Trizol according to the manufacturer's instructions from 3 biological replicates each containing 5 whole-body, female mosquitoes that were euthanized two days after dsRNA injection. cDNA was synthesized and qRT-PCR was conducted as described above (section 2.3), except that we used *CpMRJP1* qRT-PCR primers that were previously designed by Sim et al. (18), and that after normalizing *CpMRJP1* expression to the *RpL19* reference gene, *CpMRJP1* expression was again normalized to its expression in *β-gal*-dsRNA injected mosquitoes from the same photoperiodic condition (40).

To determine how *CpMRJP1* dsRNA affected seasonal phenotypes, the egg follicle lengths (n = 20) and fat content (n = 8) of randomly selected females were measured ten days following dsRNA injection as described above (section 2.4). After feeding on sugar water for one week, the lifespan of *β-gal* or *dsMRJP1* dsRNA-injected females (n = 37 – 49 females/treatment) in the absence of food was also measured as described above (section 2.5).

2.9 Data analysis

All data analyses were conducted in R version 3.3.3 (51). Two-way ANOVAs and Tukey's *post-hoc* tests were used to determine

whether dietary treatment and/or photoperiod significantly affected *CpMRJP1* mRNA abundance, egg follicle length, lipid content, and protein content in female mosquitoes. Student's T-tests were used to determine whether injecting *CpMRJP1* dsRNA effectively reduced *CpMRJP1* mRNA abundance, while two-way ANOVAs were used to determine whether dsRNA injection and photoperiod significantly affected the egg follicle length or fat content. A value of alpha < 0.05 was applied to discern statistical significance.

To determine how supplementing the diet with royal jelly and knocking down *CpMRJP1* with RNAi affected the longevity of female mosquitoes in the absence of food, we used Cox-proportional hazards models (survival package) that included the effects of diet and photoperiod. Hazard ratios were obtained from these models as an estimate of the ratio between the risk of dying between photoperiodic and dietary treatments, where negative hazard ratios (HR) indicate a protective effect. Kaplan Meier survival curves were also plotted and used to provide the median survival time or the time at which 50% of the population was still alive as defined by (52, 53), and log-rank tests were used to determine significant differences between treatments.

For analysis of NMR metabolomics data, for each spectral bin, a general linear model (GLM) was created to account for the effect of diet (sugar water or royal jelly), photoperiod (long or short day-rearing conditions), and the interaction term between diet and photoperiod. To correct resulting p-values multiple testing, we used a False Discovery Rate (FDR) of 5% (54). Signals that were significant in the GLM analysis were tested for pairwise group differences using Tukey's Honest Significant Difference (HSD) test. An Artificial Neural Network (ANN) was generated using R packages keras (version 2.11.1) and tensorflow (version 2.11.0). A dense network was generated with one input neuron per NMR bin (N=1823), 200 hidden neurons with ReLU activation function, and 2 output neurons, representing "short day" and "long day" rearing conditions. The ANN was trained on the data from the "sugar water" group using leave-one-out cross validation. Samples from the "royal jelly" group were then predicted using the trained ANN.

Pathway enrichment analysis was performed by downloading KEGG pathway maps of *Culex pipiens pallens* with KEGG code cpii (55) using the R package KEGGREST (version 1.36.3). Fisher's Exact Test was used to assess the number of matching metabolites per pathway. A pathway uniqueness score *u* was calculated as follows: For each pathway, each observed metabolite was assigned the inverse of the number of pathways in which this metabolite occurs organism-wide, then the maximum value was chosen as the pathway uniqueness score. Therefore, higher values of *u* indicate pathways with more metabolites that uniquely occur in that respective pathway, and we further investigated pathways with $u \geq 0.2$.

3 Results

3.1 Phylogenetic analysis of *Culex pipiens MRJP1*

To identify the evolutionary relationships of *Cx. pipiens* MRJP1 with MRJP/yellow homologues in other insects, we conducted a phylogenetic analysis using amino acid sequences of MRJP/yellow proteins from dipteran species. First, we conducted a BLAST search using the protein sequence of *Cx. quinquefasciatus* MRJP1 (CPIJ008700) and extracted the amino acid sequences of the top hits from dipteran species. We also obtained the protein sequences of Yellow in *Cx. quinquefasciatus* and *D. melanogaster*, as well as 13 Yellow-like proteins in *D. melanogaster* from Vectorbase (56). Multiple sequence alignment was performed to compare sequence identity between species. The sequence identity between *Cx. quinquefasciatus* MRJP1 and Yellow from *D. melanogaster* was found to be 23.41%, while it was 21.39% with Yellow from *Cx. quinquefasciatus*. This result is consistent with the high identity of 20–30% shared between MRJP and Yellow paralogs in *D. melanogaster*, suggesting a common evolutionary origin (57). Additionally, we performed a sequence identity comparison between MRJP1 proteins from *Cx. quinquefasciatus* and *Apis mellifera* to gain insights into the evolutionary and functional aspects of this protein. Our findings unveil that MRJP1 in these species share 129 identical amino acid residues, corresponding to a percent identity of 26.88%. Through alignment of the amino acid sequences between Yellow and MRJP1 in *Cx. quinquefasciatus*, *D. melanogaster*, and *Apis mellifera*, we identified a consensus conserved MRJP domain in all four sequences, indicating the preservation of the MRJP domain throughout insect evolution (Figure 1A).

We also conducted a functional analysis of *Cx. quinquefasciatus* MRJP1 using InterProScan and found that it belongs to the Royal jelly/protein yellow family in the InterPro database and the MRJP family in the Pfam database. This result is consistent with previous findings indicating that all Yellow proteins have a conserved MRJP domain (58). Subsequently, we used these sequences to create a phylogenetic tree to discover evolutionary relationships (Figure 1B). The percentage of replicate trees in which the associated H3 clustered together in 500 bootstrap replicates is shown adjacent to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. In the resulting phylogenetic tree, Yellow proteins in *Cx. quinquefasciatus* and *D. melanogaster* were found to be closely related and clustered together with all other Yellow paralogs in *D. melanogaster*. As expected, *Cx. quinquefasciatus* MRJP1 closely clustered with MRJP/Yellow-like proteins from other dipteran species identified in the BLAST top matches. However, MRJP1 was found to be farther away from Yellow from *Cx. quinquefasciatus* and *D. melanogaster* and other Yellow paralogs from *D. melanogaster* as well as MRJP1 in *A. meliferra* (Figure 1B). The distant relationship between Yellow of *Cx. quinquefasciatus* and *D. melanogaster* and MRJP1 in *Cx. quinquefasciatus* revealed by our phylogenetic analysis suggests that MRJP1 likely has different functions and targets than Yellow in *D. melanogaster*.

3.2 Measuring *MRJP1* mRNA abundance in response to rearing and dietary conditions

The relative abundance of *MRJP1* mRNA did not change significantly in response to dietary treatment or photoperiodic conditions (Figure 2A). A two-way ANOVA revealed that there

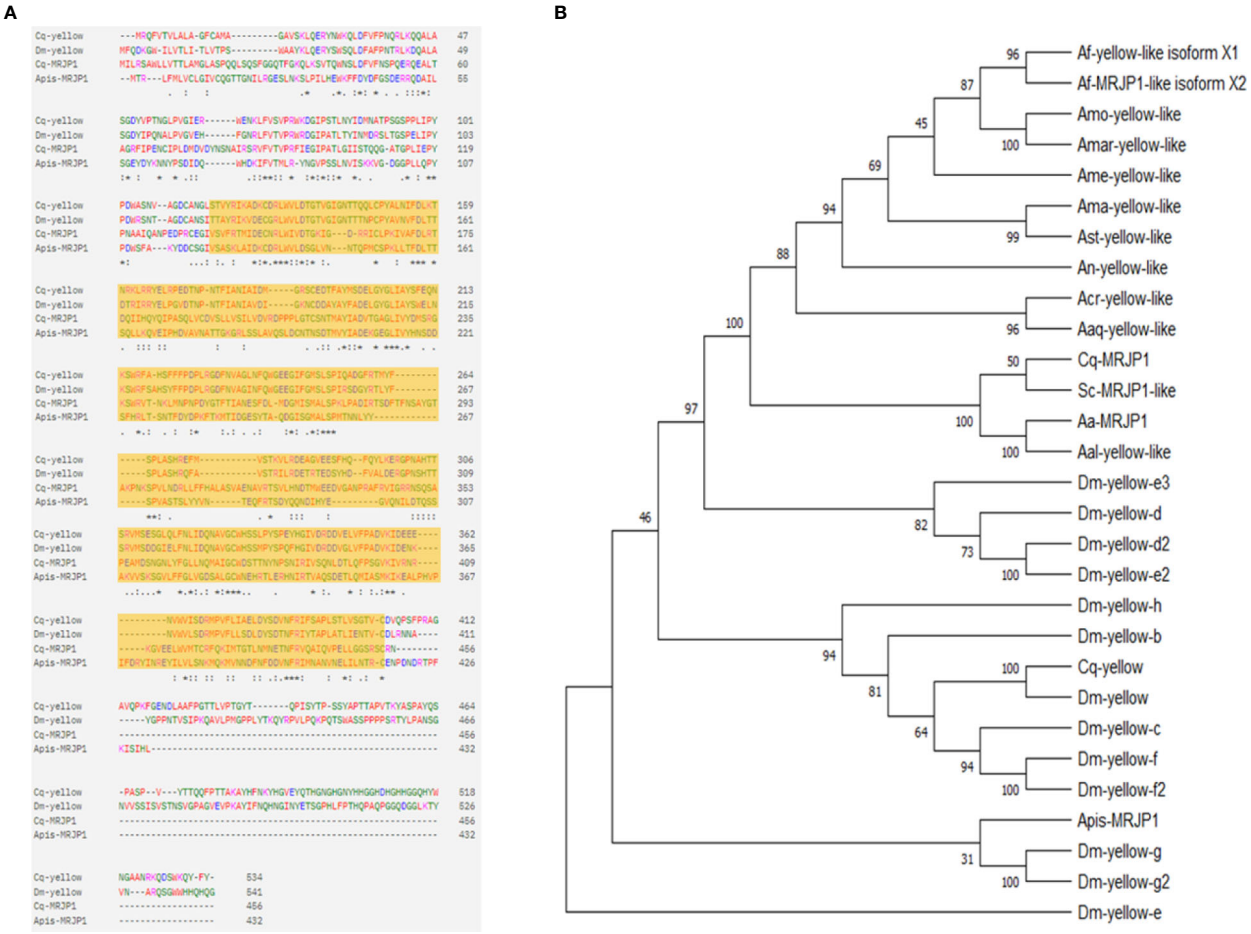


FIGURE 1
Phylogenetic analysis and sequence alignment of MRJP/Yellow family proteins' sequences in insect species. **(A)** Sequence alignment on the amino acid sequences of Yellow and MRJP1 proteins from *Culex quinquefasciatus*, *Drosophila melanogaster*, and *Apis mellifera*. The highlighted region indicates the conserved MRJP domain present in all four sequences; the . : or * indicate amino acid residues that are conserved across 2, 3 or 4 sequences. **(B)** The phylogenetic tree was generated using MEGA v.11 maximum likelihood estimation with bootstrap analysis. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test 500 replicates are shown next to the branches. Apis, *Apis mellifera*; Aal, *Aedes albopictus*; Ast, *Anopheles stephensi;* An, *Anopheles nili*; Acr, *Anopheles cruzii*; Aa, *Aedes aegypti*; Amo, *Anopheles moucheti*; Amar, *Anopheles marshallii*; Sc, *Sabethes cyaneus*; Aaq, *Anopheles aquasalis*; Af-yellow, *Anopheles funestus*; Ame, *Anopheles merus*; Cq, *Culex quinquefasciatus*; Ama, *Anopheles maculipalpis*; Af, *Anopheles funestus*; Dm, *Drosophila melanogaster*.

was not a statistically significant interaction between the effects of diet and photoperiod on the abundance of *CpMRJP1* mRNA ($F_{1,16}$ = 0.589, p = 0.458). Simple main effects analysis showed that diet did not have a statistically significant effect on *CpMRJP1* abundance ($F_{1,16}$ = 0.025, p = 0.876). Contrary to previous experiments (18), there was no significant difference in the abundance of *MRJP1* transcripts between females reared in long day or short-day conditions ($F_{1,16}$ = 2.67, p = 0.122), likely due to high levels of variation in *CpMRJP1* transcript abundance in the short-day reared females.

