Age- and sex-related ABC transporter expression in pyrethroid-susceptible and – resistant *Aedes aegypti*

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Resistance mechanisms to synthetic insecticides often include point mutations and increased expression of genes encoding detoxification enzymes. Since pyrethroids are the main adulticides used against *Aedes aegypti*, which vectors pathogens such as Zika virus, understanding resistance to this insecticide class is of significant relevance. We focused on adenosine triphosphate (ATP)-binding cassette (ABC) transporters in the pyrethroid-resistant Puerto Rico (PR) strain of *Ae. aegypti*. We investigated the expression patterns of six ABC transporters previously characterized as differentially expressed in insecticide-challenged mosquitoes, or increased mRNA expression in pyrethroid-resistant *Ae. aegypti*, by comparing PR to the Rockefeller (Rock) susceptible strain. No constitutive differential expression between strains was detected, but expression differences for these genes was influenced by sex and age, suggesting that their role is independent from resistance in PR. Instead, ABC transporters may be induced after insecticide exposure. Challenging mosquitoes with deltamethrin, with or without ABC transporter modulators, showed that Rock and PR responded differently, but a contribution of ABC transporters to deltamethrin toxicity is suspected. Moreover, the effect of dexamethasone, which enhanced the inhibition of nerve firing by deltamethrin, was observed using a *Drosophila* central nervous system preparation, showing synergy of these two compounds through the potential inhibition of ABC transporters.

*Aedes aegypti* (L.) is a mosquito species present worldwide that causes important health concerns due to its ability to vector flaviviruses such as dengue, yellow fever, and Zika viruses, as well as alphaviruses such as chikungunya virus. Global efforts to control vectors of these pathogens remain inadequate or inefficient as the range of autochthonous transmission is steadily increasing globally and threatens to spread to new regions.

Arbovirus disease mitigation predominantly relies on vector control strategies that primarily incorporate the use of synthetic insecticides, such as pyrethroids due to their enhanced safety profile. However, the prevalence of insecticide resistant mosquitoes has dramatically increased in disease-endemic countries and has spread to new regions of the world partly due to consistent and widespread use. Multiple resistance mechanisms were identified in pyrethroid-resistant populations of *Ae. aegypti*, including variable point mutations in the voltage-gated sodium channel, and increased levels of cytochrome P450 monooxygenase gene expression. Although a growing body of literature suggests that these may be the most significant mechanisms of pyrethroid resistance, they may not be the only mechanism driving the development of pyrethroid-resistance. For instance, adenosine triphosphate (ATP)-binding cassette (ABC) transporters have been linked to transport of and/or resistance to eight synthetic insecticide classes and several mode of action groups, including carbamates, macrocyclic lactones, neonicotinoids, organophosphates, chlorinated hydrocarbons, benzoylureas, phenylpyrazoles, and pyrethroids.

ABC transporters are a superfamily of transmembrane proteins that consist of two hydrophobic transmembrane domains (TMDs) and two nucleotide binding domains (NBDs). The ABC transporter proteins are involved in the transport of substrates across membranes using energy from ATP hydrolysis. All ABC proteins function as transporters due to changes of conformation of the TMDs, which open when the NBDs hydrolyze ATP.
on the cytosolic side, freeing energy to allow translocation through the membrane. Moreover, the TMDs are believed to be responsible for substrate specificity. Some ABC transporters, called permeability glycoproteins (P-glycoproteins, Pgp), have been shown to prevent anti-cancer drug accumulation and confer the phenotype of multidrug resistance (MDR) in humans and other mammals. Another important family of ABC transporters, the ABCG transporters, have been described in different animal classes, including humans and insects. They have been shown to be involved in resistance to several anti-parasitic drugs in Leishmania and in humans due to broad substrate specificity to xenobiotic compounds, conferring drug resistance in cancer cells.

