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Temporal dynamics of the ABC transporter response to insecticide treatment: insights from the malaria vector *Anopheles stephensi*

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In insects, ABC transporters have been shown to contribute to defence/resistance to insecticides by reducing toxic concentrations in cells/tissues. Despite the extensive studies about this detoxifying mechanism, the temporal patterns of ABC transporter activation have been poorly investigated. Using the malaria vector *Anopheles stephensi* as a study system, we investigated the expression profile of ABC genes belonging to different subfamilies in permethrin-treated larvae at different time points (30 min to 48 h). Our results showed that the expression of ABCB and ABCG subfamily genes was upregulated at 1 h after treatment, with the highest expression observed at 6 h. Therefore, future investigations on the temporal dynamics of ABC gene expression will allow a better implementation of insecticide treatment regimens, including the use of specific inhibitors of ABC efflux pumps.

All living organisms are exposed to potentially harmful xenobiotics and toxic endobiotics. The effects of these molecules can be mitigated by enzymatic complexes that function to detoxify cells, called drug metabolizing enzymes (DMEs)[1]. The defensive function of DMEs can be subdivided into different phases, which involve a variety of protein complexes[1–3]. In phase I, oxidation, reduction, and chemical cleavage of toxic compounds occur through the actions of cytochrome P450 carboxylesterases. In phase II, the oxidized chemicals are conjugated with endogenous molecules by enzymes such as glutathione-S-transferase (GSTs) or uridine diphosphate (UDP)-glycosyltransferases. Additionally, two other phases occur, phase 0 and phase III, which involve the action of efflux pumps located in the cellular membrane and belonging to the ATP-binding cassette (ABC) transporter family. In phase 0, ABC transporters belonging to subfamilies ABCB (P-gps) and presumably ABCG, extrude toxicants out of the cells before enzymatic modifications of the compounds, therefore preventing the accumulation of these harmful toxicants inside the cells. In phase III, ABC transporters belonging to subfamilies ABCC (MRPs) and ABCG[2] extrude toxic molecules modified by phase II enzymes from the cells[2,3]. This DME-dependent defence mechanism is conserved among animals[1].

In insects, phase I and II enzymes have been shown to play a role in the detoxification of synthetic insecticides. Numerous studies have reported that cytochrome P450 and GSTs or UDP-glycosyltransferases are overexpressed in strains of several insect species resistant to different classes of insecticides[4–7]. More recently, researchers have begun to focus on ABC transporters and genes involved in defence/resistance to insecticides in agricultural insect pests and disease vectors[8]. At present, despite our increasing awareness of the role of ABC transporters in insecticide defence, the temporal dynamics of their activation after insecticide exposure have been poorly investigated. Therefore, elucidation of the temporal dynamics of ABC transporter activation would facilitate our understanding of the interaction between ABC transporters and insecticides and would help us to develop insect-control strategies based on the inhibition of cellular defences. Several studies have shown that the combination of insecticides and ABC transporter inhibitors can increase the efficacy of the insecticide molecules[9–12]. Therefore, combined treatment with insecticides plus ABC transporter inhibitors could reduce the current dosages of insecticides, thereby preventing environmental pollution and the development of resistance[12,13].

Because the implementation of such a strategy would require gene- and species-specific inhibitors in order to avoid negative consequences in nontarget organisms, it is crucial that the activated ABC transporter genes are precisely identified and that we elucidate the temporal dynamics of their activation.

In this study, we present an investigation of the temporal expression patterns of six ABC transporter genes in the mosquito *Anopheles stephensi*. This species is the major vector of malaria in the Middle East and South Asian regions, where over 25 million people have been estimated to be affected by this disease[14]. In our previous study, we identified five genes belonging to the ABCB and ABCG subfamilies and showed that one of these genes was overexpressed in larvae treated with the insecticide permethrin at 24 and 48 h after treatment[15]. Here, after the identification of an additional ABC gene belonging to the ABCC subfamily, we investigated the expression of all six genes at seven time points from 30 min to 48 h following exposure of mosquito larvae to permethrin.