3.3 Assessing the effects of royal jelly on mosquito diapause status and lifespan

Egg follicle length can be used to determine diapause status of female mosquitoes of *Cx. pipiens* (5,8), such that an average egg follicle length of less than 75 µm indicates diapause, follicle lengths between 75 and 90 µm indicates an intermediate state, and follicles greater than 90 µm indicates nondiapause (42). We used a two-way ANOVA to evaluate the effects of both photoperiod and diet on egg follicle size. Our model revealed that both photoperiod ($F_{1,76}$ = 1008.2, p < 0.001) and diet ($F_{1,76}$ = 95.32, p < 0.001) independently affected egg follicle length. Moreover, there was a significant interaction between photoperiod and diet on egg follicle length ($F_{1,76}$ = 78.4; p < 0.001). As expected, one week after adult emergence all long-day reared females that consumed sugar water (controls) were in a clear nondiapause state (Figure 2B; average egg follicle length of 99.5 ± 1.8 µm). However, 50% of long-day reared females that consumed diets including royal jelly had egg follicles that were characteristic of being in diapause, while the remaining females were in an intermediate state, and none of the long day-reared females that had consumed diets including royal jelly had egg follicle lengths that were large enough to be considered

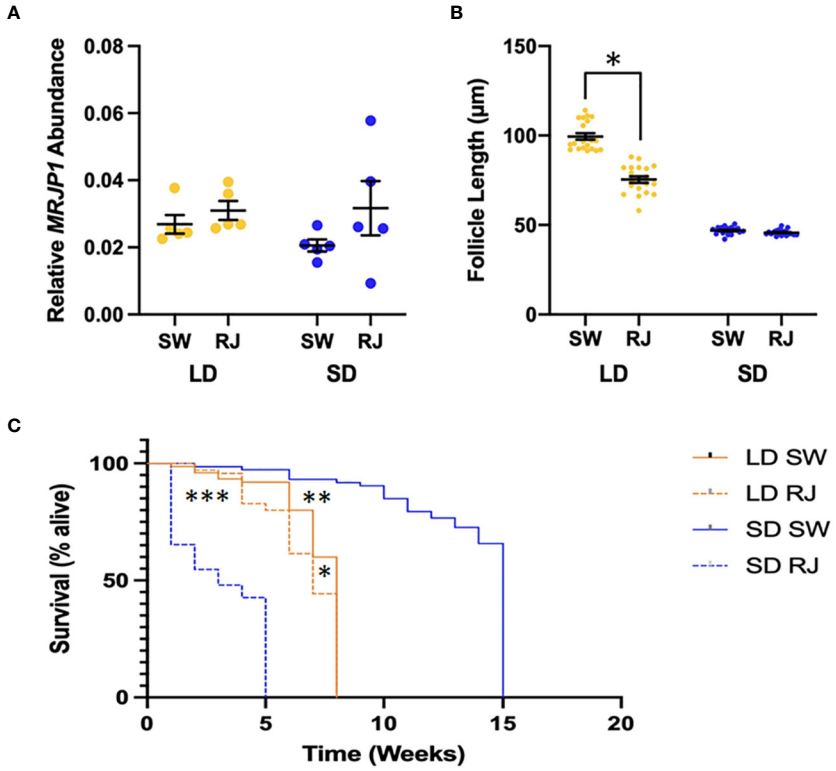


FIGURE 2
Phenotypic effects of consuming royal jelly (RJ) in mosquitoes. **(A)** Consuming RJ did not have any significant effect on relative *MRJP1* mRNA abundance in long day (LD) or short day (SD) reared mosquitoes. **(B)** Consuming RJ significantly decreases egg follicle length in long day (LD) mosquitoes. Significant difference denoted by * (Tukey's *post-hoc* test; $p < 0.001$). **(C)** The lifespan of female mosquitoes was significantly different between long day (LD) and short day (SD) mosquitoes. The consumption of royal jelly (RJ) by mosquitoes in both rearing conditions led to a significant decrease in lifespan. Significant differences denoted by *** (SD RJ to SD SW; $p < 0.001$), ** (LD SW to SD SW; $p < 0.001$) and * (LD RJ to LD SW; $p = 0.03$).

in a nondiapause state (Figure 2B). Therefore, consuming royal jelly caused a significant decrease in the overall average egg follicle length in long-day reared females (average egg follicle length of 75.4 ± 1.8 µm; Tukey's *post-hoc* test, $p < 0.001$, 95% CI = [19.3, 28.9]). Females reared in short-day conditions had significantly smaller egg follicles compared to those reared in long-day conditions (Tukey's *post-hoc* test, $p < 0.001$, 95% CI = [-43.7, -38.5]). All females reared in short-day conditions were in a clear diapause state (Figure 1B), regardless of whether they consumed sugar water only (46.9 ± 0.4 µm) or diets that included royal jelly (45.7 ± 0.3 µm); therefore dietary treatment did not effect the egg follicle length of dietary treatment did not significantly impact egg follicle length of short day-reared females (Tukey's *post-hoc* test, $p = 0.92$).

We also used a two-way ANOVA to assess whether dietary treatment and/or photoperiod affected the fat content of female mosquitoes (Supplementary Figure S2A), as previous studies demonstrate that diapausing females accumulate significantly higher levels of fat than nondiapausing mosquitoes (37, 42). Our analyses revealed that neither photoperiod ($F_{1,28} = 0.378$, $p = 0.54$) nor diet ($F_{1,28} = 0.567$, $p = 0.458$) affected fat content. However, there was a slight but non-significant interaction between photoperiod and diet ($F_{1,28} = 3.91$, $p = 0.058$). Short day-reared females that consumed sugar water had higher levels of lipid relative to those that consumed royal jelly, although this result was not statistically significant (SD RJ = 22.19 ± 2.46% lipid; SD SW = 29.48 ± 4.75% lipid; Tukey's HSD, $p = 0.822$).

A two-way ANOVA of the effects of photoperiod and diet on protein content revealed that photoperiod ($F_{1,25} = 4.97$, $p = 0.035$) but not diet ($F_{1,25} = 0.03$, $p = 0.865$) significantly affected the protein levels within female mosquitoes. Our analyses also revealed that there was no significant interaction between photoperiod and diet on protein content ($F_{1,25} = 0.03$, $p = 0.576$). Females reared in long-day conditions had roughly the same amount of protein whether they consumed sugar water or diets that included royal jelly (Supplementary Figure S2B; average protein content LD RJ = 12.16 ± 0.76 µg/mg; LD SW = 12.60 ± 1.41 µg/mg; Tukey's HSD, $p = 0.99$). Dietary treatment did not significantly affect the protein content of short-day reared females (SD RJ = 10.22 ± 0.98 µg/mg; SD SW = 9.33 ± 1.32 µg/mg; Tukey's HSD; $p = 0.95$). However, females reared in short-day conditions contained significantly less protein than long-day reared females (Tukey's HSD, $p = 0.034$, 95% CI = [-5.04, -0.200]).

As diapausing females can survive for prolonged periods without without food (reviewed in 11), we also assessed the starvation resistance of mosquitoes reared in different photoperiodic conditions that consumed either sugar water or diets including royal jelly for seven days prior to food removal (Figure 2C). Our Cox proportional hazards model revealed that photoperiod

significantly impacted survival time in the absence of food, such that short-day reared females were significantly less likely to die relative to long-day reared females (Supplementary Table S1; $z = -8.427$; $p < 0.001$). Although consuming diets that include royal jelly did not significantly impact survival time relative to feeding on sugar water (Supplementary Table S1; $z = 1.48$; $p = 0.138$), there was a strong interaction between photoperiod and diet, such that under short-day conditions, consuming diets that included royal jelly significantly increased the risk of death ($z = 12.3$; $p < 0.001$). The median survival time of long-day reared females that consumed sugar water was eight weeks of age, while the median survival time of sugar-fed, short-day reared mosquitoes was 15 weeks (Figure 2C), and the risk of dying was significantly lower for short-day reared females (Tukey's HSD, $z = 8.47$, $p < 0.001$). However, the opposite was observed with female mosquitoes that consumed diets including royal jelly; the median survival time of long-day reared mosquitoes that consumed royal jelly was 7 weeks while the median survival time of short-day reared, royal jelly-fed mosquitoes was 3 weeks, showing that under short-day conditions consuming diets that include royal jelly significantly increased the risk of dying (Tukey's HSD, $z = 10.58$; $p < 0.001$). Under long-day conditions, consuming royal jelly did not affect the risk of dying (Tukey's HSD, $z = 1.482$; $p = 0.4486$), whereas under short-day conditions, consuming diets that included royal jelly reduced the median survival time by 12 weeks and significantly increased the risk of death (Tukey's HSD, $z = 13.27$; $p < 0.001$).

3.4 Characterizing the effects of royal jelly on the metabolomic profile of long and short day-reared mosquitoes

Preliminary data collected during initial optimization of the metabolomics protocol suggests that there are largescale differences in the metabolic profile of diapausing and nondiapausing *Cx. pipiens* (Supplementary Figure S2A). A Principal Component Analysis (PCA) reveals partial group separations for both the photoperiodic conditions and consuming royal jelly, indicating that both factors affect the metabolism of female mosquitoes (Supplementary Figure S2B). It should be noted that PCA does not search for differences between groups of interest, but it rather separates samples according to signals of largest variance. Frequently in metabolomics studies, confounding factors obscure PCA group separations by adding to the overall variance of the dataset. Therefore, additional analyses were performed to find differential signals. General linear models (GLM) revealed that 168 out of 1823 spectral signals significantly changed in response to at least one of the following: photoperiod, diet, or their interaction (Supplementary Table S1). Figure 3A shows a heatmap of all signals that significantly changed. Notably, nondiapausing mosquitoes that consumed sugar water exhibit strong metabolic differences compared to diapausing mosquitoes that also consumed sugar water (Supplementary Table S1; Figure 3A). Visual inspection revealed that mosquitoes reared under long-day, diapause-averting conditions switch to a "diapause-like" metabolic profile after consuming royal jelly. In contrast, females reared under short-day, diapause-inducing conditions switched to a "nondiapause-like" metabolic state after consuming royal jelly (Supplementary Table S2; Figure 3A). To confirm these qualitative observations, an artificial neural network (ANN) was trained on the sugar water group to predict long-day or short-day rearing conditions. Using leave-one out cross validation of each SW sample (Supplementary Table S2) reveals that the ANN correctly predicted most short-day ("diapause") and long-day ("nondiapause") samples in the sugar water group. However, when using the ANN to predict the photoperiod of mosquitoes that had consumed royal jelly (Supplementary Table S3), most short-day samples were predicted as being long-day, and most long-day samples were predicted to be short-day. The ANN results thus confirm that consuming royal jelly switches the metabolic profile of long-day reared females to be "diapause-like" and short-day reared females to be "nondiapause-like."

Among the significant signals (Supplementary Table S2), six metabolites could be unambiguously identified using existing information in NMR databases (Supplementary Table S3; Figure 3B). Short day-reared, diapausing mosquitoes that consumed sugar water and long day-reared mosquitoes that consumed royal jelly had significantly higher levels of pimelic acid, asparagine, and choline; but significantly lower levels of L-alanine, histidine, and glycogen, compared to long-day mosquitoes that consumed sugar water (Figure 3B; see Supplementary Table S3 for GLM coefficients and Tukey's HSD p-values). Identical trends are seen in mosquitoes reared in short-day, diapause-inducing conditions that consumed sugar water (significant in all except asparagine). In contrast, short day-reared mosquitoes that consumed royal jelly showed opposite metabolic trends when compared to short-day, sugar-fed controls; however, this difference was only significant for choline and pimelic acid.