The observations of drug export and resistance to these compounds via increased transport by ABC transporters in multiple animals suggest that their role and ability to confer resistance may be conserved across species. ABC transporters may also be a key to understanding the mode of action of currently used insecticides, and their potential role in insecticide defense in mosquitoes has been investigated for two decades. The application of verapamil, a known ABC transporter inhibitor, increased the toxicity of cypermethrin, endosulfan, and ivermectin to susceptible and organophosphate-resistant strains of Culex pipiens and Culex quinquefasciatus larvae. However, it did not increase efficacy of the organophosphate chlorpyrifos, showing that ABC transporters may not be involved in the efflux of all classes of insecticides, regardless of resistance in the strains tested. Furthermore, exposure to a median lethal dose (LD₅₀) of permethrin has been shown to regulate expression of ABC transporter genes on a temporal scale as well as between sexes in Anopheles stephensi. These data indicate that ABC transporters play a role in the response to permethrin in mosquito and may partly contribute to the reduced insecticide sensitivity observed in resistant mosquito strains.

Porretta et al. examined the possible role of Pgps in defense against temephos, an organophosphate larvicide, and diflubenzuron, a growth regulator, in Aedes caspius larvae by applying these compounds alone or with verapamil. The LD₅₀ of these insecticides was decreased when applied with verapamil and confirmed by RNAi silencing of a Pgp sequence showing high similarity with sequences in Anopheles gambiae, Culex quinquefasciatus, and Drosophila melanogaster. This observation suggests that ABC transporters are involved in the modulation of insecticide efflux, and that there may be variations between species. ABC transporters are also suspected to be contributing to insecticide resistance in Aedes aegypti, although there is currently no confirmation of this hypothesis. The comparison between the pyrethroid-resistant Puerto Rico (PR) strain and the pyrethroid-susceptible Rockefeller (Rock) strain previously provided indications that the resistance mechanism involved multiple aspects, including target site mutation in the sequence of the voltage-gated sodium channel and increased P450 activity and gene expression. However, the role of ABC transporters in insecticide export in Aedes aegypti and the previous work done in mosquitoes exposed to combinations of verapamil and insecticides also suggest that ABC transporters are significant contributors to insecticide resistance. However, verapamil is also a calcium channel blocker and could have off-target effects. Thus, another ABC transporter modulator, dexamethasone, was used. Dexamethasone has been shown to increase ABC transporter expression in rodents. Like verapamil, dexamethasone is a substrate of ABC transporters, which could imply that they share a similar ability to compete with other substrates of the transporters, such as insecticides, and interfere with drug efflux.

The primary goal of this study was to investigate the contribution of ABC transporters to the resistance of the PR strain of Aedes aegypti mosquitoes, relative to the susceptible Rock strain. A previous study suggested the upregulation of ABC transporter-encoding genes in Aedes aegypti mosquitoes resistant to pyrethroid insecticides. However, the results of this research demonstrated no constitutive differences based on strain or exposure to deltamethrin, but did find differences based on age and sex. In toxicology experiments, treatment with ABC transporter modulators was found to alter the efficacy of deltamethrin exposure in Rock mosquitoes, and to a more limited extent in PR. Finally, a Drosophila central nervous system was used as a model to better understand the action of dexamethasone in combination with deltamethrin on the firing of the nerve. This model was used as an equivalent system to the limited success of using similar preparations with the mosquito CNS. These CNS preparations also proved their reliability, as in previous studies, to test the effect of drugs on dipteran nervous systems, including Drosophila melanogaster and Musca domestica. Dexamethasone was found to increase the rate of nerve inhibition after exposure to deltamethrin, which suggests ABC transporters are relevant to the delivery of the insecticide to the mosquito central nervous system.

