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Xenobiotic-induced expression of detoxification genes, *CYP4H28v2* and *CYP4H31v2* in the dengue mosquito *Aedes aegypti*

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Abstract. Synthetic insecticides and other xenobiotic compounds are usually used to abate the effects of insect pests/vectors of agricultural and medical importance by adversely affecting the insects. The xenobiotics are also capable of inducing the expression of detoxification genes such as the cytochrome P450 (CYP) gene in targeted insects like mosquitoes (Feyereisen, 2012). The high expression levels of CYPs in insecticide-resistant and exposed mosquitoes have been linked with a potential role in xenobiotic resistance. This study investigated the induction potential of leaf litter infusions and insecticides on the expressions of *CYP4H28v2* and *CYP4H31v2* in the dengue mosquito *Aedes aegypti* using Reverse Transcription-quantitative Polymerase Chain Reaction. Exposure of *Ae. aegypti* larvae to leaf litter infusions demonstrated that *CYP4H28v2* and *CYP4H31v2* were significantly induced by *Lagerstroemia speciosa* but not *Ficus benjamina*, *Plumeria obtusa*, *Millettia pinnata* and *Pithecellobium dulce*. None of the genes was significantly highly expressed in adult females exposed to d-allethrin and d-trans allethrin products. *CYP4H28v2* was significantly induced in *Ae. aegypti* exposed to DDT, malathion and permethrin for both 5 and 10 min. DDT, malathion and permethrin significantly induced *CYP4H31v2* only in *Ae. aegypti* exposed to the insecticides for 10 min. Exposure to the insecticides for 5 min displayed different levels of *CYP4H31v2* expression with significantly higher (DDT-exposed) and lower (permethrin-exposed) levels in the mosquito. The results show that natural and synthetic xenobiotics can induce significant expression of *CYP4H28v2* and *CYP4H31v2* in the mosquito, indicating the potential role of the genes in mediating xenobiotic resistance. This may enhance the survival capabilities of the mosquito when in contact with phytotoxins of leaf litter in their natural ecosystem and synthetic insecticides in adulticide spray regimens.

INTRODUCTION

Cytochrome P450 monooxygenases (CYPs) are a multienzymes superfamily group that metabolise a wide range of endogenous and exogenous substrates (Feyereisen, 2012). They are present in many organisms like bacteria, plants and animals (Nelson, 2013). In insects, CYPs are involved in numerous life activities such as growth and development as well as survival against xenobiotics (Scott, 1999; Liu *et al.*, 2011; Feyereisen, 2012; Nelson, 2013). Xenobiotics such as insecticides and plant toxins which are used to control biting and disease-borne mosquitoes can induce metabolic genes including CYP genes (Scott, 1999; David *et al.*, 2006; Feyereisen, 2012; Kim & Muturi, 2012). The high induction of CYPs in xenobiotic-exposed insects indicates that they may be linked to the development of xenobiotic resistance in insects (Giraudo *et al.*, 2010; Poupardin *et al.*, 2010). Xenobiotic resistance due to the production of CYP is one of the most common metabolic insecticide resistance mechanisms in insects (Li *et al.*, 2007; Feyereisen, 2012). This has been observed in several mosquito species

including the dengue mosquito *Aedes aegypti*. CYPs of families 4 and 6 have been pinpointed to be potentially involved in xenobiotic resistance in insects (Poupardin *et al.*, 2010; Feyereisen, 2012). Studies indicate that some CYPs belonging to these families are highly expressed in insecticide-treated/selected *Ae. aegypti* populations and could possibly have roles in their insecticide resistance and tolerance (Saavedra-Rodriguez *et al.*, 2012, 2014).

Aedes aegypti is a vector of arboviral diseases which militate against human health. Some of these diseases like dengue and chikungunya do not have effective vaccines or curative agents; thereby making vector control the most effective management approach. The use of insecticidal products against different developmental stages of the vector is a common control method (Ranson *et al.*, 2010), but this contributes to resistance development by inducing metabolic enzymes like CYPs (Vontas *et al.*, 2012). In addition to the insecticides commonly used for community-wide vector control, domestic insecticides like spatial repellents can also cause toxic effects on mosquitoes in controlled environments and affect the expression of CYPs (Avicor *et al.*, 2014).