Metabolic pathway enrichment results indicate that several pathways were affected by photoperiod and diet. When plotting the negative decadic logarithm of the p-value versus the uniqueness score u, pathways of interest are expected to be found toward the top ($p \leq 0.05$) or the right ($u \geq 0.2$) in the resulting scatterplot (Figure 3C). Pathways of high interest were found to be Alanine, Aspartate and Glutamate Metabolism ($p = 0.0044$, $u = 0.5$), Biotin Metabolism ($p = 0.036$, $u = 0.5$), Starch and Sucrose metabolism ($u = 1.0$), Glycerohospholipid metabolism ($u = 0.33$), and Histidine Metabolism ($u = 0.33$). Some pathways such as Aminoacyl-tRNA biosynthesis, ABC transporters, Sulfur relay system, and D-amino acid metabolism were significant. However, these significant differences were caused by repetitive metabolic reactions in these pathways and/or non-specific reactions, therefore we chose to treat these results as statistical artifacts and do not further discuss them.

3.5 Effects of *CpMRJP1* dsRNA on mosquito diapause status and survival

A knockdown confirmation analysis was performed to determine if *CpMRJP1* dsRNA significantly reduced the abundance of *CpMRJP1* mRNA transcripts (Figure 4A). We found that *MRJP1* dsRNA significantly reduced the abundance of *CpMRJP1* transcripts in both long-day (71% reduction in transcript

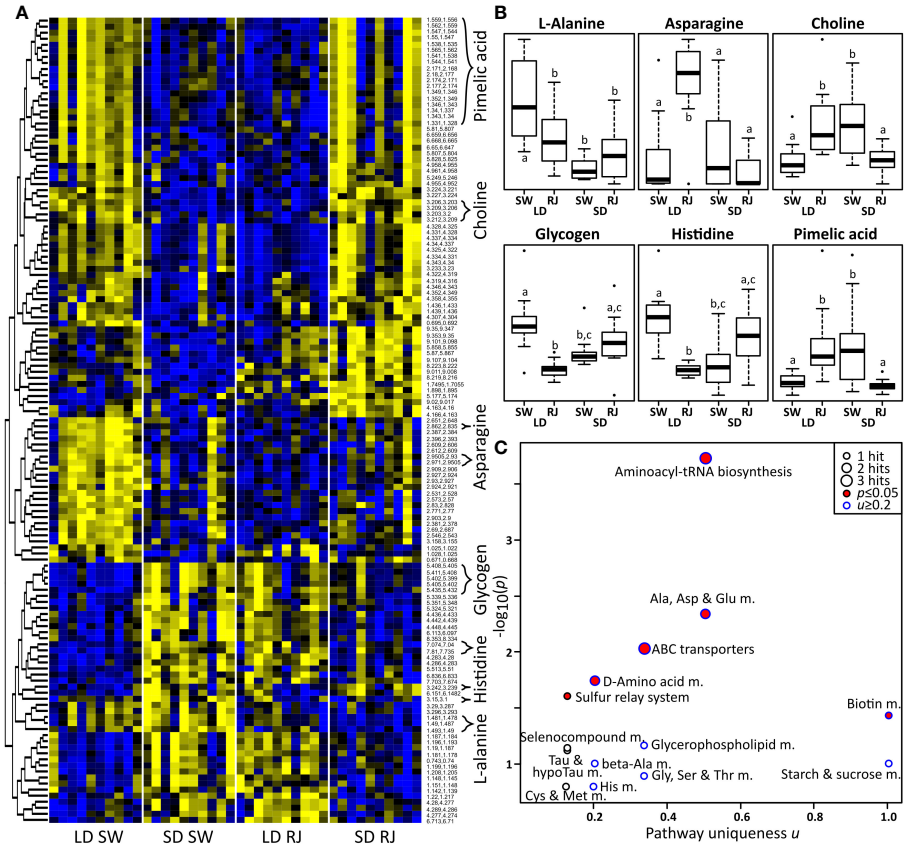


FIGURE 3
Consuming royal jelly reverses seasonal differences in the mosquito metabolome. **(A)** Heat map of NMR signals that were significantly different between treatment groups. Signals that were unambiguously identified are labeled with the respective metabolite name. Yellow represents metabolites that were highly abundant, while blue represents metabolites that were less abundant. **(B)** Boxplots of significantly altered metabolites (arbitrary units). Different superscript letters indicate differences with $p \leq 0.05$. **(C)** Pathway enrichment analysis plot. RJ, royal jelly; SW, sugar water; SD, short-day, diapause-inducing conditions; LD, long-day, diapause-averting conditions; m., metabolism; Tau, taurine.

abundance relative to *β-gal* dsRNA-injected controls; T = 4.75; p = 0.009) and short-day reared females (74% reduction in transcript abundance relative to *β-gal* dsRNA-injected controls; T = 7.40, p = 0.0018).

Knocking down *CpMRJP1* dsRNA significantly affected egg follicle length in short-day reared females (Figure 4B). A two-way ANOVA was used to determine how photoperiod and dsRNA injection affected egg follicle length, and revealed that both photoperiod ($F_{1,\ 76}$ = 394; p < 0.001) and dsRNA treatment ($F_{1,\ 76}$ = 15.4; p = 0.0019) had significant effects. Moreover, there was a significant interaction between photoperiod and dsRNA treatment ($F_{1,\ 76}$ = 28.3; p <0.001). All females reared in long-day conditions that were injected *β-gal* and *CpMRJP1* dsRNA were in a clear nondiapause state, such that the average egg follicle length of *β-gal* dsRNA-injected (100.7 ± 0.5 µm) and *CpMRJPI* dsRNA-injected mosquitoes (98.1 ± 2.5 µm) were not significantly different (Figure 3B; Tukey's HSD; p = 0.76). Females reared in short-day conditions that were injected with *β-gal* dsRNA were in a clear diapause state, with an average egg follicle length of 53.3 ± 0.4 µm. However, the average egg follicle length of females reared in short-day conditions that were injected with *CpMRJP1* dsRNA was 70.7 ± 2.7 µm, and females were found to be in a mixture of diapause (70%), intermediate (25%), and nondiapause (5%) states. Overall, knocking down *CpMRJP1* significantly increased egg follicle length in short-day reared females (Tukey's HSD; p < 0.001; 95% [CI = 10.4, 24.4]).

We also used a two-way ANOVA to determine whether photoperiod and knocking down *CpMRJP1* affected fat content (Supplementary Figure S3B). Our analyses revealed that dsRNA injection did not affect fat content ($F_{1,\ 26}$ = 2.28, p = 0.143). However, photoperiod had a significant impact on fat content ($F_{1,\ 26}$ = 4.47; p = 0.045), such that *β-gal* and *MRJP1* dsRNA-injected, short-day reared females had higher levels of fat than dsRNA-injected long-day reared females. This was largely driven by a non-significant trend where females reared in long-day conditions and injected with *β-gal* dsRNA had slightly less fat (6.45 ± 0.78%) than long day-reared mosquitoes injected with *CpMRJP1* dsRNA (10.97 ± 0.40%; Tukey's HSD, p = 0.181), and short-day reared mosquitoes injected with *β-gal* dsRNA (Tukey's HSD, p = 0.092) or *CpMRJP1* dsRNA (Tukey's HSD, p = 0.072). Our 2-way ANOVA also confirmed that that there was not a significant interaction between photoperiod and dsRNA injection ($F_{1,\ 26}$ = 2.12, p = 0.157).

We also investigated how injection with *MRJP1* dsRNA affected the lifespan of long and short day-reared females relative to *β-gal*

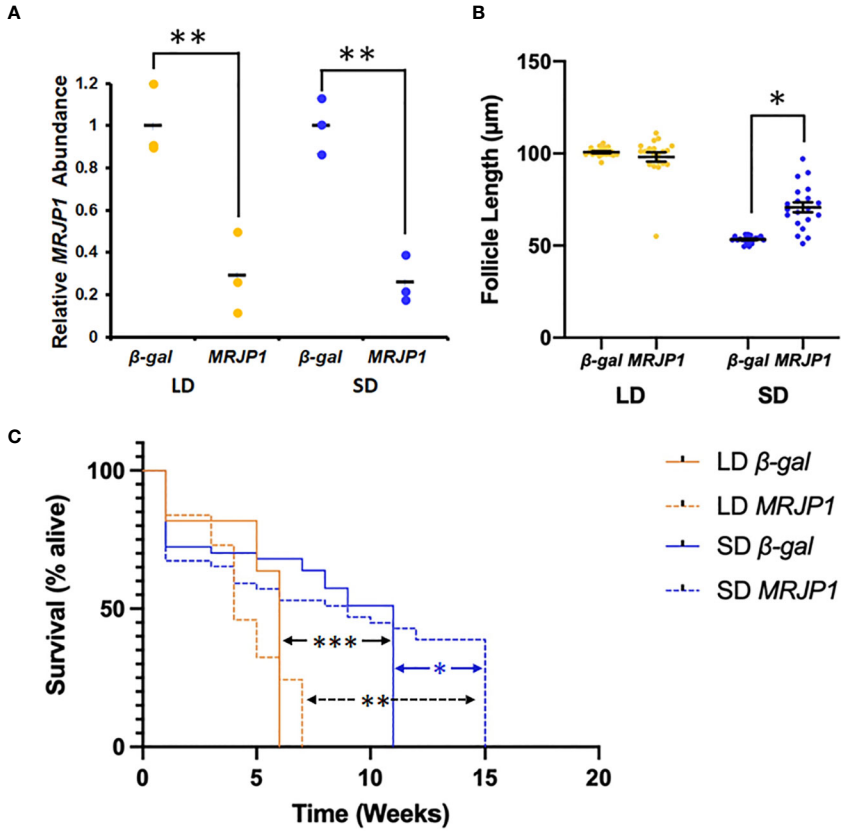


FIGURE 4
dsRNA against *MRJP1* affects seasonal phenotypes in mosquitoes. **(A)** Treatment with dsRNA for *MRJP1* significantly reduced the abundance of relative *CpMRJP1* mRNA abundance relative to *β-gal* dsRNA-injected controls in long day (LD) or short day (SD) mosquitoes. Significant difference denoted by * ($p < 0.01$). **(B)** dsRNA against *MRJP1* caused a significant increase in egg follicle length in short day (SD) mosquitoes. Significant difference denoted by * ($p < 0.001$). **(C)** dsRNA against *MRJP1* significantly affected the lifespan of (SD) conditions. Significant differences denoted by *** (LD *β-gal* to SD *β-gal*; $p < 0.001$), ** (LD *MRJP1* to SD *MRJP1*; $p < 0.001$) and * (SD *β-gal* to SD *MRJP1*; $p = 0.0187$).

dsRNA-injected controls in the absence of food (Figure 4C). A Cox proportional hazards model revealed that short photoperiods had a protective effect on female lifespan in the absence of food (Supplementary Table S1; HR = -1.31 ± 0.209; z = -6.28, $p < 0.001$), while knocking down *CpMRJP1* had a slight, but non-significant protective effect (HR = -0.307 ± 0.167; z = -1.83; $p = 0.0668$). The median survival time of long-day reared, *β-gal* dsRNA-injected females was six weeks while the median survival time of short-day reared, *β-gal* dsRNA-injected females was eleven weeks (Tukey's HSD, z = -6.28, $p < 0.001$). The median survival time of long-day reared, *CpMRJP1* dsRNA-injected females was four weeks, while the median survival time of short-day reared, *CpMRJP1* dsRNA-injected females was nine weeks (Tukey's HSD, z = -1.314, $p < 0.001$). While knocking down *CpMRJP1* did not significantly affect the likelihood of dying in females reared in long-day conditions relative to *β-gal* injected controls (Tukey's HSD, z = -1.833, $p = 0.258$), dsRNA against *CpMRJP1* significantly extended the total lifespan in short-day conditions (Figure 4C; all *β-gal*-injected females were dead at 11 weeks; all *MRJP1*-dsRNA injected females were dead at 15 weeks; Log Rank Test of survival differences: $X^2_1 = 5.9$, $p = 0.02$).