Results

Gene expression. Constitutive differences observed based on sex and age, but not strain. The gene expression analysis of six ABC transporter genes that were previously investigated in mosquitoes of various species revealed no significant differences between susceptible and resistant strains. However, significant differences in gene expression due to age and sex were observed (Fig. 1, Supplementary Table 1). More specifically, expression of the ABCG4 and ABCB6 genes showed a similar pattern in both sexes of PR and Rock, but their expression differed between the oldest age group, 5 to 7 d old, and the younger mosquitoes (Fig. 1). The expression of dABCX showed a clear difference between sexes in all age groups (Fig. 1), but the statistical analysis also highlighted a significant interaction of age, 5 to 7 d old, and sex, which can be visualized by the decrease in expression at that age in males compared to females. There were no significant differences between age groups, sexes, or strains for the gene ABCB2. The only factor with a significant effect on the expression of Pgp was sex, with a generally lower expression in females than males. The interaction of strain and sex was significant for ABCB4, which is illustrative of the larger difference in expression between sexes in PR, as females have higher expression for all age groups than Rock, where there is no difference between sexes.

Deltamethrin exposure did not alter ABC transporter expression. The expression of all genes, except Pgp (P = 0.712), was significantly different between the strains (Fig. 2, Supplementary Table 2). Four of the ABC transporter transcripts, ABCB2, ABCB6, ABCG4 and dABCX, were more highly expressed in PR compared to

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Rock, while ABCB4 was more highly expressed in Rock with a 2.6-fold difference compared to PR. Deltamethrin treatment did not have a significant effect on the expression of any of the ABC transporter transcripts ($P$-values between 0.371 and 0.961), nor did the interaction between strain and treatment ($P$-values between 0.515 and 0.955).

Figure 1. Graphical representation of the gene expression patterns, in log$_{2}$(abundance) ± standard deviation, for the six ABC transporters of interest, comparing females and males of pyrethroid-susceptible (Rockefeller) and -resistant (Puerto Rico) *Aedes aegypti*, at all age groups. Significant differences are represented by different letters and significant interactions (Strain × Sex, and Age × Sex) are represented by upper case letters.

Figure 2. Graphical representation of the gene expression patterns, in log$_{2}$(abundance) ± standard deviation, for the six ABC transporters of interest, comparing pyrethroid-susceptible (Rockefeller) and -resistant (Puerto Rico) *Aedes aegypti*, and exposure to deltamethrin versus an acetone control. Significant differences are represented by different letters.
Toxicity bioassays. Dexamethasone and piperonyl butoxide (PBO) increased deltamethrin-induced mortality in Rock and PR, verapamil only in Rock. To compare the effect of verapamil and dexamethasone (Pgp-inhibitors) pretreatments followed by deltamethrin exposure on survival of the two mosquito strains, we analyzed the overall differences of all pretreatments relative to an acetone control, as well as for each treatment separately compared to the control. We used a PBO pretreatment as positive control due to its synergistic effect with deltamethrin. Rock showed a significant decrease in survival (0 h pretreatment time: $\chi^2 = 62.35, df = 3, P < 0.0001; 4$ h pretreatment time: $\chi^2 = 87.58, df = 3, P < 0.0001$) compared to the acetone pretreatment control for all treatments regardless of pretreatment time (verapamil, 0 h pretreatment time: $\chi^2 = 21.41, df = 1, P < 0.0001, 4$ h pretreatment time: $\chi^2 = 52.88, df = 1, P < 0.0001$; dexamethasone, 0 h pretreatment time: $\chi^2 = 45.68, df = 1, P < 0.0001, 4$ h pretreatment time: $\chi^2 = 45.70, df = 1, P < 0.0001$; PBO, 0 h pretreatment time: $\chi^2 = 50.88, df = 1, P < 0.0001, 4$ h pretreatment time: $\chi^2 = 63.40, df = 1, P < 0.0001$) (Fig. 3). Furthermore, only PBO pretreatment in PR mosquitoes significantly differed from acetone pretreatment by decreasing survival compared to the control ($\chi^2 = 8.348, df = 1, P = 0.0039$) (Fig. 4). Pretreatments with verapamil and dexamethasone did not significantly differ from the acetone pretreatment (verapamil: $\chi^2 = 0.1895, df = 1, P = 0.6633$, dexamethasone: $\chi^2 = 1.618, df = 1, P = 0.2033$). A 4 h pretreatment time with PBO significantly decreased survival of PR relative to acetone pretreatment ($\chi^2 = 5.708, df = 1, P = 0.0105$), as did 4 h pretreatment with dexamethasone ($\chi^2 = 26.99, df = 1, P < 0.0001$), while survival after a 4 h pretreatment with verapamil did not differ from the survival after pretreatment to acetone ($\chi^2 = 1.137, df = 1, P = 0.2862$).