Results

Identification of a novel ABCC transporter gene in *An. stephensi*. Within the transcriptome of *An. stephensi*, a sequence of 1014 base pairs was found to share 60.98% identity (Fig. 1) with the ABCC11 multidrug transporters (Q7Q4K3) of *Anopheles gambiae*. This sequence was named *Anst*ABCC11 and was deposited in the EMBL nucleotide sequence database (accession number LK392618). Supplementary Table S1 shows the Dayhoff PAM distances between *Anst*ABCC11 of *An. stephensi* and the homologous ABC transporters of the mosquitoes *An. gambiae*, *Anopheles darlingi*, *Aedes aegypti*, and *Culex quinquefasciatus* (the species that showed the highest per cent identity following a BLAST search).

Expression profile after insecticide treatment. Polymerase chain reaction (PCR) amplicons obtained from each of the six examined ABC transporter genes were sequenced. The sequences obtained were identical to those retrieved from the transcriptome of *An. stephensi*, confirming the specificity of the amplification. The relative expression of ABC transporter genes at different times after permethrin treatment of larvae at the lethal dose required to kill 50% of population (LD_{50}; 0.137 mg/L, determined in the same colony of the Liston strain, as described in a previous study[15]) is shown in Figure 2 and Table S2. Initial upregulation was observed after 2 h of treatment for the *Anst*ABCG4 gene and after 4 h of treatment for the *Anst*ABCmember6 gene. The relative expression of these two genes reached a maximum at 6 h after treatment, with 20- and 4-fold increases, respectively (Fig. 2, Table S2). Moreover, the *Anst*ABCG4 gene was upregulated by about 3- and 10-fold compared to the control at 24 and 48 h after treatment, respectively, consistent with the results of the study by Epis and colleagues[15].

Discussion

In a previous, study we identified five ABC transporter genes in the larval transcriptome of the mosquito *An. stephensi* and showed that inhibition of ABC transporters increases the efficacy of permethrin insecticide treatment in this species[15]. In particular, we observed increased mosquito larvae mortality in bioassays conducted with permethrin in combination with the ABC transporter inhibitor verapamil and increased expression of the *Anst*ABCG4 gene in larvae exposed to permethrin at the LD_{50} for 24 and 48 h. In the current study, we identified an additional ABC transporter gene belonging to the ABCC subfamily (ABCC11) and analysed the expression patterns of all of the identified ABC transporters after permethrin exposure for 30 min to 48 h. Members of the ABCB, ABCC, and ABCG subfamilies have been shown to be associated with transport and/or resistance to pyrethroid insecticides in several insect species[8]. Our results showed that permethrin treatment of *An. stephensi* larvae lead to overexpression of the ABCG4 and ABCBmember6 genes, while no overexpression was observed for the other genes (Fig. 2, Table S2).

In this study, we observed no overexpression of ABCB subfamily genes at 24 and 48 h after treatment, despite the fact that genes from this subfamily are activated in other species at these time points (e.g., in the mosquito *Ae. aegypti*, the *ABCB2* gene was shown to be upregulated by 8-fold in larvae 48 h after temephos treatment[12]). Instead, by analysing more time points, we found that the *ABCBmember6* gene was overexpressed at 4 and 6 h after insecticide treatment.

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AnstABCC11      DTHQNYIYHNNSANRWIGIRLEFIGAMVIYYVALLTVSNQSMVGLAFVGIIVSYVLRLIP 60
AGAP008436      DTHQNYIYHNRFANRWIGIRLEFIGAIVIYYVALLTVSNQSMVGFAFVGIIVSYVLRLIP 60
                **********. **************:*****************:***************

AnstABCC11      SLNSLLLASGLLEENIISFERVAQYLDLPRETNDETGVGYPTSGQEKLPVRGPIEYRDVS 120
AGAP008436      SLNSLLLALGALEENIISFERVAQYLDLQRETNDDTGVDYPTSGMDKHPVLGPIIYRDFS 120
                ******** * ***************** *****:***.***** :* ** *** ***.*

AnstABCC11      LTYSDGSTVLHNVTLTIGAGEKLGIVGRTGSGKSSFIGTLFRFYPKNTSGTILIAGVGLN 180
AGAP008436      LTHADGSTVLHNVTLTIAAGEKLGIVGRTGSGKSSFIGTLFRFYPKHTTGYISIAHVELG 180
                **::**************.****************************:*:* * ** * *.