4 Discussion

Our phylogenetic analyses show that CpMRJP1 forms a unique clade with MRJP's in the paddled-beauty mosquito, *Sabethes cyaneus*, and *Ae. aegypti* (Figure 1B). Additionally, our results demonstrate that ingesting royal jelly reverses some, but not all, of the phenotypes associated with diapause in female mosquitoes. Specifically, female mosquitoes reared in long-day, diapause-averting conditions that consumed a diet including royal jelly enter a diapause-like state with small egg follicles (Figure 2B). In contrast, consuming a diet that includes royal jelly significantly reduced the lifespan of females reared in short-day, diapause-inducing conditions (Figure 2C). While consuming a diet containing royal jelly does not cause significant differences in fat content within long or short day-reared mosquitoes (Supplementary Figure S1A), it alters the metabolic profile of mosquitoes (Figure 3; Supplementary Figure S2; Supplementary Table S1). Specifically, long-day reared mosquitoes that consumed a diet containing royal jelly were metabolically similar to diapausing controls, and there were no significant differences in the relative abundance of 5/6 metabolites we identified between these two

groups (Figure 3B; Supplementary Table S2). In contrast, short-day reared mosquitoes that consumed a diet containing royal jelly were metabolically similar to nondiapausing controls as there were no significant difference in any of the identified metabolites between long-day reared females that consumed sugar and short-day reared females that consumed diets with royal jelly (Figure 3B; Supplementary Table S2). As royal jelly is a complex mixture containing several proteins, carbohydrates, lipids and vitamins, it is currently not clear how royal jelly might be mediating these effects. However, it is likely that some of the traits we observed are induced through the action of MRJP1. This is because knocking down *CpMRJP1* significantly increased the egg follicle lengths of short-day reared females (Figure 4B) and allowed them to live significantly longer than *β-gal* dsRNA-injected controls in the absence of food (Figure 4C).

Transcripts encoding CpMRJP1, an ortholog of MRJP1 in *Apis mellifera* that is the most abundant protein in royal jelly, were upregulated in diapausing females (18). Another study demonstrated that consuming royal jelly increased the likelihood that alfalfa leaf cutting bees would enter diapause (25). Therefore, we hypothesized that consuming diets that include royal jelly, and thereby artificially increasing the levels of AmMRJP1 within mosquitoes, would induce diapause phenotypes in long-day reared mosquitoes. Although our current study did not show any significant differences in *CpMRJP1* mRNA abundance between diapausing and nondiapausing, sugar-fed mosquitoes (Figure 2A), we found that long day-reared females that consumed diets containing royal jelly had significantly smaller egg follicles (Figure 2B). This indicates that feeding female mosquitoes royal jelly in conditions that typically prevent diapause causes them to arrest reproductive development. However, in contrast to our results, consuming royal jelly promotes reproductive development in honey bee queens (15) and fruit flies (23). As other species also become more virile and fecund upon consuming royal jelly (22, 23), there must be a separate, unique pathway through which royal jelly acts in *Cx. pipiens* to confer reproductive arrest. We believe these effects are mediated through CpMRJP1 because knocking down this transcript stimulated reproductive development in mosquitoes reared in short day, diapause-inducing conditions (Figure 4B).

Reproductive arrest is not the only indicator of diapause, as diapausing females of *Cx. pipiens* also display an increase in fat content (37, 42, 59). We observed a trend where long-day reared females that consumed royal jelly had slightly higher fat content than females that consumed sucrose solution (Supplementary Figure S1A). However, due to high variation within our samples, the fat content was not significantly different between rearing conditions or food source. Typically, the increase in fat content is a consequence of the feeding habits of female mosquitoes; short-day reared females gorge on nectar that is rich in sugar (5, 6, 9) which they then convert into lipids (9). In contrast, nondiapausing mosquitoes do not accumulate substantial levels of fat (9, 37, 42). These differences are not likely not driven by lipid deposition into egg follicles as other researchers found that female mosquitoes of *Ae. aegypti* only deposit lipids into their egg follicles several hours after blood-feeding (60), and none of the mosquitoes in our study were given access to vertebrate blood. Although it is unclear why we did not observe significant increases in the fat content of diapausing females relative to nondiapausing sugar-fed controls, the absence of an effect of consuming diets that include royal jelly could be the result of nutritional differences in royal jelly (14), and/or because female mosquitoes in the royal jelly treatment may not have consumed as much food as the sugar-fed, control mosquitoes. Unfortunately, we did not measure how much food the mosquitoes consumed in our experiments. However, we conducted follow-up experiments demonstrating that long and short-day reared mosquitoes die within six days of adult emergence if they are provided access to only water. All our phenotypic measurements were collected from females that were seven-days old, indicating that they must have consumed at least some of their respective diets.

As royal jelly contains a high proportion of protein (13, 14), we chose to examine whether protein content changed between females that consumed diets that included royal jelly relative to those who fed on only sucrose (Supplementary Figure S1B). Consuming diets containing royal jelly did not affect the protein content of long or short-day reared mosquitoes, possibly because females in the royal jelly treatment may not have consumed as much food as those in the sucrose treatment as discussed above. However, we did find that females reared in long-day conditions have significantly greater protein content than those reared in short-day conditions. Early in diapause, females of *Cx. pipiens* produce fewer proteins than they do upon diapause termination (61). Furthermore, diapausing female mosquitoes are relatively inactive and take refuge in protected shelters (62), so they would not require as much protein to power their flight muscles.

In addition to determining how supplementing the diet of mosquitoes with royal jelly would affect seasonal phenotypes, we used RNAi to elucidate the functional role of CpMRJP1 in diapausing females of *Cx. pipiens*. We were able to successfully knock-down the relative abundance of *CpMRJP1* transcripts in both long and short-day reared *Cx. pipiens* (Figure 4A). However, knocking down *CpMRJP1* only induced phenotypic effects in short-day reared females, where it significantly increased the egg follicle length of 7-day old mosquitoes such that approximately 30% of the females sampled were categorized as being in an intermediate or nondiapause state (Figure 4B). Therefore, knocking down *CpMRJP1* causes short-day reared females to avert diapause, and again suggests that the gene encoding CpMRJP1 plays a critical role in arresting egg follicle development during diapause induction in females of *Cx. pipiens*.

Knocking down *CpMRJP1* did not lead to a significant change in fat content of females reared in short-day conditions (Supplementary Figure S3B), although, we observed a trend in which the long day-reared females that were injected with *CpMRJP1* dsRNA had a slight, but non-significant, higher level of fat compared to *β-gal* dsRNA-injected controls. This is surprising, seeing as *CpMRJP1* mRNA is upregulated in diapausing mosquitoes that acquire high levels of fat (18), but we found that the fat content slightly increased when *CpMRJP1* was knocked down with RNAi. Overall, the female mosquitoes that were injected with *CpMRJP1* or *β-gal* dsRNA had lower levels of fat (Supplementary Figure S3B) than the female mosquitoes that were not injected and allowed to

consume sugar water in the initial dietary experiment (Supplementary Figure S1A). Therefore, we conclude that injecting mosquitoes likely caused some minor injuries that impaired their ability to consume the sucrose source that was within their cage. These results, combined with the effects of consuming royal jelly, suggest that CpMRJP1 may not be directly involved in accumulating fat during diapause and rather that this protein regulates reproductive development and/or starvation resistance.

Consuming diets that contained royal jelly for one week prior to food removal significantly reduced the median survival time in the absence of food for both long and short-day reared mosquitoes, relative to mosquitoes that consumed 10% sucrose for one week before food removal (Figure 2C). The decrease in survival was most dramatic and pronounced in mosquitoes reared in short-day, diapause-inducing conditions where consuming diets that included royal jelly reduced the median lifespan by 12 weeks. This is a surprising result, seeing as royal jelly increases the lifespan of honey bees and fruit flies (14, 23). Additionally, in short-day conditions, knocking down *MRJP1* with RNAi increased lifespan (Figure 4C). Although *CpMRJP1* dsRNA-injected mosquitoes initially died sooner and had a lower median survival time than *β-gal* dsRNA injected controls, knocking down *CpMRJP1* in short day-reared mosquitoes extended their lifespan by four weeks. Taken together, our data suggest that a factor within royal jelly, possibly MRJP1, may reduce the starvation resistance of diapausing mosquitoes.

We found six metabolites that were significantly differentially abundant between diapausing and nondiapausing mosquitoes as well as those that had consumed royal jelly (Figure 3B). It is obvious that consuming royal jelly caused largescale changes in whole mosquito metabolomes that partially reversed the seasonal phenotypes of the mosquitoes (Figure 3A), and these results were further supported by artificial neural network predictions. In addition, mosquitoes reared in short-day, diapause-inducing conditions that consumed diets that included royal jelly had a metabolic profile that was similar to nondiapausing mosquitoes, such that both long-day reared, sugar-fed controls and short-day reared, royal jelly-fed mosquitoes had significantly lower levels of asparagine, choline, and pimelic acid as well as higher levels of glycogen, and histidine (Figure 3B). These metabolic results are consistent with the phenotypes we observed, where ingesting diets that contained royal jelly both induced diapause phenotypes in long-day reared mosquitoes (e.g., reduced egg follicle length) and caused short-day reared females to exhibit nondiapause phenotypes (e.g., reduced starvation tolerance).

A pathway enrichment analysis (Figure 3C) revealed several metabolic pathways that were affected by day length and/or royal jelly consumption. One of the affected pathways is the biotin metabolism pathway, with its metabolite pimelic acid being upregulated during diapause and in long day-reared mosquitoes that consumed royal jelly (Figure 3B). Pimelic acid is a precursor of biotin (vitamin B7) that can be made during fatty acid synthesis, specifically, it has been linked to the FabF enzyme in the bacteria *Bacilus subtilis* (63). While FabF is also part of fatty acid synthesis in *Cx. pipiens*, and fatty acid synthesis is upregulated in diapause (5), no pimelic acid synthesis has been previously reported for this organism, and further experiments would be required to investigate this possibility. Pimelic acid is also a metabolite in mosquito microbiomes (64). Therefore, microbial contributions may explain the observed change. Supplementing the diets of honey bee with pimelic acid has been shown to decrease stress responses and increasing survival times (65). Decreased levels of pimelic acid could thus partially explain the decreased life expectancy of short day-reared mosquitoes that consumed royal jelly. The observed increase in pimelic acid in diapause-inducing conditions also indicates a potential microbial contribution to mosquito diapause.

Our analyses also show that the starch and sucrose metabolism pathway was similarly affected (Figure 3C), with glycogen being less abundant in diapause and in long-day reared mosquitoes that consumed diets that included royal jelly (Figure 2B). A previous study reports metabolic flux of dietary glucose toward glycogen in diapausing *Cx. pipiens* (66). However, that study did not analyze glycogen in nondiapausing animals and thus cannot be directly compared to our data. In accordance with our results, Zhou and Meisfeld (67) found that glycogen decreases during the first weeks of diapause in *Cx. pipiens* as compared to nondiapausing mosquitoes, with a simultaneous increase in body fat. Our findings suggest that consuming diets that contain royal jelly significantly reduced the diapause-associated catabolism of glycogen in short-day reared mosquitoes.

The alanine, aspartate and glutamate metabolism (AAGM) pathway was also significantly affected. One metabolite in this pathway, L-alanine, was less abundant in diapausing mosquitoes and females reared in long-day conditions that consumed royal jelly (Figure 3B). Alanine can, via pyruvate and the glycolysis/gluconeogenesis pathway, feed into starch and sucrose metabolism. Therefore, our finding that the abundance of L-alanine decreased diapausing mosquitoes is consistent with the observed drop in glycogen as discussed above. Alanine is also downregulated in diapausing *N. vitripennis* (28), but is more abundant in diapausing flesh flies, where it likely functions as a cryoprotectant (27). We also found that asparagine, another metabolite of the AAGM pathway, was significantly upregulated in long-day reared mosquitoes that consumed royal jelly (Figure 3B). Asparagine was upregulated in diapausing pupae of the moth *Antheraea pernyi* (68) but was less abundant in diapausing larvae of the parasitoid *N. vitripennis* (28). Together with the downregulation of L-alanine, these results suggest that L-aspartate is preferably converted into L-asparagine rather than L-alanine in diapausing *Cx. pipiens* to meet their differing metabolic needs.

Glycerophospholipid metabolism also changed across photoperiods and dietary treatments (Figure 3C), such that choline was upregulated in diapausing mosquitoes and in long-day reared mosquitoes that consumed diets including royal jelly (Figure 3B). As choline is a precursor of phosphatidylcholine, the observed differences suggest that there are differences in phospholipid synthesis during diapause. This could indicate that diapausing insects are catabolizing phospholipids to generate precursors for triglycerides, which would be consistent with previous studies that have shown that triglyceride stores increase during diapause in *Cx. pipiens* (5, 9) and other insects

(reviewed in 69). However, this finding could also indicate that diapausing insects are synthesizing more and/or different phospholipids. Previous studies have demonstrated that diapausing insects increase the concentration of unsaturated fatty acids within cell membranes to increase membrane fluidity and enhance cold tolerance (69–71). Future studies are necessary to distinguish whether phospholipid catabolism and/or anabolism are occurring within diapausing *Cx. pipiens*.