**Figure 3.** Survival of 5 to 7 d old adult, female pyrethroid-susceptible (Rockefeller) *Aedes aegypti* treated with verapamil, dexamethasone, or piperonyl butoxide (PBO) for (A) 0 h, or (B) 4 h prior to introduction into deltamethrin-coated bottles. Data presented as Kaplan-Meier survival curves with points representing mean values ± standard error for 60 mosquitoes (6 replicate groups of 10 mosquitoes).

Exposure to *Drosophila melanogaster* central nervous system (CNS). Potency of deltamethrin and dexamethasone to firing rate of *Drosophila* CNS. Deltamethrin was found to be highly potent to *Drosophila* CNS with a concentration required to inhibit 50% of firing (IC$_{50}$) of 72 nM (95% CI: 46–81; Hillslope: $-1.8; r^2$: 0.81), which is shown in Fig. 5A. Importantly, dexamethasone was found to have negligible influence on the spike discharge frequency with a maximal concentration of 300 µM increasing firing rate by 13% ± 7% (Fig. 5B,D). Based on the toxicology data, we operated around the premise that inhibition of Pgps by dexamethasone will...
increase the potency of deltamethrin. To test this, we pretreated an intact CNS with 30 µM dexamethasone followed by subsequent co-exposure to a range of deltamethrin concentrations. The inclusion of dexamethasone increased deltamethrin potency by 2.2-fold (IC50: 32 nM; 95% CI: 25–38; Hillslope: −4.1; r²: 0.94), which is shown in Fig. 5A. In addition to increased potency, we observed a 2.1-fold increase in Hillslope value with co-exposure of deltamethrin and dexamethasone (Fig. 5A).

Dexamethasone increases rate of inhibition to CNS firing after deltamethrin exposure. In addition to increased potency, we speculated that the inhibition of Pgps would reduce deltamethrin efflux from the CNS and, thus, increase the bioavailability of deltamethrin in the CNS. To test this, we measured the rate of inhibition of CNS firing after exposure to 80 nM deltamethrin, which is the approximate IC50 value. Data show complete inhibition of CNS firing after exposure to 80 nM deltamethrin at 30 ± 5 min post-exposure. Importantly, the rate of nerve inhibition was increased by 2-fold after co-exposure to 30 µM dexamethasone and 80 nM deltamethrin, which is a statistically significant (P < 0.01) increase (Fig. 5C). Representative traces showing the rate of nerve inhibition for deltamethrin alone and after co-exposure to deltamethrin and dexamethasone are provided in Fig. 5E,F.

Discussion

This study primarily focused on determining if ABC transporters could be involved in pyrethroid resistance in PR mosquitoes by comparing various parameters to a susceptible counterpart, including constitutive gene expression, and expression after deltamethrin exposure. Additionally, the study aimed to determine if ABC transporters are involved in the response to deltamethrin exposure via toxicity in Ae. aegypti mosquitoes and electrophysiology bioassays via ABC transporter pharmacology with a prepared Drosophila CNS.