AnstABCC11      AISLRKLRNELTLVPQSTSLFSGLVQNFIDPWKAHTVEQLQTIMRECELASVSLGATLQE 240
AGAP008436      RISLQKLRGELTLVPQSTSLFSGVVQNFIDPRNGHTDEELIRCLRECGLGNVHLATPLEN 240
                ***:***.**************:******* :.** *:*   :*** *..* *.:.*::

AnstABCC11      LSDGQRQLLCLVRGLLRRKPIIILDEATSGLNADTEDLILKVFHKQFHDRTVLMIAHHLN 300
AGAP008436      LSVGQCQLLCLVRGFLRKKPIIILDEATSALDEATEDLILKVLDKQFHGRTVLMIAHHLN 300
                ** ** ********:**:***********.*:  ********:.****.***********

AnstABCC11      TVKCCDRILWLQEGRVRKIAPLSEYTVEERVELGFR 336
AGAP008436      TLRNCHRVLWLQEGRVRKIAPLQDYTVEERAELGFR 336
                *:: *.*:**************.:******.*****
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Figure 1 | Alignment of the amino acidic sequences of the ABCC11 gene of *Anopheles stephensi* and *Anopheles gambiae* (AGAP008436) by ClustalX, inferred from nucleotide sequences. Asterisks: conserved amino acid residues; colons: conserved substitutions; dots: semiconserved substitutions.