Both photoperiod and diet affected histidine metabolism (Figure 3C), such that histidine was significantly downregulated in diapausing *Cx. pipiens* and long-day reared mosquitoes that consumed diets containing royal jelly (Figure 3B). Although histidine is upregulated in diapausing *N. vitripennis* (28), histidine is less abundant in diapause-destined larvae of the cotton bollworm, *Heliocoverpa armigera* (72). In pre-diapausing *H. armigera*, down-regulating histidine likely leads to lower levels of its byproduct histamine, an inhibitory neurotransmitter, that may alter the photoperiodic responses necessary for diapause induction (72).

Notably, royal jelly contains multiple proteins, lipids, vitamins, and carbohydrates (13), and any of these components could have directly or indirectly altered mosquito reproductive development, starvation resistance and metabolic profiles. Our RNAi experiments support the findings of our feeding experiments; specifically, consuming royal jelly (thereby artificially increasing the abundance of AmMRJP1 within mosquitoes) decreased egg follicle length in long-day reared mosquitoes, whereas knocking down *CpMRJP1* increased egg follicle length short-day reared females. Taken together, this suggests that higher levels of MRJP1 promotes reproductive arrest. However, at this time we still do not know precisely where and when *CpMRJP1* is being expressed or how it might be mediating these effects. It is also unclear if consuming AmMRJP1 on its own or other components in royal jelly promotes the metabolomic switch that we observed. Therefore, future studies are necessary to characterize the mechanism by which MRJP1 promotes reproductive arrest in *Cx. pipiens* and to delineate the effects of MRJP1 from other components in royal jelly.

5 Conclusions

This study demonstrates that consuming diets that include royal jelly has opposing effects on phenotypes associated with diapause in *Cx. pipiens*. In long-day reared mosquitoes, these include suppressing reproductive development and causing the metabolomic profile to resemble that of diapausing females. In contrast, in short-day reared females, consuming diets that include royal jelly reduces starvation resistance and shifts the metabolomic profile to be more similar to long-day reared mosquitoes. As royal jelly is a complex mixture of multiple proteins, sugars, lipids, vitamins and other substances (13), it is currently unclear how consuming royal jelly switches seasonal phenotypes in *Cx. pipiens*; but we can conclude that this effect on diapause was mediated at least in part by MRJP1. This is because knocking down *CpMRJP1* caused females that were reared in short-day, diapause-inducing conditions to avert diapause and develop significantly larger egg follicles and to live significantly longer than *β-gal* dsRNA injected controls. As diapausing females of *Cx. pipiens* do not bite humans and other animals (6), they do not transmit debilitating diseases (62). Future work should investigate whether it would be possible to develop control measures that use royal jelly to induce diapause in female mosquitoes during the long days of summer to reduce disease transmission. Additionally, future work should be done to elucidate whether AmMRJP1 alone or other components of royal jelly induce reproductive arrest, cause largescale metabolic shifts and alter mosquito starvation resistance. Such studies will not only uncover the underpinnings of the interesting results we observed in this study, but may also lead to exciting insights on the molecular regulation of seasonal responses in other insects and animals.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author/s.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

OB: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. AA: Investigation, Methodology, Writing – review & editing. MK: Data curation, Formal Analysis, Investigation, Methodology, Software, Supervision, Visualization, Writing – review & editing. XW: Investigation, Methodology, Software, Visualization, Writing – review & editing. CS: Investigation, Methodology, Software, Supervision, Visualization, Writing – review & editing. MM: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Context-specific variation in life history traits and behavior of *Aedes aegypti* mosquitoes

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Aedes aegypti, the vector for dengue, chikungunya, yellow fever, and Zika, poses a growing global epidemiological risk. Despite extensive research on *Ae. aegypti*'s life history traits and behavior, critical knowledge gaps persist, particularly in integrating these findings across varied experimental contexts. The plasticity of *Ae. aegypti*'s traits throughout its life cycle allows dynamic responses to environmental changes, yet understanding these variations within heterogeneous study designs remains challenging. A critical aspect often overlooked is the impact of using lab-adapted lines of *Ae. aegypti*, which may have evolved under laboratory conditions, potentially altering their life history traits and behavioral responses compared to wild populations. Therefore, incorporating field-derived populations in experimental designs is essential to capture the natural variability and adaptability of *Ae. aegypti*. The relationship between larval growing conditions and adult traits and behavior is significantly influenced by the specific context in which mosquitoes are studied. Laboratory conditions may not replicate the ecological complexities faced by wild populations, leading to discrepancies in observed traits and behavior. These discrepancies highlight the need for ecologically relevant experimental conditions, allowing mosquito traits and behavior to reflect field distributions. One effective approach is semi-field studies involving field-collected mosquitoes housed for fewer generations in the lab under ecologically relevant conditions. This growing trend provides researchers with the desired control over experimental conditions while maintaining the genetic diversity of field populations. By focusing on variations in life history traits and behavioral plasticity within these varied contexts, this review highlights the intricate relationship between larval growing conditions and adult traits and behavior. It underscores the significance of transstadial effects and the necessity of adopting study designs and reporting practices that acknowledge plasticity in adult traits and behavior, considering variations due to larval rearing conditions. Embracing such approaches paves the way for a comprehensive understanding of contextual variations in mosquito life history traits and behavior. This

integrated perspective enables the synthesis of research findings across laboratory, semi-field, and field-based investigations, which is crucial for devising targeted intervention strategies tailored to specific ecological contexts to combat the health threat posed by this formidable disease vector effectively.

KEYWORDS

Aedes aegypti, context-specific variation, life history traits, behavioral plasticity, transstadial effects

1 Introduction

The global epidemiological risk associated with *Aedes aegypti* is a significant concern. This invasive mosquito species is a crucial vector for several mosquito-borne viruses causing dengue, chikungunya, yellow fever, and Zika. These viruses are responsible for frequent outbreaks of diseases, leading to morbidity and mortality and substantial economic burdens worldwide. Dengue, in particular, has become a significant public health concern over the past decade, with approximately 3.9 billion people at risk of infection in over 128 countries (1, 2). Dengue incidence in the Americas, Southeast Asia, and the Western Pacific regions surged to approximately 5.5 million in 2020, as reported by the Pan American Health Organization (3). This worrisome upward trajectory has persisted, with 4.6 million dengue cases in the Americas alone in 2023, and over 9.3 million cases already reported as of June 2024 (4). In the United States, where cases were typically associated with international travel, the local transmission of dengue fever was reported in Arizona, California, Florida, Hawaii, and Texas in 2023, raising new challenges for local public health departments. Besides posing health risks, the cumulative economic costs of mitigating *Aedes*-borne diseases from 1975 to 2020 are estimated at 310.8 billion USD worldwide (5).

Ae. aegypti is the primary vector of dengue, chikungunya, Zika, and yellow fever viruses. *Ae. albopictus* also serves as a vector for these arboviruses, contributing to their transmission in various regions (6, 7). Since 1920, the estimated global abundance of *Ae. aegypti* has risen by approximately 9.5%, and future projections indicate a 30% increase by the end of the 21st century (8). By 2080, *Ae. aegypti* is predicted to be reported in as many as 162 countries, including countries documenting their presence for the first time (9).

Given these escalating risks, understanding the adaptability of *Ae. aegypti* to changing conditions becomes paramount for understanding its success as one of the most invasive mosquito species. Adaptability in *Ae. aegypti* encompasses genetic variation and phenotypic plasticity, each playing crucial roles in the mosquito's ability to respond to environmental changes. Genetic variation provides the raw material for natural selection, enabling populations to evolve over time (10, 11). Influenced by larval environments, phenotypic plasticity allows individual mosquitoes to adjust their traits in response to immediate conditions. Moreover, plasticity can evolve within populations over time, potentially interacting with genetic differences. This means that the degree of genetic differentiation underlying various traits can vary within a population, leading to intrapopulation diversity in plastic responses that enhance adaptability (12, 13).

The plasticity of traits throughout their life cycle allows them to respond dynamically to environmental changes (14, 15). However, despite the acknowledged importance of adaptations and trait plasticity in *Ae. aegypti*, significant gaps persist in how we investigate and perceive behavior and life history trait variations, given the contextual complexity arising from heterogeneous study design and methodology. It is important to note that not all plastic responses are adaptive; some may arise from physiological or environmental constraints (16). Understanding genetic and plastic contributions to adaptability provides a comprehensive view of how *Ae. aegypti* can thrive in diverse environments.

While this review primarily focuses on *Ae. aegypti*, it is essential to consider insights from studies on other mosquito species to understand phenotypic variation and adaptability comprehensively. For instance, studies on *Ae. albopictus* and other mosquito species have highlighted similar adaptive responses to environmental pressures, suggesting broader patterns that can inform our understanding of *Ae. aegypti* (7). These comparisons can reveal fundamental principles of mosquito biology and adaptation, enhancing our ability to predict and manage vector populations.

This review highlights such knowledge gaps, specifically leveraging findings across laboratory, semi-field, and field-based investigations (17). By integrating insights from various *Aedes* species and other mosquitoes, this review emphasizes the importance of adopting study designs and reporting practices that acknowledge plasticity in adult behavior while also considering variation arising from differences in larval and adult traits due to larval growing conditions, also referred to as transstadial effects. Embracing such approaches paves the way for a comprehensive understanding of contextual variation in mosquito life history traits and behavior. This integrated perspective enables the synthesis of research findings across different study contexts, ultimately improving our capacity to devise targeted intervention strategies in the field tailored to specific ecological contexts to effectively combat the health threat posed by this formidable disease vector.

2 Variation in life-history traits

Ae. aegypti exhibits considerable variation in life history traits, contributing to its adaptability and vector potential. This review focuses on several key life history traits, including longevity, fecundity, adult body size, age of reproduction, and reproductive effort. These traits are influenced by both genetic and environmental factors, with phenotypic plasticity playing a crucial role in the mosquito's adaptability. Understanding these variations is crucial because they provide insights into the mechanisms driving the adaptability and invasiveness of *Ae. aegypti*.

Ae. aegypti is a globally distributed and highly variable species, with significant variation in traits both within and among populations. Differences in genetic composition and environmental conditions across various geographical regions can lead to substantial differences in traits such as fecundity, longevity, development time, and vector competence (18, 19). This intra-species variability influences how different populations respond to environmental pressures and control measures, and it can affect disease transmission dynamics and invasion potential (20).

Mosquito life history traits and behavior have been well studied across multiple species (Figure 1A). Among these studies, population-specific variation in traits have been documented, showing that mosquitoes from different regions exhibit varying levels of insecticide resistance, which can impact the effectiveness of control strategies (21). Additionally, the genetic diversity within populations can influence their capacity to adapt to new environments, making some populations more successful invaders than others (22). Therefore, it is essential to consider genetic and environmental factors when studying the life history traits and vector potential of *Ae. aegypti*.

Several studies have documented adaptive plasticity in life-history traits, resistance to desiccation and insecticides, preference towards urban environments, and degree of anthropophily. However, the influence of environmental and physiological factors (i.e., context-specificity) in shaping the variations in these plastic traits (i.e., the direction and magnitude of effects) needs more attention. Many studies have investigated the influence of temperature on plasticity in life history traits, so we will discuss these findings considering the abundance of available data (Figure 1B). Additionally, we will discuss context-specificity in neuroethological studies because sensory processes involved with host detection and location have been extensively studied.

Ae. aegypti predominantly thrives in habitats with temperatures ranging from 18°C to 38°C, with the median temperature ranging between 25°C and 32°C (23, 24). While they are also found in much colder and warmer habitats, temperatures between 18°C and 38°C facilitate their complete metamorphosis, survival, and reproduction. These temperatures account for approximately two-thirds of their current geographical range (25, 26). Not surprisingly, the poleward shifts in their global distribution are predicted to covary strongly with mosquitoes' adaptations to more extreme temperatures (26, 27).