ABC transporters of the families ABCB and ABCG were considered good candidates for the export of pyrethroids out of neural tissue because they were differentially expressed in mosquitoes in response to insecticide exposure57,58,36,38,39 or as potential resistance genes19. In particular, ABCB transporters are known to be specific for hydrophobic substrates59, such as the pyrethroid deltamethrin.
Overall, there were no differences in expression of the ABC transporter transcripts of interest between PR and Rock in the experiment comparing sexes, ages and strains, but there were significant expression differences between males and females, and between age groups. Age dependent regulation of ABC transporter genes, as well as an involvement in embryo development, were previously shown in rats. Sex was also a predominant factor of expression level variability, with a higher expression in female rats compared to males, resulting in quicker drug efflux by hepatic Pgps in females. It was also observed that sex influenced the expression of the homologous genes in An. stephensi.

A follow-up exposure using 5 to 7 d old female mosquitoes showed that all the ABC transporter transcripts investigated, except the transcript Pgp, exhibited significantly different expression levels between strains (Fig. 2 and Supplementary Table 2). A particularly interesting result was the expression of ABCB4, which was lower in PR mosquitoes than in Rock, contradicting previously published results. This experiment also illustrates the importance of accounting for the appropriate parameters in a gene expression experiment. There were many aspects to consider in the model for the analysis of the constitutive difference that did not detect more fine-tuned strain differences in the oldest age group, highlighted in the second experiment. These results may show that the higher expression of some of the ABC transporters tested is part of the resistance profile of PR mosquitoes, but it would have to be observed in males as well since they are also pyrethroid-resistant, and at all age groups, which was not the case in the previous experiment.

This follow-up transcript expression assay also revealed that the exposure to deltamethrin did not have a significant effect on the expression of the genes of interest. This could be the result of using the LD25, respective to each strain, which may be too low at the 2 h timepoint to elicit a strong stress response and significantly alter...
Ae. aegypti decreased overall expression of the ABC transporter transcripts of interest either. The expression patterns were genes in PR or in case of exposure to deltamethrin, but the constitutive gene expression analysis did not show a resistance mechanisms in PR, the expression analysis did not show a consistent higher expression of the select here as a positive control, which significantly increases mortality in both PR and Rock mosquitoes. Regarding the strain may not rely as heavily on ABC transporter export compared to the susceptible Rock strain. The resistance in the PR strain, and their involvement would be delayed compared to Rock. It can be hypothesized that since dexamethasone to induce nerve death.

Dexamethasone has been described in mammals as a stimulant of ABC transporter expression, as well as a substrate of ABCB and ABCG transporters, especially those involved in cancer resistance44,45, and is readily transported by MDR1 in mouse brain and human cells46,48. Thus, verapamil and dexamethasone share the ability to be transported by ABC transporters, hence, potentially sharing a similar role. In arthropods, however, it was thought to be an inducer of cytochrome P450 expression43. In Pediculus humanus for instance, dexamethasone induced tolerance to ivermectin, whereas verapamil increased ivermectin toxicity49, which led the authors to hypothesize that decreasing ABC transporter export with verapamil would increase toxicity, and that dexamethasone ultimately increased P450 activity. However, based on the results obtained in the present study using PR and Rock mosquitoes, as well as previous work42, increased P450 activity induces tolerance to deltamethrin and is likely part of the resistance mechanism in PR mosquitoes, whereas the toxicity assay exposing PR mosquitoes to deltamethrin did not increase survival in presence of dexamethasone. Survival was decreased when mosquitoes were exposed to these drugs, suggesting that increased P450 is either not the major modification induced by the exposure to dexamethasone, or is not the effect of this drug in Ae. aegypti. The effect of dexamethasone suggests that in Ae. aegypti, its role is more likely similar to the role of verapamil in Rock mosquitoes and can act as an ABC transporter competitive inhibitor. Dexamethasone and verapamil may differ in certain functional aspects, which would explain the divergence of results in PR after a 4 h pretreatment, as well as the temporal dimension of the effect of dexamethasone. Additionally, the results of the CNS preparation with exposure to dexamethasone show that it decreased the IC\textsubscript{50} of deltamethrin by increasing firing rate at the nerve, which suggests a direct role of dexamethasone at the blood brain barrier. Dexamethasone also significantly decreased the time needed for deltamethrin to induce nerve death.