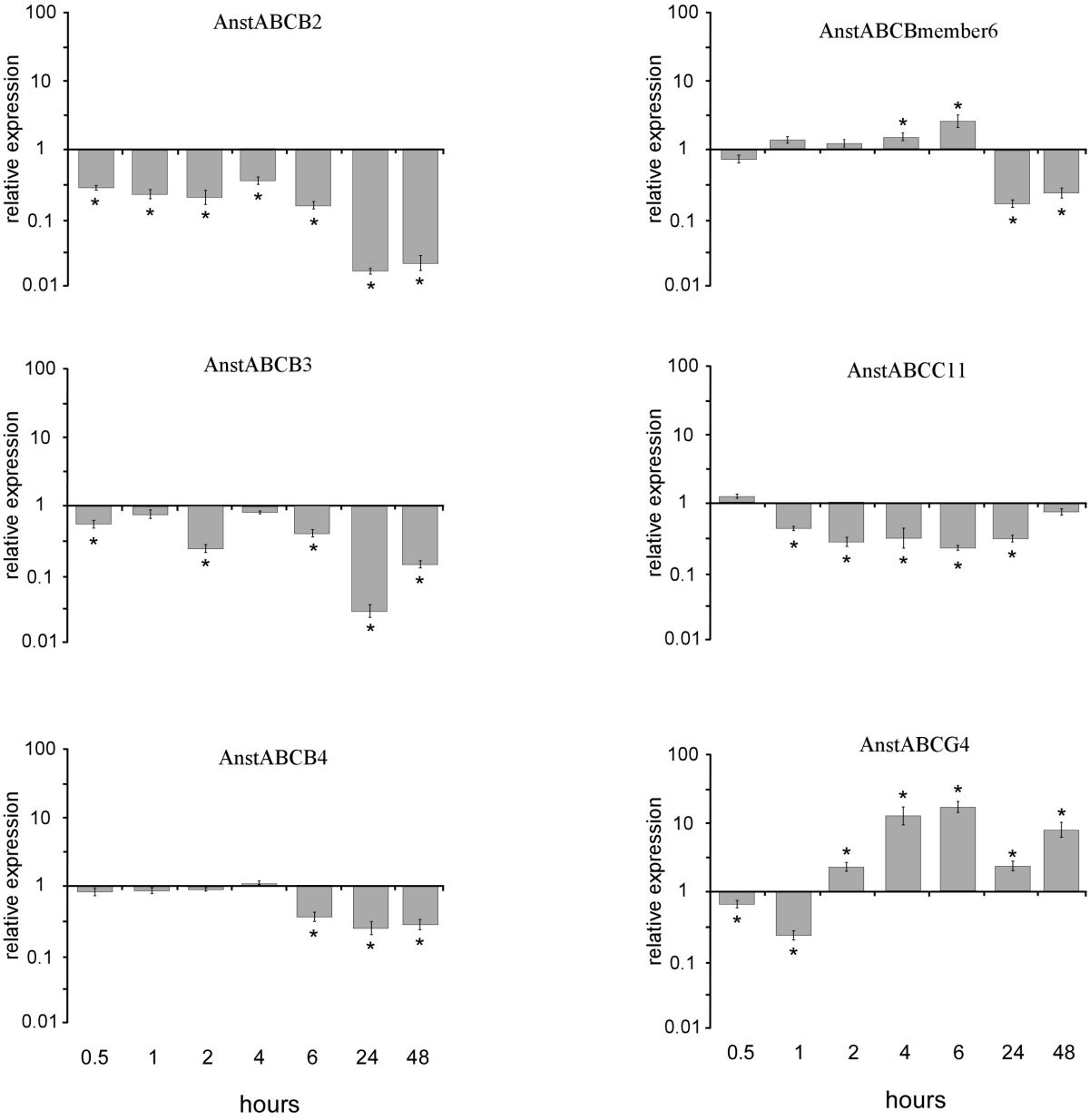

Figure 2 | Relative expression of ABC genes from *Anopheles stephensi* measured by quantitative PCR after exposure to permethrin for different times. The expression level in untreated larvae was considered the basal level, which was set to 1. The internal reference gene *rps7* for *An. stephensi* was used to normalize expression levels. The values are expressed as means of two values. The error bars show the minimum and maximum values observed. Asterisks show significant differences in overexpression between treated and control groups ($P < 0.05$).

Thus, the data from the current study highlighted the importance of investigating the expression profiles of ABC transporter genes at different time points after insecticide exposure; the study of a single time point could fail to reveal an association between ABC transporters and insecticide defence.

In addition, the analysis of temporal dynamics of ABC gene expression could help to elucidate how ABC transporters that belong to the same or different subfamilies interact. In humans, ABC transporters appear to operate in the context of a network of transporters, with cross-regulation of their activity. Each member would thus respond to the presence of transporter network partners, with coordinated regulation of the expression of related pump proteins[2]. For example, an increase in MDR1 protein expression was observed in ABCG2-knockout animals[16]. However, this mechanism has not been studied in insects. Interestingly, in *An. stephensi*, we observed that permethrin induced overexpression of some genes, but downregulation of other genes, suggesting that a cross-regulation mechanism of ABC transporter expression could occur in insects, as has been observed in humans. Further studies are needed to test this hypothesis.

Analysis of the temporal dynamics of ABC gene expression could also facilitate our understanding of the timing of ABC transporter engagement in the overall detoxification process. ABC transporters are thought to act in phases 0 and III of the detoxification process. In phase 0, ABCBs would transport toxicants that do not have enzymatic modifications out of the cell. In phase III, ABCC and ABCG transporter subfamilies would transport toxicants that were modified by detoxifying enzymes during phases I and II (e.g., CYP450, GSTs, or UDP-glycosyltransferases) out of the cell[2,3]. Our data appeared to support the role of ABCB transporters early after insecticide treatment. In particular, the ABCBmember6 transporter was overexpressed at 4 h after treatment, with the highest expression

