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SCIENTIFIC NOTE

ADENOSINE TRIPHOSPHATE–BINDING CASSETTE TRANSPORTERS ARE NOT INVOLVED IN THE DETOXIFICATION OF *AZADIRACHTA INDICA* EXTRACTS IN *ANOPHELES STEPHENSI* LARVAE

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ABSTRACT. Detoxifying pathways of mosquitoes against the neem (*Azadirachta indica*) extracts are still unclear. The aim of the present study was to investigate the role of adenosine triphosphate–binding cassette (ABC) transporters in this process in *Anopheles stephensi*, one of the main malaria vectors in southern Asia. Third-stage larvae of *An. stephensi* were fed with fish food alone or in combination with neem extract at 0.5%, 1%, 5%, and 10%. Six ABC-transporter genes from 3 different subfamilies (B, C, and G) were analyzed to assess their relative expression compared with controls. A bioassay was also performed to assess larval mortality rate at different concentrations and in combination with verapamil, an ABC-transporter inhibitor. No significant variation in the expression levels of any transporter belonging to the B, C, and G subfamilies was detected. Furthermore, the use of verapamil did not induce an increase in mortality at any of the tested neem extract concentrations, indicating that ABC transporters are not involved in the detoxification of neem extracts in *An. stephensi* larvae.

KEY WORDS Detoxification, mosquito defenses, natural insecticides, neem tree, vector control

Malaria is a major health problem in developing countries. According to the World Health Organization, about 216 million cases occurred in 2016, with 445,000 deaths (WHO 2017). Long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS), and artemisinin-based therapies are the main interventions aimed at preventing malaria infection and spread. Vector control through insecticides is a core component of malaria control programs, but the continuous use of chemical compounds has led to the insurgence of resistance in different vector populations, thus threatening the global malaria control efforts (Alonso and Tanner 2013). Out of the 73 malaria-endemic countries providing data to the WHO, 60 reported resistance to at least 1 insecticide class, while 50 reported resistance to 2 or 3 classes (WHO 2017). For this reason, new, effective insecticides are needed. In this context, botanical sources represent a promising alternative to synthetic insecticides. *Azadirachta indica* (Juss), commonly known as neem tree, has been used for centuries in traditional medicine (Soh and Benoit-Vical 2007). This is probably due to the wide effects that this plant has on parasites and other agents of infection (Soh and Benoit-Vical 2007). *Azadirachta indica* and other Meliaceae species have shown strong larvicidal, antiemergence, repellency, and antioviposition effects in different mosquito species (Mulla and Su 1999). It is now known that, in several mosquito species, part of the detoxification process against xenobiotics is mediated by adenosine triphosphate–binding cassette (ABC) transporters on *Anopheles stephensi* Liston (Epis et al. 2014a, 2014b; Porretta et al. 2016; De Marco et al. 2017; Mastrantonio et al. 2017); *Anopheles gambiae* Giles (Nkya et al. 2014); *Aedes aegypti* (L.) (Bariami et al. 2012); and *Aedes albopictus* (Skuse) (Esquivel et al. 2016). In particular, on *An. stephensi* it has been demonstrated that, among the 8 subfamilies (from A to H) of ABC transporters existing in insects, the B and G subfamilies play a major role in the detoxification of permethrin, showing a pattern of response that varies with time (Epis et al. 2014a, 2014b; De Marco et al. 2017; Mastrantonio et al. 2017). Despite their importance against pyrethroids, these genes are not differentially expressed in response to larval exposure to temephos, a widely used larvicide, highlighting an insecticide-specific involvement of the transporters in this mosquito species (Porretta et al. 2016). For these reasons, the objective of this study was to thoroughly investigate the potential role of ABC transporters in *An. stephensi* defense against the neem extract.