The results highlight that ABC transporters may not play an important role in the efflux of deltamethrin in the PR strain, and their involvement would be delayed compared to Rock. It can be hypothesized that since PR mosquitoes carry mutations that confer target-site insensitivity22 and exhibit increased P450 activity42, this strain may not rely as heavily on ABC transporter export compared to the susceptible Rock strain. The resistance mechanism would be relying on P450 activity instead42, which can be observed through the effect of PBO, used here as a positive control, which significantly increases mortality in both PR and Rock mosquitoes. Regarding the resistance mechanisms in PR, the expression analysis did not show a consistent higher expression of the select genes in PR or in case of exposure to deltamethrin, but the constitutive gene expression analysis did not show a decreased overall expression of the ABC transporter transcripts of interest either. The expression patterns were particularly interesting for the gene ABCB4, because it was previously found to be more highly expressed in other pyrethroid-resistant strains of mosquitoes when compared to the New Orleans susceptible strain of Ae. aegypti39, but does not follow the same pattern of expression here. Two hypotheses can be offered to explain the discrepancies between studies in terms of gene expression: (1) comparing a resistant strain to a separate susceptible strain, rather than a susceptible isogenic strain, can pose a challenge since there are likely other genetic differences between the two that could confound the results of such an experiment; (2) susceptible New Orleans and Rock strains can also present genetic differences resulting in diverging expression patterns when a resistant strain is compared. Interestingly, the expression pattern of ABCB4 may be part of a compensatory mechanism between P450s and ABC transporters, which could be selected to avoid fitness costs in resistant strains, due to a higher P450 activity that is energetically costly. However, if such a mechanism exists, then more experiments would need to be conducted to gain an understanding of this phenomenon and verify our hypothesis.

Overall, the competitive inhibition of ABC transporter export function by verapamil, and potentially dexamethasone, increased mortality, although no expression level change after exposure to deltamethrin was detected. These can be interpreted as discrepancies between mosquito species or insecticide classes, since the genes chosen for this study were either involved in response to other modes of action for Aedes species39,40, or extracted from work done with An. stephensi exposed to permethrin35,36,38, and may not be involved in the response to deltamethrin exposure. ABC transporter genes involved in the response to deltamethrin may be different from those genes examined in this study and their differential expression could explain the survival curves observed for Rock, and PR to a lesser extent. Consequently, this study contributes to the large body of work demonstrating
the heterogeneity of the role of ABC transporters in insecticidal challenge, especially across species. Overall, we observed differences between strains due to treatment with ABC transporter modulators, illustrated by the effect of verapamil in particular. There is also a temporal aspect to their contribution towards deltamethrin challenge, especially in PR, and in the expression of the tested ABC transporters, which was variable between age groups. Additionally, a different role in females and males was suggested by the differences between sexes, as previously observed. 

Although the genes involved in deltamethrin export were not clearly delineated, the toxicity assays in *Ae. aegypti*, complemented by the CNS preparation study in *D. melanogaster*, highlighted the participation of ABC transporters in deltamethrin cellular export, with an increased efficiency when using competitive inhibitors in Rock, and potentially in PR. This work also provided insight into the function of dexamethasone when applied in conjunction with deltamethrin, presumably allowing increased penetration, and eventually, increased nerve inhibition and death. These findings highlight the complicated nature of insecticide resistance by demonstrating the likely role of other proteins involved in insecticide detoxification or transport, emphasizing the need to continue investigating the mechanisms that confer resistance in different species.

Methods

Subjects. *Aedes aegypti*. Two strains of mosquitoes were used in this study: (1) a susceptible laboratory population, Rockefeller (Rock), established in 1937 (obtained through BEI Resources, NIAID, NIH: *Aedes aegypti*, Strain ROCK, MRA-734, contributed by David W. Severson), and (2) a pyrethroid-resistant strain, Puerto Rico (PR), collected from San Juan, Puerto Rico and maintained in the laboratory under selection pressure since 2012 (provided by Centers for Disease Control and Prevention (CDC) for distribution by BEI Resources, NIAID, NIH: *Aedes aegypti*, Strain Puerto Rico, Eggs, NR-48830). Our mosquito-rearing protocol can be found in Rault et al. 