observed at 6 h. Similarly, our results supported the role of ABCG4 at later time points after treatment: ABCG4 was overexpressed until 48 h, at which time ABCB transporters exhibited decreased expression. Interestingly, ABCG4 was also overexpressed at early time points after treatment, with the highest value observed at 6 h. This observation indicated that this ABC subfamily could play a role during the initial phase of the detoxification process. Moreover, while studies have shown that the detoxification process can be subdivided into different phases in which different enzymatic complexes act, the time after insecticide treatment at which each single phase starts is unknown[2,3]. Future genome-wide expression studies, carried out at different time points after insecticide treatment, could help to elucidate the interplay among the protein complexes involved in the detoxification process, including phase I and phase II enzymes and ABC transporters.

An understanding of the temporal dynamics of *ABC* gene expression is relevant to the field of insect pest control. Studies on ABC transporters in insect pests have been fuelled by the possibility of increasing the susceptibility of target individuals to pesticides used in control programs, e.g., by silencing *ABC* transporter genes[10–12]. Combined treatment with insecticides plus ABC transporter inhibitors could reduce the required dosages of insecticides, thereby minimizing environmental pollution and preventing the development of resistance[17]. To avoid the negative consequences of exposure in non-target organisms, implementation of such a strategy would require gene- and species-specific inhibition of ABC transporter genes. Successful techniques for sequence-specific suppression of gene expression based on RNA interference (RNAi) in insects have been developed in recent years[18], and silencing of ABC transporters by RNAi has recently been conducted in the blood-sucking insects *Cimex lectularius*[7], *Ae. aegypti*[12], and *Pediculus humanus humanus*[13]. However, the development of optimal RNAi protocols remains a highly empirical process[18]. The temporal expression patterns of ABC transporter genes, as determined here in *An. stephensi*, should thus be considered in the implementation of control strategies against insect pests and vectors.

Mosquitoes, including the Asian malaria vector *An. stephensi*, represent an important health concern because they transmit diseases, such as malaria, yellow fever, and filariasis, which affect hundreds of millions of people around the world[19–21]. Human activities and global climate change have contributed to the diffusion of vectors, which increases the risk of disease epidemics[22–26]. For the time being, pesticides remain a main tool for vector and pest control[21,27,28]. Concerns for environmental pollution due to pesticide use, as well as the insurgence of resistance to insecticides in pest populations, highlight the need to identify strategies that optimise the use of chemicals[29–33]. Improving our understanding of insect population dynamics[24,30,31,34–36] and of the molecular basis of the interaction between cellular defences and insecticides[37] is therefore paramount for our ability to control populations of insect pests and vectors. ABC transporters thus represent an interesting research subject, considering that they act as resistance factors against a wide range of molecules. Studies of ABC transporters at the gene level and in terms of temporal dynamics is therefore relevant both for our understanding of their actual role as a defence mechanism and for the development of tools for their inhibition, with the goal of implementing novel strategies for pest and disease vector control.

Methods

Mosquito samples. The mosquito larvae used in this study were obtained from an *An. stephensi* colony (the Liston strain) maintained for 4 years in the insectary at the University of Camerino[15]. The colony was maintained following standard conditions as follows. Adult insects are reared at 28 ± 1°C and 85%–90% relative humidity with 12-h light/12-h dark photoperiods and a 5% sucrose solution. Adult females were fed mouse blood for egg laying. Eggs were placed in spring water to obtain larvae. Larvae were maintained in spring water and fed daily with fish food (Tetra, Melle, Germany) at the same temperature and photoperiods of the adults.

Identification of ABCC transporter genes. To identify genes encoding ABCC transporters, we used a previously described approach[15]. Briefly, the transcriptome of *An. stephensi* was compared with Blastx (e-value = 0.00001) to the annotated transcriptome of *An. gambiae*, available in the VectorBase database, and the sequences of ABCC transporters were extracted automatically and manually controlled. Based on published results obtained by Bonizzoni and colleagues[38], we selected one *ABCC* gene (*Anst*ABCC11).