Mosquito larvae interact with leaf litter in multiple ways including feeding and as part of/in their larval habitats. The interactions can affect the growth and development of the mosquito as well as inducing toxicity in mosquito larvae (Boyer *et al.*, 2006; David *et al.*, 2006; Kim & Muturi, 2012) and hence can serve as a tool for mosquito control. However, the leaf litter toxins can also induce the expression of CYPs, thereby increasing larval tolerance to plant toxins (David *et al.*, 2006; Kim & Muturi, 2012).

Although the toxicity of leaf litter and spatial repellents on *Ae. aegypti* are well known (Boyer *et al.*, 2006; David *et al.*, 2006; Kim & Muturi, 2012; Ogoma *et al.*, 2012; El-garj *et al.*, 2015), studies about their effects on metabolic enzymes such as CYPs are either limited or unknown respectively. Recently two CYP family 4 genes, *CYP4H28v2* and *CYP4H31v2* were isolated from a Malaysian strain of *Ae. aegypti* and although studies detailing the identification of *CYP4H28v2* (Elgarj & Wajidi, 2013) and *CYP4H31v2* and their characterisation in larvae induced with different synthetic insecticides have been conducted (El-garj *et al.*, in press), the effects of leaf litter and synthetic toxic substances (insecticides and insecticide based products) on expression of the genes in the larvae and adults respectively were not examined. Since exposure of mosquitoes to phytotoxins like leaf litter toxins in larval habitats and anthropogenically introduced synthetic toxic substances impose toxic stress on mosquitoes and can lead to an increase in detoxification genes of insects (Boyer *et al.*, 2006; David *et al.*, 2006; Poupardin *et al.*, 2010; Kim & Muturi, 2012; Avicor *et al.*, 2014), characterisation of the induction potential of these xenobiotics on *CYP4H28v2* and *CYP4H31v2* will provide useful information on their potential involvement in xenobiotic resistance in *Ae. aegypti*. Hence, in this study, a laboratory strain of *Ae. aegypti* susceptible to insecticides was used to investigate the induction of two representative CYPs in larvae and adults exposed to leaf litter infusions and insecticides respectively to evaluate their potential involvement in xenobiotic resistance.

MATERIALS AND METHODS

Aedes aegypti strain

Larvae (4th instar) and adult females (3-5 days old unless stated otherwise and fed on 10% sucrose solution) of a susceptible laboratory reference strain of *Ae. aegypti* from the Vector Control Research Unit of the School of Biological Sciences, Universiti Sains Malaysia were used for xenobiotic (leaf litter infusions, spatial repellents and insecticide-impregnated papers) inductions. All treatments including the controls were performed in triplicates unless stated otherwise.

Plant species

Leaf litter of the Weeping fig (*Ficus benjamina*; family Moraceae), Giant crape myrtle (*Lagerstroemia speciosa*; family

Lythraceae), Singapore graveyard flower (*Plumeria obtusa*; family Apocynaceae), Manila tamarind (*Pithecellobium dulce*; family Fabaceae) and Pongamia tree (*Millettia pinnata*; family Fabaceae) were selected for the study. The plants contain tree holes and other water holding cavities which are potential habitats for mosquitoes as observed in some species of these genera (Omlin *et al.*, 2007; Chitra *et al.*, 2014; Chowdhury *et al.*, 2014). The plants can grow to various heights ranging from 10 to 25 m and are used as ornamentals (CABI, 2015; EOL, 2015; USDA-ARS, 2015) and for medicinal purposes (Sangwan *et al.*, 2010; Singh *et al.*, 2010; Stohs *et al.*, 2012; Ali *et al.*, 2014; Imran *et al.*, 2014).

Leaf litter infusions and induction

Leaves of *F. benjamina*, *L. speciosa*, *P. obtusa*, *P. dulce* and *M. pinnata* which were visibly fungi free were collected within the Universiti Sains Malaysia main campus and infusions prepared according to Kim & Muturi (2012). Leaf litter infusions and treatment period that caused mortality of less than 10 % were used to observe the induction effect. Powdered leaf litter (2g) was immersed in 1L of deionised water for eight days with constant agitation and filtered. The infusion (360mL) was decanted into a 400mL plastic container and 40 fourth instar larvae of *Ae. aegypti* were added, followed by the addition of 0.25g of leaf powder. This was monitored for 48 h at 25 ± 2 ºC and a relative humidity (RH) of 70 ± 5 %. The control was larvae exposed to only deionised water.