The relationship between temperature and life history traits in *Ae. aegypti* is complex and often nonlinear. Many researchers view traits such as egg viability and larval survival as having optimal temperature ranges where the traits are maximized, with reduced viability and survival at temperature extremes (28, 29). For instance, egg viability typically peaks at intermediate temperatures and decreases at both lower and higher extremes (30). Similarly, larval survival rates tend to be highest within a moderate temperature range and drop off at temperatures outside this range, reflecting a nonlinear response (31). It is essential to recognize that temperature effects on most life history traits are better described by nonlinear or non-monotonic relationships, with trait performance often peaking at optimal temperatures and declining at suboptimal extremes.

While development rate is one of the few traits that might exhibit a more linear relationship with temperature within a limited range, even this relationship can become nonlinear at higher temperatures where development may fail due to mortality (32). The slope of this linear relationship corresponds to the cumulative effect of temperature variations on the development rate, and the intercept represents the theoretical temperature at which development ceases to occur, also known as the developmental zero (32, 33). However, empirical data from multiple studies suggests that this linear relationship is likely only true for mosquitoes developing within the median temperature range, i.e., 25°C and 32°C (34).

Outside the median temperature range, as temperatures approach the warmer or colder extremes, the magnitude of temperature-mediated effects scale non-linearly per unit change in temperature. For example, the egg hatch rates exhibited a non-linear decline with rising temperature, decreasing to 1.6% at 35°C. Similarly, lower temperatures also have had a non-linear impact on egg hatchability, albeit with a lesser magnitude of decline compared to higher temperatures: from 72% at 20°C to 55% at 18°C, 60% at 16°C, 53% at 14°C, and 43% at 12°C (30). Likewise, larval rearing at 27°C resulted in a pupation rate of 98.5% seven days post egg hatch, but this decreased to 97.2%, 87.4%, and 74.2% at 30°C, 33°C, and 35°C, respectively (35).

Even within the median temperature range, the relationship is often nonlinear when the effects of temperature have been studied in interaction with other environmental factors. For instance, larval competition and resource availability affect the temperature dependence of *Ae. albopictus*'s fitness (36). In particular, in resource-scarce or high-competition environments, the temperature facilitating optimal development and fitness drops by ~6°C. Furthermore, these interactive effects result in a ~10°C reduction in the width of *Ae. aegypti*'s thermal niche, *i.e.*, the range of temperatures that facilitates the species' survival and reproduction (37). To better visualize this context, Figures 2A–C presents a hypothetical illustration highlighting the differences between modeling the environment-trait-fitness relationship as linear versus nonlinear. Figure 2A depicts a classical linear environment-trait relationship. Figure 2B illustrates the nonlinear relationship, as discussed in the examples above on temperature-mediated effects on life history traits. Figure 2C shows how these environmentally-mediated trait variations shape mosquito fitness.

In addition, the interactions between temperature and relative humidity, together with variation in adult body size, strongly correlate with the longevity of adult *Ae. aegypti* mosquitoes (38). Low resource larval environments at 26.4°C resulted in females with shorter lifespans (6.9 days) compared to larval environments with

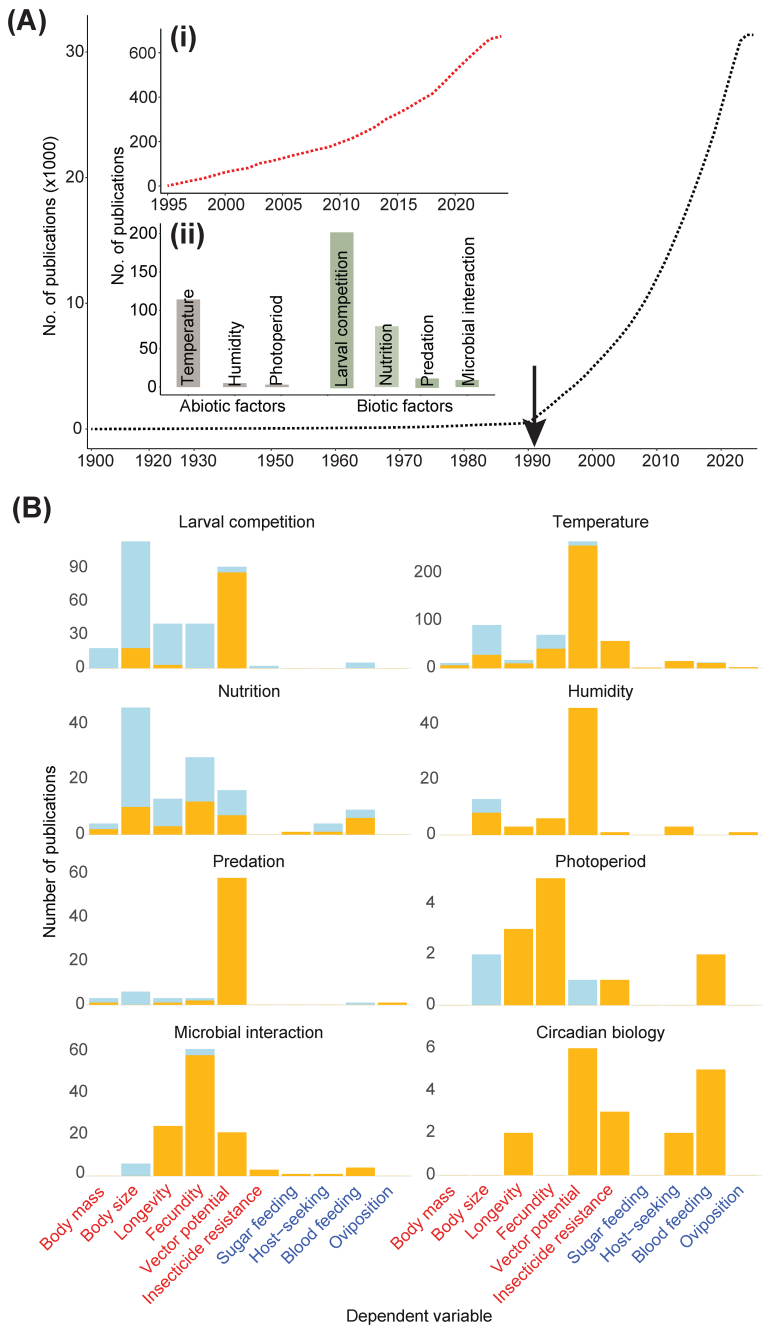

FIGURE 1
Trends in Mosquito Research Publications: **(A)** The cumulative number of publications from 1900 to 2024 focusing on life history traits and behavior of all mosquito species. The black arrow indicates the first description of the influence of transstadial effects. Insets: (i) Cumulative number of publications until 2024 specifically focused on *Ae. aegypti* traits and behavior. (ii) Cumulative number of publications investigating the impact of specific abiotic and biotic factors on *Ae. aegypti* life history and behavior. **(B)** A visualization of the literature trend investigating the effects of abiotic and biotic factors (independent variable) on larval and adult traits and behavior across all mosquito species. The bar plots in the left column represent the data on the number of studies investigating the effects of five biotic (larval competition, nutrition, predation, microbial interaction, circadian biology) and three abiotic factors (temperature, humidity, photoperiod) across all mosquito species. Along the X-axis are the most commonly investigated dependent variables; names denoted in red and blue denote adult traits and adult behavior, respectively. Yellow-shaded bars denote studies that have not considered the influence of transstadial effects on adult traits and behavior stemming from larval growing conditions. Blueshaded bars denote studies that have factored in transstadial effects. Data source: Clarivate Web of Science.

similar resource availability at 30.1°C (10.7 days) and 35.1°C (8.5 days) (38). Similarly, the time taken to reach pupation decreased progressively, exhibiting a non-linear decline, with durations of 21.97 days at 15.2°C, 14.46 days at 17.9°C, 9.83 days at 21.6°C, and 8.67 days at 25.3°C (39). These non-linear temperature-mediated effects highlight the complexity of *Ae. aegypti*'s response to temperature fluctuations and suggest potential analogous trends in other life-history responses to various biotic and abiotic factors.

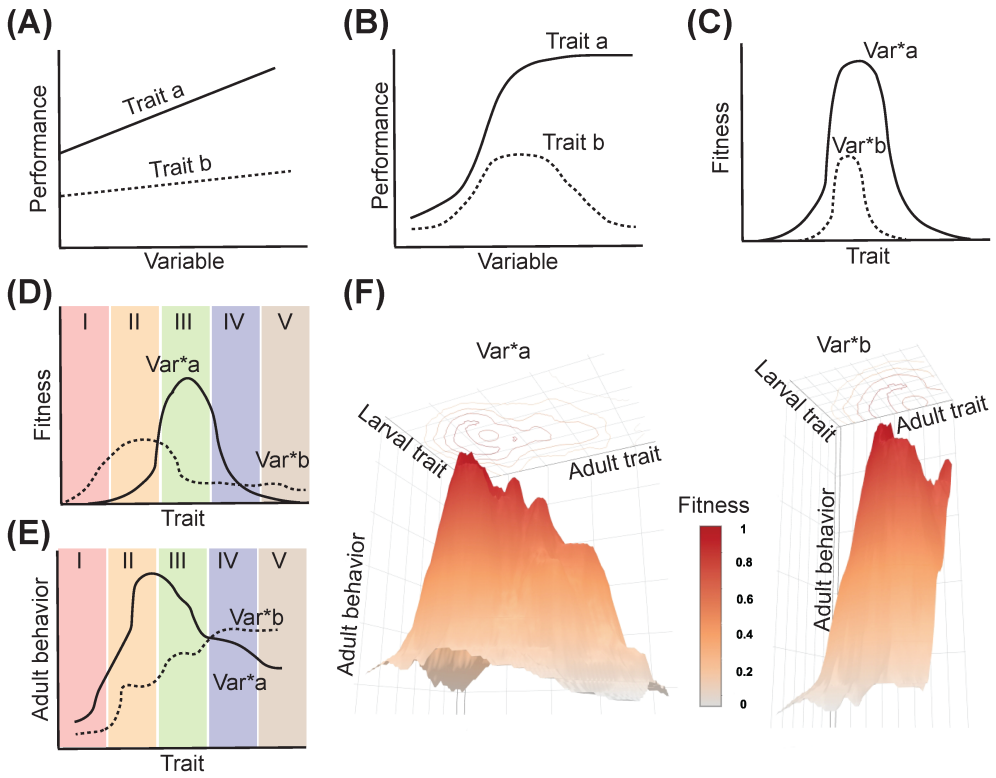

FIGURE 2
Adaptive trait-environment relationships in *Ae. aegypti*: **(A)** Classic representation of trait plasticity in response to environmental variables, **(B)** Representation of non-linear relationships between traits and environmental variables, **(C)** Influence of non-linear trait-environment relationships on mosquito fitness, **(D, E)** Existing methods fail to consider the covariation in larval and adult traits and its impact on adult behavior and fitness within the framework of environment-trait relationships. Sections I-V illustrate hypothetical segments of the overall trait distribution and demonstrate how sampling only a subset of this distribution affects the interpretation of relationships between fitness, adult behavior, and life-history traits, **(F)** A comprehensive framework that visualizes the data in **(D, E)** by depicting the environment-trait-fitness relationship while accounting for the covariations in larval and adult traits influenced by transstadial effects. This visualization assumes a 1:1 correlation between environment-trait variables and fitness; therefore, the color gradient mirrors the 3D surface. However, this correlation may vary across specific experiments. Asterik (*) symbol in **(C–F)** denote the interaction between two variables.

In the context of reproduction, higher temperatures lead to reduced egg production in *Ae. aegypti* females, reduced the latency to oviposition, and altered oviposition patterns. For instance, at 25°C and 80% humidity, *Ae. aegypti* females lived twice as long and produced 40% more eggs than at 35°C and 80% humidity. At high temperatures and high humidity, mosquitoes survived less and produced fewer eggs. At 35°C and 60% humidity, only 15% of females laid more than 100 eggs, and 45% of the females did not oviposit any eggs. Egg fertility also decreased with rising temperatures at lower humidity levels (40). The interaction between temperature and humidity plays a crucial role in the survival of eggs. High humidity levels enhance egg viability and hatching rates at optimal temperatures, while low humidity leads to desiccation and reduced viability, even if the temperature is within a favorable range (41). This interaction highlights the complexity of environmental factors affecting mosquito life history traits. Moreover, the magnitude of any larval environmental effect on adult traits differs between male and female mosquitoes due to protandry, whereby female mosquitoes exhibit a slower growth rate than their male counterparts (42, 43). Consequently, females spend more time in larval habitats, rendering the quality of larval growing conditions significantly more influential on female larval and adult traits than males. For example, suboptimal larval growing conditions generally lead to higher female mortality rates and a skewed sex ratio towards males (43). Density-dependent female larval mortality is also a critical determinant of adult body size and other traits (44). Thus, evaluating plasticity in mosquito traits requires a sex-specific approach.