All the mosquitoes used in this study derived from a susceptible *An. stephensi* colony held at the insectary of the University of Camerino, Italy. The

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colony is maintained at standard conditions (28 ± 1°C, 85% relative humidity, 12:12 h light:dark photoperiod) and fed with fish food (FF) (Tetra, Melle, Germany). Third-stage larvae of the test mosquito were used for bioassays and molecular analysis, as described by Epis et al. (2014a, 2014b). Experimental groups were fed with FF containing neem seed extract (FF + neem) at different concentrations: 0.5%, 1%, 5%, and 10%. To obtain these concentrations, *A. indica* seeds were crushed and homogenized to 1 g FF in 50 ml chloroform (Sigma-Aldrich, St. Louis, MO), mixed for 10 min, and then evaporated at a reduced pressure (37°C, 3 mmHg) with a Büchi R 200 rotavapor. The powder obtained was left at room temperature for 24 h.

For the bioassay, 5 groups of on average 25 3rd instars (range: 24–28) were put in 100 ml of spring water and fed with FF + neem at different concentrations (0%, 0.5%, 5%, and 10%), alone or in combination with a sublethal dose of the inhibitor verapamil (100 μM), as reported in previous studies (Epis et al. 2014a, 2014b). Verapamil is a blocker of calcium channels that competes with toxic compounds for the extrusion by transmembrane pumps. Control groups with FF alone or verapamil with FF were included. Mortality was assessed every 24 h for 3 days.

To investigate the effect of different treatments on larval mortality, we ran a generalized linear mixed model with Poisson error structure, using the number of dead larvae as dependent variable and considering replicates as a residual-type random component. We explored the effect on the response variable of the concentration of neem extract (i.e., 0%, 0.5%, 1%, 5%, 10%), addition of verapamil (yes/no), time of treatments (24, 48, or 72 h) and their 2nd-order interactions. The initial number of larvae of each replicate was included in the model as a covariate. Interactions were excluded from the final model when they were found nonsignificant. Interpretation of effects with more than 2 levels was based on pairwise *t*-tests of differences of least square means, applying the Tukey correction for multiple comparisons. The analyses were carried out through PROC GLIMMIX in SAS/STAT 9.4 software (SAS Institute Inc., Cary, NC).

We analyzed the expression of 6 genes known to encode for ABC transporters in *An. stephensi* (*Anst*ABCB2, *Anst*ABCB3, *Anst*ABCB4, *Anst*ABCBmember6, *Anst*ABCG4, *Anst*ABCC11). The expression profile of these genes in the larvae was evaluated after 0.5, 24, 48, and 72 h of treatment at different neem concentrations. The RNA extraction, cDNA synthesis, and quantitative real time polymerase chain reaction (RT-PCR) were performed following the protocol described by Epis et al. (2014a, 2014b) to determine cycle threshold (Ct) values and relative expression levels for each gene. Two different genes, RPS7 and GAPDH, were used as reference genes to normalize the relative expression. To detect any significant effect of neem treatment on the expression of ABC genes, RT-PCR data were analyzed through nonparametric Wilcoxon 2-sample tests, due to the nonnormal distribution of some samples (Yuan et al. 2006). For each of the 6 genes and each of the dose–time combinations, differences in ΔCt ($Ct_{target} - Ct_{housekeeping}$) between treated and control (i.e., dose 0) samples were compared. Estimates of ΔΔCt values and their 95% confidence limits were obtained through the Hodges–Lehman method. All the analyses were carried out using PROC NPAR1WAY in SAS® 9.4 Software (SAS Institute).