Mosquitoes were aspired and transferred to a new cage within 24 h of emergence in order to maintain age-matched cohorts of each strain. Mosquitoes were anesthetized by chilling on ice and decapitated before dissecting out CNS in Schneider’s medium (Invitrogen, Paisley, Scotland, UK).

Gene expression. *Deltamethrin exposure*. Two experiments were performed in order to 1) identify constitutive differences between susceptible and resistant strains, as well as sex and age differences, in the expression of ABC transporter transcripts, and 2) identify deltamethrin-induced changes in the expression of the same transcripts. The specimens used were either 1) untreated, in triplicates of 10 mosquitoes of the 2 strains, of 3 age groups for both sexes, or 2) 5 to 7 d old females that received their respective LD25 (at 24 h) calculated based on weight differences observed by strain (resistant or susceptible). Deltamethrin was applied topically on the pro- notum, then mosquitoes were placed in Petri dishes in groups of 10. Each condition was repeated in 3 replicates. Rock females received 0.2 µL of a stock solution of 22.5 nM in acetone and PR females received 0.2 µL of a stock solution of 1.5 µM of deltamethrin in acetone. The specimens were placed on ice before recovery after a 2 h exposure, and subsequently flash-frozen in liquid nitrogen and stored at −80°C.

**RNA extraction and cDNA synthesis.** The RNA extraction area and materials were treated with RNase AWAY™ (Molecular BioProducts™, San Diego, CA). RNA extraction was performed using Tri Reagent RT (Molecular Research Center, Inc., Cincinnati, OH) and 1 µg of RNA per 20 µL reaction volume was reverse transcribed using the iScript cDNA reverse transcription kit following manufacturer indications (BioRad, Hercules, CA). The resulting cDNA was diluted 2-fold (constitutive experiment) or 3-fold (deltamethrin exposure) to accommodate the volumes of reaction.

Quantitative reverse transcriptase polymerase chain reaction. The primers for ABCB2, ABCB4, ABCB6, and ABCG4 genes were originally designed for *An. stephensi* in Local Alignment Search Tool “BLAST” and also work in *Ae. aegypti* due to the conserved Walker A- Walker B region across species. Other primer sequences were gathered from Bariami et al. (Supplementary Table 3). All primers were synthesized by Sigma-Aldrich (Saint Louis, MO).

The quantitative reverse transcriptase (qRT) polymerase chain reaction (PCR) experiments were performed using a BioRad CFX Connect Real-Time System with iTaq Universal SYBR® Green Supermix (BioRad). The PCR cycle protocol program consisted of an initial step of 3 min at 95°C for denaturation, followed by 40 cycles of the following steps: 10 s at 95°C for denaturation, 30 s at 59°C for primer annealing, and 30 s at 72°C for primer extension. After a final step of 72°C for 5 min, a melting curve was taken from 65°C to 95°C by increments of 0.5°C for 5 s. All reactions consisted of 2 µL of cDNA, 0.5 µL of each primer at 10 µM, 5 µL of SYBR® green and 2 µL of water, for a total volume of 10 µL. Each condition was repeated in technical triplicates.
The statistical analysis of the qRT-PCR results was performed using the R\textsuperscript{\textregistered} package MCMC.qpcr\textsuperscript{49}. This method uses a generalized linear mixed model (GLMM). GLMM permits the analysis of complex designs by an ANOVA-type analysis. The model also considers primer efficiencies and allows the calculation of molecule counts from C\textsubscript{T} values for all conditions. Prior to analysis, outliers of technical replicates were removed after visualization of the melt curve and amplification curve. All transcripts of interest and two housekeeping genes were analyzed. The housekeeping genes selected for the analysis were β-actin and ribosomal protein S17 (RPS17)\textsuperscript{69}, however, these genes were not specified as “control” genes since no assumption was made about their stability, which is one of the features of the package used. The effect of (1) age, sex, and strain, or (2) strain and treatment, on the expression of the selected genes was measured. P-values were corrected for multiple tests by applying the method of Benjamini and Hochberg\textsuperscript{70} with a false discovery rate of 5%.