Spatial repellents and induction

Mosquito coils (0.2 and 0.3 % (w/w) d-allethrin and 0.1 and 0.15 % (w/w) d-trans allethrin) whose efficacies have been previously evaluated (El-garj *et al.*, 2015) were used in this study. These were exposed separately to 100 adult females in a Peet-Grady chamber for 1 hour (WHO, 2009) as in El-garj *et al.* (2015) at 29 ± 2 ºC and 70 ± 5 % RH. The mosquitoes were then collected into paper cups and supplied with 10 % sucrose solution for 24 h. Adult females placed in the chamber without any coil exposure was the control.

Insecticide-impregnated papers and induction

Three-day old adult females (4 replicates of 25 mosquitoes each) were exposed to World Health Organization (WHO) papers impregnated with DDT (4 %), malathion (0.8 %) and permethrin (0.25 %) at 5 and 10 min using WHO insecticide exposure tubes at 25 ± 2 ºC and 70 ± 5 % RH. The respective WHO non-insecticide oil papers were used as control. Mosquitoes were provided with 10 % sucrose solution for 24 h post-treatment.

Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from mosquitoes (30mg of larvae or adult females per sample) that survived the induction assays using Qiagen® RNeasy® Mini Kit and treated with DNaseI (Fermentas®) according to the products' protocols. cDNA was prepared from total RNA (10µg) using 5X iScript Reverse Transcription Supermix (Bio-Rad®) according to the product's protocol in a PTC-100® Thermal cycler.

Aedes aegypti 40S ribosomal protein S17 gene (*rpS17*, GenBank accession number: AY927787.2) was used as the reference gene as in Clemons *et al.* (2011). The target genes were *CYP4H28v2* (GenBank accession number: KC481237.1) and *CYP4H31v2* (GenBank accession number: KF779931.1). Primers were designed using the online-tool Primer3Plus (Untergasser *et al.*, 2007). The expected product lengths of the genes (Table 1) were 117 bp (*rpS17*), 214 bp (*CYP4H28v2*) and 186 bp (*CYP4H31v2*). The primers were checked for secondary structure, melting temperature and complementarity using the OligoCalc: Oligonucleotide Properties Calculator (Kibbe, 2007).

The optimised PCR mix (10µL) contained 2µL of cDNA, 5µL of iQ™ SYBR® Green Supermix, 2µL of nuclease-free water and 0.5µM each of the forward and reverse primers. The optimised cycling condition of 95 ºC for 3 min, then 40 cycles of 95 ºC for 15 s and 62.5 ºC for 30 s was performed in a CFX96™ Real-Time System (Bio-Rad®). Product homogeneity was confirmed by melting curve analysis (from 55-95 ºC, in

Table 1. List of primer sequences used for RT-qPCR

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
rpS17	AGACAACTACGTGCCGGAAG	TTGGTGACCTGGACAACGATG
CYP4H28v2	GGTTCTGCTGTCCAAGGTCAC	GCCAACGTGCTGCTCTATCTT
CYP4H31v2	GAATGCCCAGAAGAATCCAA	TCGATCACCGAAATTGTGAA

increments of 0.5 °C for 5 s per step). Electrophoresis of amplified products was conducted for 45 min at 70V. The gel was stained with ethidium bromide and observed under ultra-violet (UV) light in the UVP GelDoc-It® Imaging System. Three biological and three PCR replicates were performed for all treatments. Serial dilution of calibrator cDNA was performed to determine the amplification efficiency of the primer sets. The relative expression of each gene was calculated using the amplification efficiency and quantification cycle according to Pfaffl (2001). One-way analysis of variance (ANOVA) was used to analyse the treatment data using IBM SPSS version 20.0 (IBM Corp., 2011) with a significance level at $p<0.05$. Genes with a fold expression greater than or equal to 2 or less than or equal to -2 relative to the control group were considered as having a significantly higher or lower expression respectively (Strode *et al.*, 2008).