Oligonucleotide primers were designed from the selected sequence to be used in quantitative PCR (qPCR) experiments. The sequence of the identified *Anst*ABCC11 transporter was then translated to amino acids and compared against the Uniprot database[39] using Blastp. ClustalX was used to align homologous proteins, and PROTDIST software with the PHYLIP package was used to estimate distances among homologous proteins using the Dayhoff PAM matrix.

Bioassays. Permethrin (PESTANAL, $C_{21}H_{20}Cl_2O_3$, Sigma-Aldrich S.r.l., Milan, Italy) was diluted in acetone (ACS reagent, ≥99.5%, Sigma-Aldrich S.r.l.) to a final concentration of 5 mg/mL and then stored at 4°C as stock solution. This stock solution was diluted with water to obtain a 100× solution (13.7 mg/L), to be distributed into the test vials (250-mL plastic glasses). The assays were conducted on *An. stephensi* mosquito larvae at the third instar, collected from the same breeding site and at the same time[15]. Test vials containing 25 larvae in 99 mL of spring water were used for the assays, and experiments were performed in quadruplicate. For insecticide exposure, 1 mL of 100× permethrin solution was added to each of the four vials at a final concentration of 0.137 mg/L in 100 mL. For the control, 1 mL of water plus acetone (at the same concentration as in the insecticide test) was added to each vial. Two pools of 10 living larvae were collected at different times (0.5, 1, 2, 4, 6, 24, and 48 h) after permethrin exposure and from the control vials. The larvae were stored in RNAlater for molecular analysis.

Gene expression profile after insecticide treatment. Gene expression profiles were investigated at seven times (0.5, 1, 2, 4, 6, 24, and 48 h) after permethrin exposure for all of the six ABC genes from *An. stephensi*. RNA was extracted from each pool of larvae using an RNeasy Mini Kit (Qiagen, Hilden, Germany) including an on-column DNase I treatment (Qiagen), according to the manufacturer's instructions. Briefly, total RNA was eluted into nuclease-free water, and the concentration of RNA was determined at 260 nm[40] using a NanoDrop ND-1000 (Thermo Scientific, DE, USA). cDNAs were synthesized from 150 ng of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen) with random hexamers. The cDNA was used as template for reverse transcription (RT)-PCR using previously reported primer pairs[15] for *Anst*ABCB2, *Anst*ABCB3, *Anst*ABCB4, *Anst*ABCmember6, and *Anst*ABCG4. For the *Anst*ABCC11 gene, the primers (forward: ABCC11_F 5′-GGTTGGATTGGCT-TTCGTG-3′ and reverse: ABCC11_R 5′-ATAACCGACTCCCGTTTCG-3′) were derived from the sequence identified in the context of this study (see above). The amplification fragments obtained using standard PCR conditions and cycles (see below) were sequenced in order to confirm the specificity of the amplifications.

Quantitative RT-PCR of target ABC cDNAs was performed using a Bio-Rad iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with iQ SYBR Green supermix (Bio-Rad), under the following conditions: 50 ng of cDNA; 300 nM of forward and reverse primers; 98°C for 30 s, followed by 40 cycles of 98°C for 15 s and 59°C for 30 s; fluorescence acquisition at the end of each cycle; melting curve analysis after the last cycle. The cycle threshold (Ct) values were determined for each primer pair, and the expression levels of target genes were calculated relative to the internal reference gene *rps7* for *An. stephensi*[41]. The expression levels of the ABC transporter genes in the control groups were considered as the basal level (set equal to 1). The relative expression levels of the treated larvae were reported as means. Significant differences in expression between treated and control groups have been evaluated as implemented in the CFX Manager software (Bio-Rad).

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Author contributions

S.E., D.P., V.Ma and V.Me. contributed to conceiving and designing experiments, running the experiments, analyzing data associated with the experiments, and in writing the manuscript. D.S. and L.D.M. performed the bioinformatic analyses; I.R. and M.M. contributed to sample collection. G.F., C.B. and S.U. contributed to data interpretation and manuscript writing. All authors read and approved the final version of the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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