Understanding the distribution of life history traits is essential for comprehending the biological and ecological factors contributing to *Ae. aegypti*'s success as an invasive species and a vector. Longevity and fecundity, for example, are directly related to the mosquito's ability to sustain and spread infections over time (45). Variation in the age of reproduction can lead to differences in generation time, affecting how quickly populations can grow and adapt to new environments (46). Adult body size is another critical factor influencing mosquitoes' survival, fecundity, and vector competence (23). Larger adult mosquitoes generally have higher fecundity and longer lifespans, making them formidable disease vectors (1). However, body size is highly influenced by larval rearing conditions, such as temperature, food availability, and density. For example, larval competition and limited resources can lead to smaller adult sizes, which may reduce individual survival and reproductive success (47). Notably, these factors can result in similar phenotypic outcomes through different mechanisms. The

reproductive effort reflects the balance between the number of offspring produced and the investment in each offspring's quality, significantly impacting population dynamics and resilience (1). These life history traits are influenced by both genetic and environmental factors, with phenotypic plasticity playing a crucial role in how *Ae. aegypti* adapts to varying conditions (14, 43). Therefore, deciphering the interplay between these environmental factors and life history traits enhances our comprehension of the complex biology of *Ae. aegypti* and sheds light on the adaptive mechanisms that make them such formidable disease vectors.

3 Genetic variation and phenotypic plasticity

While phenotypic plasticity plays a crucial role in the adaptability of *Ae. aegypti*, it is essential to recognize the genetic components underlying these traits. Phenotypic traits such as host choice, behavior, and adult size exhibit significant genetic variation, which interacts with environmental factors to shape the observed phenotypic outcomes (48, 49). This interaction between genetic variation and phenotypic plasticity is pivotal for understanding the adaptability and vector potential of *Ae. aegypti* (15, 50).

Host choice exemplifies a trait influenced by both genetic and environmental factors. Studies have shown that *Ae. aegypti*'s preference for human hosts has a strong genetic basis, with certain populations exhibiting innate tendencies towards anthropophily (51). Specific genetic loci associated with the preference for human odors underscore the genetic underpinnings of this behavior. However, environmental conditions, such as the availability of hosts and habitat characteristics, also modulate this preference, showcasing epigenetic plasticity.

Behavioral traits, including feeding and oviposition behaviors, also exhibit genetic variation. For instance, the genetic differentiation between sylvatic and domestic forms of *Ae. aegypti* influences their behavior and habitat preferences (52). Sylvatic populations tend to feed on a broader range of hosts and oviposit in natural habitats, while domestic populations strongly prefer human hosts and artificial containers for oviposition. These inherent genetic differences are further influenced by environmental factors, such as the availability of breeding sites and host density, leading to context-specific behavioral adaptations.

Adult size is another trait where genetic variation and phenotypic plasticity intersect. Body size is determined by both genetic factors and larval rearing conditions, such as temperature and resource availability (53). Genetic differences between populations can result in varying growth rates and adult size at emergence (15). Heritability plays a significant role in determining adult body size, yet environmental factors like larval density and nutrition levels also induce plastic responses, affecting size-related traits such as longevity and fecundity. This interaction between genetic predisposition and environmental conditions underscores the complexity of size variation and its implications for vector competence (15).

Understanding the interplay between genetic variation and phenotypic plasticity is crucial for predicting how *Ae. aegypti* populations might respond to changing environments (49). This knowledge is vital for devising effective vector control strategies, as both genetic adaptation and plastic responses can influence the success of interventions. For example, genetic differences in insecticide resistance can interact with environmental factors, such as exposure levels and habitat characteristics, to shape resistance dynamics (48). Recognizing the contributions of both genetic variation and phenotypic plasticity will enhance our ability to anticipate and manage the evolutionary responses of *Ae. aegypti* to control measures.

4 Behavioral plasticity

The success of *Ae. aegypti* as an invasive species is largely attributed to its anthropophilic behavior, with a strong preference towards human hosts for blood meals. While its host-seeking behavior has been extensively studied, its sugar-feeding habits and preferences, crucial for metabolic sustenance, remain relatively unexplored (54). Given the exclusive sugar-feeding diet of males and the importance of carbohydrates for females' metabolism, this is a crucial contributor to the species' invasion potential (55). Despite *Ae. aegypti* mosquitoes predominantly inhabiting human-dominated areas, it is essential to acknowledge the existence of known sylvatic populations that defy this trend and also feed on non-human hosts (52).

The variation between sylvatic and domestic forms of *Ae. aegypti* is a key example of both genetic differentiation and behavioral plasticity. Sylvatic *Ae. aegypti* primarily inhabit forested areas and utilize natural water containers for breeding, while domestic *Ae. aegypti* thrive in urban environments, breeding in artificial containers and frequently entering human dwellings. Multiple studies provide substantial evidence for a genetic basis underlying the plasticity in mosquito behavior (51, 56–58). These studies demonstrate that genetic differences are responsible for variations in behaviors such as host preference, habitat selection, and oviposition site choice. In addition to genetic differentiation, both *Ae. aegypti* and *Ae. albopictus* exhibit considerable flexibility in their feeding behaviors, which further reflects their adaptability to varying environmental conditions. For instance, while longevity typically decreases in the absence of sugar, fecundity can actually increase when females rely solely on human blood without sugar supplementation (59). This plasticity in feeding and reproductive strategies underscores the adaptability of these species, allowing them to thrive under different environmental pressures. Such flexibility is a testament to the complex interplay between diet, genetic predispositions, and life history traits that enable these mosquitoes to exploit a wide range of ecological niches. This combination of genetic factors and behavioral plasticity is central to the success of *Ae. aegypti* in invading tropical and subtropical regions. The inherent genetic differences between sylvatic and domestic forms are a result of their adaptation to specific environments, while behavioral plasticity enables them to respond dynamically to varying habitat conditions.

To identify and locate their hosts, *Ae. aegypti* females rely on the synergistic integration of sensory cues, including olfactory, visual, thermal, and gustatory cues (60). Olfactory cues and carbon dioxide facilitate long-range attraction, and other sensory cues enable medium-to-short-range attraction. Finally, thermal cues are primarily effective at short distances since temperature gradients created by convection around a human host diminish quickly (61–63). While there is a consensus on the relative significance of these sensory cues, the contribution of thermal cues in host-seeking behaviors is still debated, most likely due to contextual differences in the experimental paradigms used in studies. For example, some studies found that the attraction of *Ae. aegypti* to thermal cues depend on their ability to sense CO_2 (64), while others showed that their attraction to thermal cues could also occur independently of CO_2 (61). In the absence of CO_2, the sensory integration of thermal and chemosensory cues (host volatiles) likely drives the host-seeking behavior (65). Nevertheless, there is evidence that thermal cues alone (specifically convective but not radiative heat) are sufficient for females to locate heat sources in the range of potential hosts' temperature (66).

In the context of long-range attraction, the response to CO_2 predominantly drives host-seeking and reduces the response threshold to human-derived odors. *Ae. aegypti*'s preferences for visual cues of specific wavelengths are also gated by the detection of CO_2 (60). However, within a short range to the human host, orientation and landing behaviors are mediated by olfactory cues, not CO_2 or a co-located visual cue (67, 68). Further, several hundred human-derived chemoattractants have been identified (69, 70). The valence of these chemoattractants can vary significantly, both in isolation and in synergistic combinations, and these variations are influenced by physiological and environmental factors (51, 71, 72).

While host-seeking is typically regarded as a female mosquito trait, *Ae. aegypti* males also display attraction to humans (73, 74). Mature males are particularly drawn to human-derived chemosensory cues, which drive their swarming behavior in pursuit of potential mates and contribute to their mating success in natural populations (66, 75). Amidst debates on mosquitoes' attraction to sensory cues and multimodal sensory integration, responses to environmental cues in *Ae. aegypti* are often interpreted as phenotypic plasticity, where organisms modify their behavior or traits in response to changing environmental conditions (13). For instance, variations in host-seeking behavior can be influenced by larval rearing conditions, indicating plastic responses to environmental cues (23). However, some responses are considered innate, meaning they are hardwired into the organism's genetic makeup and are not easily altered by the environment (51). These innate responses can still exhibit context-specific variations, as the expression of innate traits can be modulated by environmental factors (76). Thus, distinguishing between plastic and innate responses requires careful consideration of both genetic and environmental influences (77). Unfortunately, many studies overlook this context specificity, focusing on mechanistic effects under controlled conditions. While mechanistic insights are valuable, the broader relevance of these mechanisms in real-world contexts is needed to bridge findings from the lab to their applicability in field conditions.

Extrinsically, factors like temperature, humidity, and photoperiod significantly shape mosquito behavior. As poikilotherms, the ambient environmental temperature dictates mosquitoes' body temperature and activity. Warmer temperatures, for example, increase their metabolism, leading to heightened activity and increased sugar feeding from plants to meet their nutritional needs (78). Besides elevated activity levels, temperature influences the sensitivity to chemosensory cues as *Ae. aegypti* females were more attracted to CO_2 when tested at 30°C compared to 20°C and 25°C (79). Electrophysiological recordings indicate that odorant-specific changes in antennal sensitivity to odors mediate this effect of temperature on olfactory behavior (79). The differences in response to odorants could also be partly due to temperature-induced alterations in the characteristics of odorant compounds. Indeed, one can expect temperature changes to affect the chemicals' partial vapor pressure, impacting their diffusion and subsequent interaction with odorant binding receptors in the chemosensory organs of *Ae. aegypti* (80, 81).

Intrinsically, as observed in many other insect models, the olfactory sensitivity of *Ae. aegypti* varies with the time of day (82), with several genes involved in olfactory processes being regulated by the mosquito's circadian clock (83, 84). Furthermore, sleep deprivation detrimentally affects both host-seeking and blood-feeding behaviors in *Ae. aegypti*, potentially linked to alterations in time-dependent olfactory sensitivity (85). It is thus critical to synchronize mosquitoes to test behavioral and physiological responses to host cues in the proper (or most relevant) temporal context. It is also essential to report temporal information in publications as the norm. Overall, the physiological state of the insect (*e.g.*, age, reproductive status, feeding state, chronobiology, sleep patterns, prior experience, *etc.*) significantly affects its responses to resource-associated cues (86). For example, older females, who typically exhibit a higher propensity for seeking hosts, display increased sensitivity to CO_2 compared to their younger counterparts in the first few days post-emergence. (87). Additionally, mating and blood-feeding suppress host-seeking behavior, with a return to baseline levels after oviposition (88–90).

Altogether, this underscores the importance of considering the interplay between mosquitoes' physiology and behavior. Overall, extrinsic and intrinsic factors modulate mechanisms at peripheral and central levels to drive behavioral variation tailored to different physiological and ecological contexts.

5 Using transstadial effects to navigate contextual complexity in studying life history and behavior

The variation in life history traits and behavior of *Ae. aegypti* described in the literature are primarily influenced by decisions made by experimenters, *i.e.*, the study design employed to quantify traits. While simple experimental designs offer more explicit contexts, their capacity to fully capture the breadth of variation in mosquito life history and behavior is debatable. On the other hand, complex study designs reporting multivariate and interactive effects encounter challenges in discerning the relative contribution of each independent variable to the magnitude and direction of observed effects on traits and their underlying distribution (91). These studies also hinder experimenters' ability to dissect the mechanistic

underpinnings of the observed behaviors (49). This complexity arises when multiple experimental variables are manipulated simultaneously, potentially interacting in ways that obscure context-dependent effects unless addressed explicitly in the study design (92–94).