RESULTS

Expression of *Aedes aegypti* CYPs in leaf litter infusions-treated larvae

The effects of leaf litter infusions on the expression of the two detoxification genes in *Ae. aegypti* were examined using RT-qPCR. When treated with leaf litter infusions, significantly different expression patterns of *CYP4H28v2* and *CYP4H31v2* were detected in the larvae ($p<0.05$, Figure 1). Infusions of *F. benjamina* induced significant lower expression of the two genes, with greater effect on *CYP4H31v2*. Significant lower expression of *CYP4H28v2* was also detected in larvae treated with *P. obtusa* and *M. pinnata* (Figure 1). Although *P. dulce* induced lower expression of the two genes, this was not significant (less than 2-fold lower expression). Water infusion of *L. speciosa* leaf was the only infusion that induced a significantly higher expression of *CYP4H28v2* and *CYP4H31v2* in the larvae.

Expression of *Aedes aegypti* CYPs in spatial repellents-treated females

Expressions of *CYP4H28v2* and *CYP4H31v2* in adult *Ae. aegypti* females treated with mosquito coils showed patterns that were significantly different ($p<0.05$), although neither of the genes was significantly highly expressed (Figure 2). Contrasting expression patterns were detected in 0.15 % d-trans allethrin (lower expression for both genes) and 0.2 % d-allethrin (~1 fold for *CYP4H28v2*) treated females, although the expression levels were not significant. Significantly lower expression of *CYP4H28v2* was only detected in the 0.3 % d-allethrin treated females. However, significantly lower expression of *CYP4H31v2* was detected in two products, 0.1 % d-trans allethrin and 0.3 % d-allethrin coils.

Expression of *Aedes aegypti* CYPs in adult females treated with insecticide-impregnated papers

CYP4H28v2 expression in adult *Ae. aegypti* females treated with insecticide-impregnated papers was significantly different ($p<0.05$) at 10 min but not ($p>0.05$) at 5 min induction (Figure 3). In contrast, the expression pattern of *CYP4H31v2* (Figure 4) was significantly different at 5 min ($p<0.05$) but not ($p>0.05$) at 10 min. The *CYP4H28v2* gene was significantly highly expressed after treatment with DDT, malathion and permethrin for 5 and 10 min (Figure 3). Malathion induced the highest relative fold expression for both induction periods (>3-fold to >5-fold). Expression of *CYP4H28v2* was higher when *Ae. aegypti* was induced with

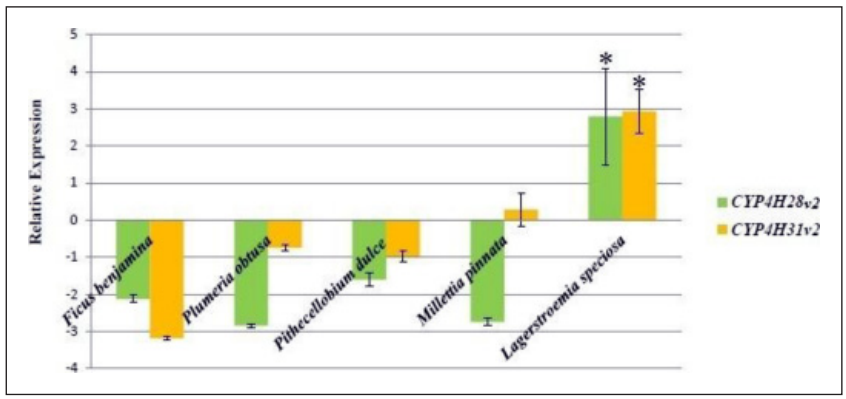

Figure 1. Expression analysis of *CYP4H28v2* and *CYP4H31v2* in fourth instar larvae of *Aedes aegypti* induced with leaf litter infusions relative to the control. Error bars represent standard error. Asterisks show genes that have significantly higher levels of expression.