**Toxicity bioassays.** The reagents used were acetone (Sigma Aldrich, Saint Louis, MO), verapamil hydrochloride, 99% (ThermoFisher Scientific, NJ), dexamethasone (Sigma Aldrich, Saint Louis, MO), and technical grade deltamethrin (Chem Service, Inc., PA).

A toxicity bioassay was performed with two pretreatment times: (1) Rock and PR female mosquitoes between 5 and 7 d old received a topical application of 0.2 µL of either acetone as a control treatment, PBO as a control (10 mM), verapamil (10 mM), or dexamethasone (8 mM), diluted in acetone, on the pronotum, and were immediately placed in bottles coated with deltamethrin. A sub-lethal concentration of PBO, verapamil and dexamethasone was used for each bioassay. Rock were exposed to 0.1 mg/bottle (1 mL/bottle) and PR were exposed to 1 mg/bottle (1 mL/bottle). (2) All conditions were identical, but the topical application of the four compounds tested was performed 4 h before the transfer into the coated bottles. Controls were running simultaneously to account for natural mortality. Six replications were used for all the conditions. Mortality was recorded every 15 min for 2 h. The resulting survival curves were compared between treatments, separately for each pretreatment time and strain, and analyzed with a log-rank Mantel-Cox test. All statistical tests were carried out at a significance level of P ≤ 0.05. Analyses were performed with GraphPad Prism version 8.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

**Electrophysiological studies of Drosophila melanogaster neural systems.** Neurophysiological recordings were performed on the CNS of 3\textsuperscript{rd} instar D. melanogaster larvae and slightly modified from previously described methods\textsuperscript{50,51,71,72}. One primary difference in this study is the recordings were performed on intact CNS to ensure the blood brain barrier was fully functional. The CNS was excised from the larvae and placed in a separate dish with physiological saline (200 µL) containing: 157 mM NaCl, 3 mM KCl, 2 mM CaCl\textsubscript{2}, and 4 mM HEPES, pH = 7.25. Peripheral nerve trunk s were drawn into a recording suction electrode and electrical activity was monitored from descending nerves originating from the ventral ganglia, with amplification by an AC/DC amplifier (Model 1700, A-M Systems, Inc., Carlsborg, WA, USA). Descending electrical activity was subjected to window amplitude discrimination and converted on-line into a rate plot, expressed in Hertz (Hz), using LabChart7 Pro (ADInstruments, Colorado Springs, CO, USA). Noise (60 Hz) was eliminated using Hum Bug (A-M Systems, Sequim, WA, USA). Activity was monitored for a 5 min time period to establish a constant baseline firing rate, as the spike frequency typically increased from 0 to 10 min before stabilization. After a baseline was established, the CNS preparation was directly exposed to test compounds by adding 200 µL of solution to the bath containing 200 µL of saline. The final concentration of solvent in the bath was 0.1% DMSO. Frequencies were measured for 3–5 min for each concentration prior to the addition of the next drug concentration. Mean spike frequencies over the 3–5 min recording period for each concentration were used to construct concentration-response curves in order to determine IC\textsubscript{50} values, which were calculated by nonlinear regression (variable slope) using GraphPad Prism\textsuperscript{39} (GraphPad Software, San Diego, CA, USA). Each drug concentration was replicated 5–10 times.

**Data availability**

The datasets generated during and/or analyzed during the current study are contained within the paper and its Supplementary Information Files, or can be obtained from the corresponding author on reasonable request.

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The authors declare no competing interests.

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