Numerous studies have explored the impact of larval competition and nutrition on adult mosquito body size at emergence. Typically, heightened larval competition and lower nutrition reduce per capita resource availability, resulting in smaller adult mosquitoes (95, 96). Consequently, a diverse range of mosquito size distributions resulting from varying levels of larval competition and nutrition have been reported in the literature (43, 97–99). However, it remains challenging to determine if these varying size distributions across studies, often overlapping, genuinely reflect the ecologically relevant limits (*i.e.*, in larval density and food availability). To address this concern, some studies integrate field-derived preliminary data on larval density or food availability in natural habitats, ensuring that experimental variables are manipulated within ecologically relevant bounds (100, 101). Others employ experimental variables that may not strictly adhere to these limits but conduct standardization trials under controlled conditions to establish the range within which variables can adequately capture a trait's distribution (102, 103). When manipulated individually, varying larval densities and food levels could still produce adults with similar size distributions, although due to different physiological responses. Nevertheless, more needs to be understood about whether these adults, despite their similar size distribution, share similarities in their physiological, behavioral, and life history characteristics. When these variables interact, untangling their influence on adult size distribution becomes complex and challenging. Nonetheless, these intricacies are frequently disregarded in many studies, highlighting the significance of interpreting effect sizes on mosquito traits and behavior while considering the distribution's shape, especially at the tails, where sampling efforts may be limited. These gaps in approach will likely impact the perceived relationship between environmental factors and life history traits, the extent of variation, and the plasticity window for those traits (Figures 2D, E).

It is important to consider whether the source of size variation, or any other phenotypic variation, influences how these traits relate to fitness and disease transmission. Different sources of variation, whether genetic, physiological, or environmental, might have distinct effects on these outcomes. Investigating these differences could provide valuable insights into the adaptive strategies of *Ae. aegypti*. This question merits further exploration in the context of understanding vector competence and developing effective control strategies. For example, resource limitation and competition during larval development often result in smaller adults, which can exhibit greater susceptibility to several arboviruses and, in some cases, higher oral transmission rates (47, 104). Conversely, other studies have found that smaller individuals from high-density rearing conditions may have reduced vector competence (105, 106). Additionally, research on *Ae. albopictus* has indicated that higher temperatures during larval development can produce smaller adults with lower susceptibility to some arboviruses.

Transstadial effects, such as resource limitation, fluctuations in temperature and humidity, predator presence, parasites and several other factors, during larval development, significantly influence the adult size of mosquitoes, primarily through phenotypic plasticity. As previously noted, these factors may lead to similar phenotypic outcomes through different mechanisms. However, the role of selection among individuals with inherent genetic differences in size in shaping adult size distributions under various larval growing conditions has been less frequently considered. Additionally, size distributions can vary among populations due to inherent genetic differences (107). Future research investigating the specific contributions of genetic and environmental factors to mosquito size variation, mediated by transstadial effects, is crucial for understanding their impact on fitness traits and vector competence in *Ae. aegypti* and other mosquitoes.

Multiple studies have revealed intriguing trends linking variation in temperature with the host-seeking behavior of female *Ae. aegypti*. However, a crucial aspect is often overlooked: mosquitoes' complex life history and the influence of larval growing conditions that cascade to modulate adult traits and behavior (43, 76, 108, 109). Temperature-driven variation in host-seeking behavior have often been reported during behavioral studies (110, 111). The complex life cycle of *Aedes* mosquitoes involves distinct habitats for larvae and pupae versus adults, which often results in them experiencing different thermal environments. Larvae and pupae are confined to aquatic environments, typically in small volumes of water (single containers, tree holes, etc.), with a narrow thermal range during their development. In contrast, adult females are highly mobile and traverse various aerial and terrestrial environments, exposing them to a wide range of microclimatic conditions as they seek sugar sources, hosts for blood meal, oviposition, and resting sites (112, 113).

Given these differing thermal exposures, it is essential to consider how transstadial effects might influence sensory and behavioral responses. The thermal mismatch or match between larval and adult environments could significantly impact adult mosquitoes' phenotypic traits and behaviors. For example, a stable larval thermal environment might lead to different developmental outcomes compared to the variable thermal exposures experienced by adults, affecting their survival, reproduction, and vector competence (28 and 114–116). Studies have shown that discrepancies in thermal environments between life stages can alter adult behavior and physiology, emphasizing the need for experimental designs to account for these differences (114). Therefore, the thermal history of both larval and adult stages is crucial for accurately interpreting the effects of temperature on mosquito biology and behavior. This consideration helps ensure that experimental results are ecologically relevant and reflective of the natural conditions experienced by mosquitoes throughout their life cycle. However, the adult mosquitoes used in most behavioral studies often originate from larval environments chosen primarily for optimizing the yield of colonies, needing more alignment with the experimental context. (64, 117). Also, it is essential to note that most behavioral studies need to accurately report the larval growing conditions, which hinders contextualizing the reported effect sizes on adult behavior.

Laboratory studies often focus on controlled conditions to ensure repeatability and reliability of results. However, these studies might not fully capture the complexity and variability of mosquito behavior

in the wild. For instance, despite artificial blood feeding over many generations, laboratory strains of *Ae. aegypti* maintain a strong preference for human hosts, demonstrating that some behaviors are robust and persist even under artificial rearing conditions (19, 56, 118). Nevertheless, many other behaviors and responses might be context-specific and influenced by the natural environment (*e.g.*, circadian rhythms, activity patterns, etc.), which laboratory settings fail to replicate completely (119). Therefore, a hypothetical framework is needed to interpret the environment-trait-fitness relationship, considering the covariations in larval and adult traits influenced by transstadial effects (Figure 2F). This framework emphasizes the importance of visualizing the covariations in larval and adult traits, considering the influences of independent variables across developmental stages along the two axes. Such an approach is crucial for accurately interpreting their effects on adult behavior (third axis) and its implications for mosquito fitness (fourth dimension). By accounting for these relationships between environmental variables, transtadially-mediated trait variation and adult fitness, we can better contextualize experimental findings and highlight their ecological relevance.

Furthermore, adult females selected for behavioral assays from laboratory colonies may exhibit trait distributions specific to their rearing conditions, such as larval crowding, feeding regime, temperature, and humidity (120–124). As described in several studies' methodology, the typical "standard larval rearing condition" often does not yield adult *Aedes mosquitoes* representing the full spectrum of their trait distributions for use as subjects in behavioral experiments (28, 32, 125–127). Critically, this limitation extends beyond laboratory-reared mosquitoes to field-collected mosquitoes utilized in laboratory and semi-field experiments (128, 129). In such instances, the comprehensive trait distribution is frequently disregarded, resulting in the unintentional selection of mosquitoes with a subset of trait values or characteristics as experimental subjects. Due to the strong covariation between life-history traits and several adult behaviors, studies conducted with these mosquitoes, representing only a subset of the overall trait distribution, may only incompletely capture the variation associated with a specific behavioral repertoire. This limitation not only narrows the range of contexts in which study results can be interpreted but also affects the magnitude and direction of reported effect sizes, presenting challenges for reproducibility and generalization to broader contexts.

6 Discussion

From a vector-borne disease control perspective, studies on mosquito life history traits and behavior aim to elucidate their significance in influencing vector potential, insecticide resistance, and invasion potential. This review highlights how neglecting context-specific effects significantly undermines the accuracy of the relationships quantified between experimental variables. By integrating findings from studies on other species, we can draw broader conclusions and identify patterns that may apply across mosquitoes. For example, similar phenotypic plasticity and adaptability mechanisms observed in *Ae. albopictus*, another important vector species, can provide comparative insights that enhance our understanding of *Ae. aegypti*. Such cross-species comparisons are particularly valuable for identifying generalizable principles of mosquito biology and vector management, which can inform strategies to control multiple species simultaneously.

Moreover, considering genetic and phenotypic variation across mosquito species helps us recognize the evolutionary pressures and environmental factors shaping these traits. This understanding is crucial for anticipating how mosquito populations may respond to environmental changes, such as climate variability, urbanization, and habitat modification. For instance, the genetic diversity within populations can influence their capacity to develop insecticide resistance, necessitating the development of dynamic and adaptable control measures. Additionally, recognizing the role of phenotypic plasticity in facilitating rapid adaptation to new environments can help predict and mitigate the spread of invasive mosquito species.

The lack of context specificity in reported effects, along with difficulties in experimentally quantifying population-specific variation in mosquito traits and behavior, complicates the parameterization of data for predicting mosquito demography, distribution, and disease transmission dynamics (44, 49, 130–132). For instance, the impact of larval rearing conditions on adult traits is often studied using laboratory-bred strains under controlled laboratory conditions, which may not accurately reflect the variability encountered in natural settings. This discrepancy can lead to over- or underestimation of the effects of environmental factors on mosquito populations.

Unfortunately, interactive effects are frequently viewed as epistemically precarious due to their variability, sometimes leading to the belief that observed effect sizes are unpredictable (133–136). This perspective stems from the challenge of isolating specific variables in multifactorial experiments and the inherent complexity of ecological interactions. However, this review stresses that while interactive effects vary significantly across contexts, their variability does not imply unpredictability. With appropriate study design measures established *a priori*, or at least detailed, *a posteriori* reporting of experimental methods, their variability across contexts can be systematically studied, allowing knowledge to be rigorously extrapolated. Incorporating field-derived data into laboratory experiments is one such approach, enabling researchers to better simulate natural conditions and account for context-specific effects.

There are, however, notable exceptions to this issue. Some studies have successfully accounted for interactive effects, offering valuable insights into mosquito life-history traits. For example, Carrington et al. (2013) critically analyzed the effects of fluctuating daily temperatures on *Ae. aegypti*, emphasizing the interaction between mean temperatures and temperature fluctuations. Similarly, Muturi et al. (137) examined the interactive effects of temperature and insecticide exposure on the life-history traits of *Culex restuans* and *Ae. albopictus*, providing a nuanced understanding of how these factors together influence development time, survival, and adult size. Additional studies by Alto and Juliano (138) on temperature and larval density and Yeap et al. (139) on temperature and *Wolbachia* infection further illustrate how these environmental factors jointly shape mosquito traits and vector competence. These examples highlight the

importance of considering interactive effects in mosquito research to avoid misinterpretation of biological outcomes and to ensure the ecological validity of laboratory findings.

By addressing these interactive effects in mosquito research, we can fully leverage the extensive knowledge gained from laboratory and semi-field studies, which outnumber field-based studies, to apply these findings effectively in field contexts. This integration is essential for developing accurate mosquito behavior and population dynamics models, which are critical for predicting or, eventually, mitigating vector populations and vector-borne diseases. Furthermore, understanding the interaction between genetic variation and phenotypic plasticity can help identify potential targets for genetic modification or biological control strategies, offering new avenues for disease prevention.

Overall, this review underscores the necessity of a holistic approach incorporating genetic, phenotypic, and environmental factors to understand mosquito ecology comprehensively. Such an approach will enhance our ability to develop robust, context-sensitive interventions that can adapt to the dynamic nature of mosquito populations and the environments they inhabit.

Author contributions

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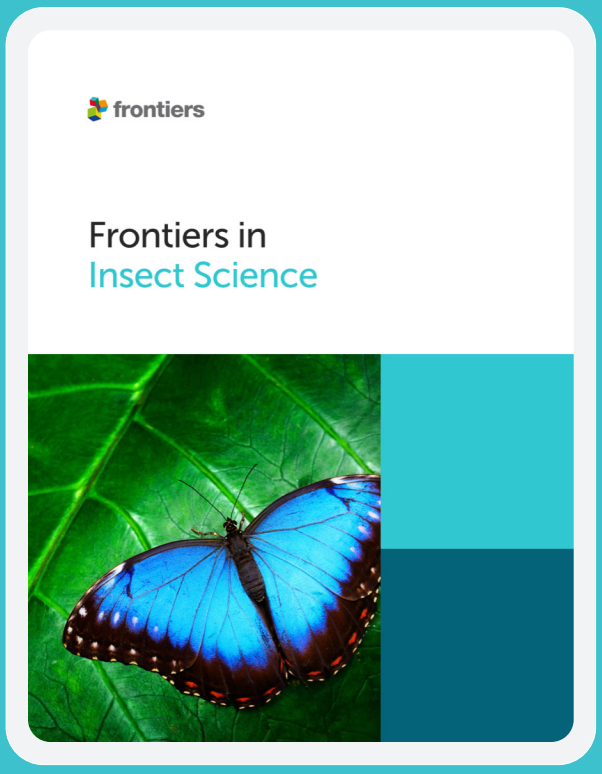

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