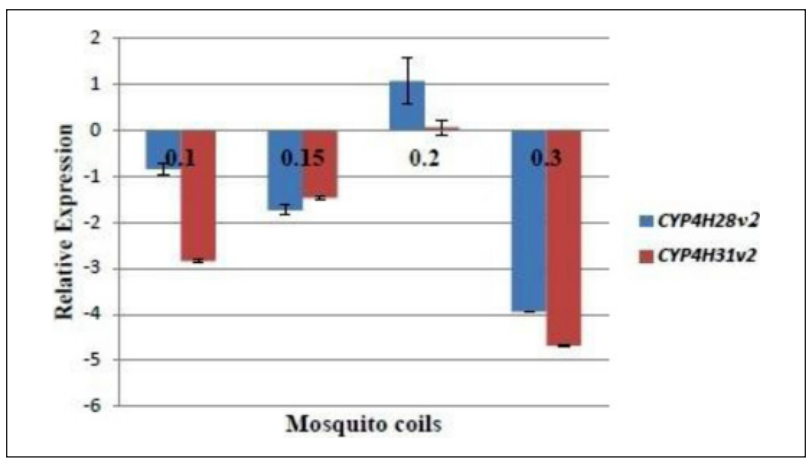

Figure 2. Expression analysis of *CYP4H28v2* and *CYP4H31v2* in adult females of *Aedes aegypti* induced with d-trans allethrin (0.1 and 0.15 %) and d-allethrin (0.2 and 0.3 %) mosquito coils relative to the control. Error bars represent standard error.

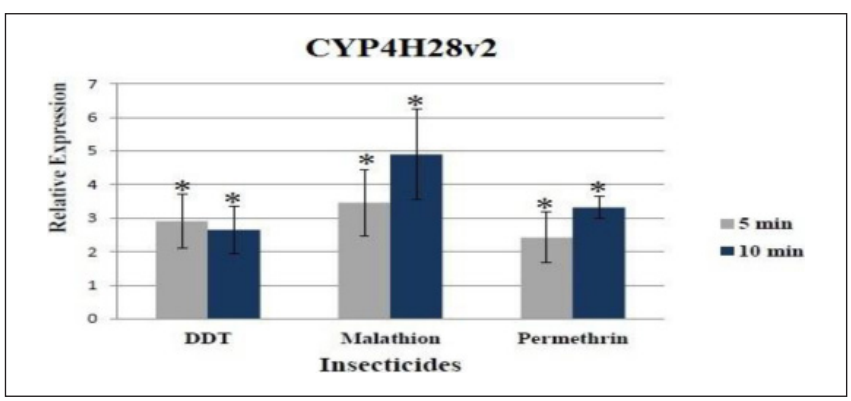

Figure 3. Expression analysis of *CYP4H28v2* in adult females of *Aedes aegypti* after treatment with insecticide-impregnated papers at 5 and 10 min relative to the control. Error bars represent standard error. Asterisks show genes that have significantly higher levels of expression.

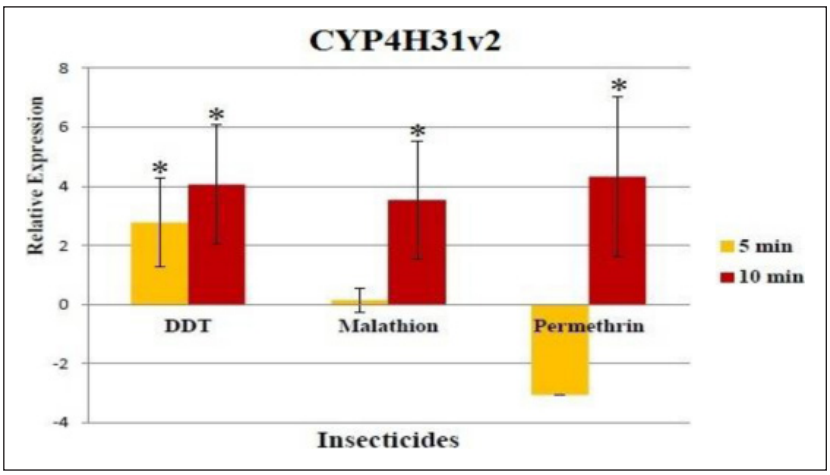

Figure 4. Expression analysis of *CYP4H31v2* in adult females of *Aedes aegypti* after treatment with insecticide-impregnated papers at 5 and 10 min relative to the control. Error bars represent standard error. Asterisks show genes that have significantly higher levels of expression.

insecticides for 10 min than 5 min except for DDT-treated female.