Statistical analysis of bioassay data revealed that mortality of larvae (Fig. 1) increased significantly with time ($F_{2,18} = 41.4$; $P < 0.0001$) and at higher concentrations of insecticide ($F_{4,36} = 16.8$; $P < 0.0001$), with no interaction between the 2 explanatory variables. In detail, time had a continuous positive effect on mortality, with the highest proportion of dead larvae recovered at 48-h post-treatment. Comparing mortality at subsequent concentrations of neem extract, the number of dead larvae was higher at 1% as compared with 0.5% ($P_{adj} = 0.025$) and at 10% to 5% concentration ($P_{adj} = 0.038$), while there was no significant difference between controls and 0.5% and between 1% and 5%. These results are in accord with those presented in previous studies on the effect of neem extract on mosquito larvae (Vatandoost and Vaziri 2004, Dua et al. 2009). However, addition of verapamil had no effect on larval mortality, either as a single factor or in interaction with the neem extract (both $P > 0.13$). This lack of differential mortality suggests that ABC transporters are not involved in the cellular response of *An. stephensi* to neem extracts. Porretta and colleagues (2016) obtained similar results treating *An. stephensi* larvae with the insecticide temephos alone or in combination with verapamil. Their study failed to detect any effect of the inhibitor on larval mortality, excluding the role of the transporters in temephos detoxification. On the other side, Epis et al. (2014a) used verapamil in combination with permethrin to demonstrate ABC's involvement against pyrethroids. The combined treatment could lower the 50% lethal dose from 0.137 mg/liter (permethrin alone) to 0.025 mg/liter (permethrin + verapamil). Their mortality results were supported by RT-PCR data, showing a differential expression of the genes analyzed, in particular ABCG4 and ABCmember6. The overexpression peak of these 2 genes was detected after 6 h exposure, but the up-regulation persisted after 24 h. The other genes taken into account were down-regulated or not differentially expressed at the different time points (Epis et al. 2014a, 2014b; De Marco et al. 2017). In the present study, the analysis of RT-PCR data did not reveal any effect of neem treatment on ABC genes' expression: treated sample ΔCt values were not significantly different from controls, for any of the 6 target genes and any of the dose–time combinations (all $P > 0.05$). Gene expression analysis confirmed the

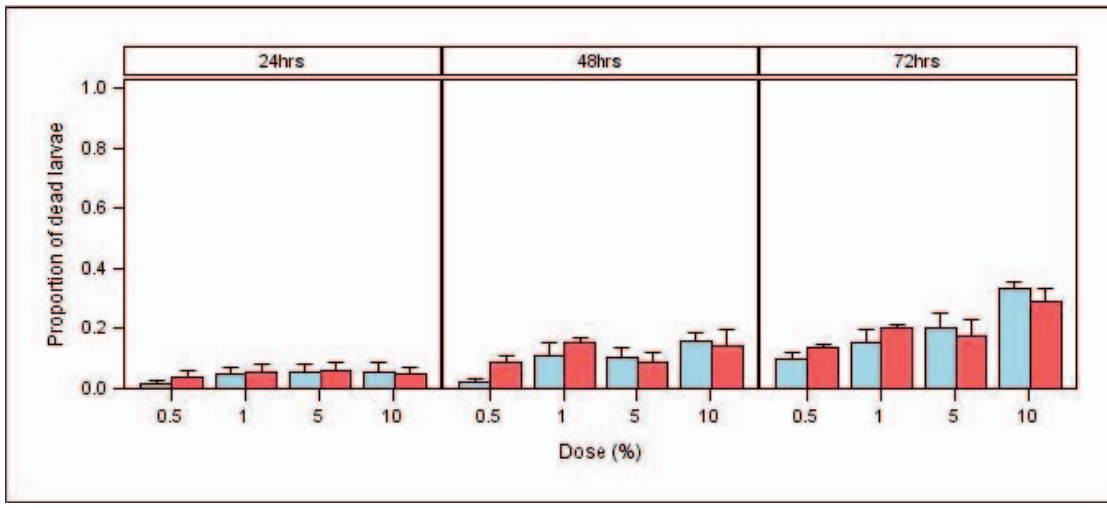

Fig. 1. Proportion of dead larvae at different times and insecticide concentrations, with (blue bars) and without (red bars) verapamil addition. Error bars indicate standard errors.

bioassay data, demonstrating that ABC transporters were not involved in the cellular response of *An. stephensi* to neem extracts. Also, similar expression results were shown by Porretta et al. (2016), where none of the investigated genes were differentially expressed after temephos exposure. All together, these results indicate that different compounds can induce different responses in the *An. stephensi* ABC transporters.

In conclusion, the present study demonstrates that the analyzed ABC transporters are not involved in response/defense to neem extracts in *An. stephensi* larvae. However, we cannot exclude other mechanisms involved in neem extract's detoxification, and, for this reason, further investigations are needed to clarify the response of *An. stephensi*. In particular, future studies should focus on phase I and phase II detoxification enzymes, such as cytochrome P450, carboxylesterases, UDP-glucoronosyltransferases, and glutathione S-transferases, known to be differentially expressed in response to various xenobiotics used for vector control.

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