Insecticide treatment for 5 min produced contrasting *CYP4H31v2* expression in the *Ae. aegypti* females. DDT treatment caused a significantly higher *CYP4H31v2* expression (~2.8-fold) while permethrin caused a significantly lower expression (~-3.1-fold). All three insecticides induced significantly higher expression levels of *CYP4H31v2* (~4.1-fold for DDT, ~3.5-fold for malathion and ~4.3-fold for permethrin) after a treatment period of 10 min.

DISCUSSION

This study investigated the induction potential of xenobiotics on the *Ae. aegypti* detoxification genes, *CYP4H28v2* and *CYP4H31v2*. Infusions of the various leaf litters induced different expression levels of the two CYP genes in *Ae. aegypti* larvae. Of the plant species tested, leaf infusions of *L. speciosa* significantly induced *CYP4H28v2* and *CYP4H31v2*. The *L. speciosa* tree is a perennial plant with tree holes that can collect water. The tree holes phytotelmata can serve as larval habitat for container mosquitoes like *Ae. aegypti* and this adduces to the sampling of *Lagerstroemia* plants for tree hole inhabiting mosquitoes (Mangudo *et al.*, 2011; Sultana *et al.*, 2012). Allochthonous leaf litter of *L. speciosa* may also contribute to detritus in the habitat of litter inhabiting larvae, exposing the larvae to *L. speciosa* toxicity which has been reported to effect larvicidal activity on the brine shrimp *Artemia salina* (Rahmatullah *et al.*, 2010). Hence the significant induction of *CYP4H28v2* and *CYP4H31v2* is a nascent indicator of their possible involvement in detoxification of *L. speciosa* leaf toxins. A single CYP gene can metabolise several substrates (Feyereisen, 2012), hence tree hole inhabiting *Ae. aegypti* (Anosike *et al.*, 2007; Mangudo *et al.*, 2011; Tubaki *et al.*, 2010) with an evolved resistance to *L. speciosa* toxins due to the high induction of CYPs may also develop resistance to other flora in their larval habitats. Leaf litter infusion of *F. benjamina* significantly suppressed expression of the CYP genes. The infusions of *P. obtusa* and *M. pinnata* also significantly suppressed expression of *CYP4H28v2*. Findings of this study show that the CYP genes are differentially induced by the various leaf litter infusions. The results are congruent with previous studies in *Ae. aegypti* using infusions from different plant leaves (David *et al.*, 2006; Kim & Muturi,

2012) which suggested that the induction of CYPs may enhance the metabolism of toxic products in the leaf litter. Hence the significant higher levels of *CYP4H28v2* and *CYP4H31v2* in larvae exposed to *L. speciosa* imply that the larvae may be expressing these detoxification genes in response to leaf litter toxins. According to David *et al.* (2006), the increased expression of an *Ae. aegypti* CYP, *CYP6AL1* in larvae exposed to leaf litter may influence their ability to detoxify plant toxins found in larval environments. Hence, the high induction of *CYP4H28v2* and *CYP4H31v2* in this study indicates that they probably play roles in detoxification of plant toxins to enhance the tolerance capabilities of larvae.

The use of personal insecticides such as d-allethrin and d-trans allethrin products for anti-mosquito purposes is a common phenomenon due to their activity on mosquitoes, especially under controlled conditions (Avicor *et al.*, 2013, 2015; Ogoma *et al.*, 2012). These products can induce the expression of detoxification genes like CYPs in organisms as observed in rats and *Aedes albopictus* (Vences-Mejía *et al.*, 2012; Avicor *et al.*, 2014). Expression profiles of *CYP4H28v2* and *CYP4H31v2* showed that none of the genes were significantly highly expressed in *Ae. aegypti* adult females treated with the spatial repellents. Adult females treated with 0.2 % d-allethrin expressed *CYP4H28v2*, although this was not at a significantly higher expression level. These findings are in contrast with studies by Vences-Mejía *et al.* (2012) and Avicor *et al.* (2014) in rats (*CYP2E1* and *CYP3A2*) and mosquitoes (*CYP4H42* and *CYP4H43*) exposed to similar active ingredients. The spatial repellents used in this study could be poor inducers of the two *Ae. aegypti* CYPs. Also, these CYPs may probably not be involved in detoxifying the active ingredients of these products.

Significant lower expression of *CYP4H28v2* (in 0.3 % d-allethrin treated females) and *CYP4H31v2* (in 0.1 % d-trans allethrin and 0.3 % d-allethrin females) were detected. The absence of significant increased expression of the two genes bodes well for the usage of these products since they pose low risk in increasing the levels of detoxifying enzymes that may reduce their insecticidal activity. This finding however does not exclude the possibility of the induction of other CYPs by these spatial repellents. Although expression of *CYP4H28v2* was ~1-fold in 0.2 % d-allethrin treated females, expression of this gene was significantly lower (>-3-fold) in 0.3 % d-allethrin treated females. This shows that although expression of CYPs may predicate on active ingredient concentration, the relationship between insecticide dose and CYP induction is unclear since it has been shown that lower doses can also induce CYPs at levels higher than those induced by higher insecticide doses (Liu *et al.*, 2011).

Resistance to the major insecticide classes extensively used for mosquito control is rife in *Ae. aegypti* (Ranson *et al.*, 2010; Vontas *et al.*, 2012). Metabolic insecticide resistance ruins the effectiveness of insecticide-based vector control through the expression of metabolic enzymes. The insecticide-impregnated papers used in this study significantly induced *CYP4H28v2* at both 5 and 10 min of exposure. However, for *CYP4H31v2*, there were different expression responses. Expression of *CYP4H31v2* was significantly higher in DDT-treated females at 5 and 10 min. Malathion significantly induced the expression of *CYP4H31v2* at higher levels in females which were treated with the insecticide for 10 min but not 5 min. For permethrin-treated females, there was a contrasting effect (significant lower expression at 5 min and significant higher expression at 10 min exposure) at the induction periods. Studies have shown that CYPs are highly expressed in insecticide-resistant and induced insects and these may potentially be involved in the emergence of insecticide resistance (Amenya *et al.*, 2008; Poupardin *et al.*, 2010). The expression of *CYP4H28v2* in *Ae. aegypti* treated with insecticide-impregnated papers in this study was over 2-fold irrespective of the treatment period, with the highest expression in the malathion-treated mosquitoes. The induction potential of these insecticides on the expression of *CYP4H28v2* can impair the

effectiveness of these synthetic toxicants on *Ae. aegypti*. The increase in expression of *CYP4H28* in response to insecticide treatment has been shown in *Ae. aegypti* treated with permethrin (Saavedra-Rodriguez *et al*., 2012). Permethrin can also induce CYPs belonging to other families in insect populations (Poupardin *et al*., 2012; Gong *et al*., 2013; Reid *et al*., 2014). Previous studies also indicate that DDT and malathion are capable of inducing expression of CYPs from other families in insects (Festucci-Buselli *et al*., 2005; Huang *et al*., 2013). These findings are comparable with the results of this study. Exposure duration of permethrin influences the expression of *CYP4H31v2*. A relatively brief (5 min) exposure significantly reduced *CYP4H31v2* expression while a longer exposure (10 min) significantly increased expression (over 4-fold) of the gene. It is suggested that the shorter exposure period was below the threshold potent to trigger *CYP4H31v2* expression. Expression of *CYP4H28v2* and *CYP4H31v2* in response to *L. speciosa* and insecticides in this study and other previous studies on CYP inducers (Poupardin *et al*., 2010; Kim & Muturi, 2012; Huang *et al*., 2013) highlights the possibility of overlapping substrates of these genes, a characteristic of CYPs (Scott, 1999; Scott & Wen, 2001; Li *et al*., 2007; Feyereisen, 2012). The high levels of both genes in xenobiotic-induced mosquito can be considered as an indicator of the likely involvement of these genes in metabolic resistance. However, the roles of *CYP4H28v2* and *CYP4H31v2* in the metabolism of xenobiotics remain to be resolved and would require the support of *in vitro* studies to confirm their involvement.

In conclusion, expression studies of *Ae. aegypti CYP4H28v2* and *CYP4H31v2* demonstrated that the genes were significantly induced in *L. speciosa*, DDT, malathion and permethrin-treated mosquitoes. Plasticity of expression of the two detoxification genes depends on the type of insecticide, dose and duration of exposure. These findings necessitate characterisation of these genes to elucidate their involvement in insecticide resistance. Further investigations are also needed on the mechanism of induction and the relationship between the induced genes and their inducer in insects.

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