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Sublethal effects of chronic exposure to chlorpyrifos or imidacloprid insecticides or their binary mixtures on *Culex pipiens* mosquitoes

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Abstract. Mosquitoes represent one of the most significant threats to human and veterinary health throughout the world. Consequently, improving strategies for the control of mosquitoes is essential. In the present study, juvenile *Culex pipiens* (Diptera: Culicidae), the common house mosquito, are chronically exposed to sublethal concentrations of chlorpyrifos (20% of LC_{50}) and imidacloprid (5% of LC_{50}), both separately and as a mixture. Developmental time, the emergence rate of adults and the expression of five selected genes involved in detoxification and resistance to pesticides are assessed. To assess the effects on oviposition choice, gravid females are forced to oviposit into cups containing water with added chlorpyrifos, imidacloprid or a mixture of both. The time required for the development of second- and third-instar larvae is observed to differ significantly between treatments. Adults of *C. pipiens* fail to emerge from larvae hatched in both imidacloprid and the binary mixture. The expression of the four quantified detoxification genes differs significantly in third-larval instars exposed to chlorpyrifos and/or imidacloprid compared with controls. Gravid females also fail to lay eggs on water to which either of the insecticides or the binary mixture is added, although they do lay eggs in cups containing water only. Chronic exposure to sublethal concentrations of chlorpyrifos or imidacloprid has significant adverse effects on development and thus the reproductive fitness of *C. pipiens* and, accordingly, could be used in the population control of these mosquitoes.

Key words. Detoxification, Diptera, gene expression, mosquitoes, neonicotinoides.

Introduction

Mosquitoes can spread diseases that infect more than 600 million people per year (Tehri & Singh, 2015; Whitehorn, 2015). *Culex pipiens* (Diptera: Culicidae), the common house mosquito, is a vector of several human pathogens, including West Nile virus, Rift Valley fever virus and *Bancroftian filariasis* (Meegan *et al*., 1980; Thomas & Callaghan, 1999). *Culex pipiens* has a wide geographical distribution, although it is most common in temperate and tropical regions, where eggs are laid in rafts of 150–350 eggs in stagnant or foul water in a variety of water-filled containers or areas. Consequently, developing and improving strategies and tactics for the control of mosquitoes is essential.

The concept of integrated mosquito management is central to the goal of their control and the prevention of disease. Integrated pest management is defined as a synergistic, ecosystem-based strategy that focuses on the long-term suppression of pests or their damage via a combination of techniques, including biological control, trapping, habitat manipulation and chemical control (American Mosquito Control Association, 2017). However, when the control of the source of mosquitoes is not feasible, it

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might be necessary to use larvicides to prevent larval mosquitoes from hatching and becoming adults (Yuan *et al*., 2015; Campolo *et al*., 2015; Centers for Disease Control). The efficacy of insecticides, however, is threatened by the development and spread of resistance of insects to insecticides. Therefore, the use of combinations of sublethal concentrations of insecticides to control mosquitoes could be a promising strategy for avoiding the development of resistance and minimizing any effects on human health and nontarget animals.

Neurotoxic insecticides, particularly organophosphates (OPs) and pyrethroids are most frequently used against adult mosquitoes (Gamal, 2014; Sarwar, 2016). Neonicotinoids (NIs) are a class of neurotoxic insecticides developed for use against agricultural pests and mosquitoes (Paul *et al*., 2006). The development of resistance of mosquitoes to insecticides threatens the effectiveness and sustainability of the control of mosquitoes in various parts of the world. For example, in 1955, the World Health Organization began a global campaign aiming to kill mosquitoes using DDT [1,1′-(2,2,2-trichloroethane-1,1-diyl)bis(4-chlorobenzene)] but, within a few years, the treatment became less effective as resistance developed (Ranson *et al*., 2000). There are also concerns regarding adverse effects to nontarget species, including humans (Ben Cheikh *et al*., 2008; Fouad *et al*., 2016). Consequently, to avoid and minimize the development of resistance, the application of insecticides to control juvenile mosquitos needs to be as efficient as possible, with the use of minimal amounts to achieve effective control (Insecticide Resistance Action Committee, 2011).

Exposure of larval and developing mosquitoes to insecticides in natural environments is dependent upon many factors, including rates of dissipation and degradation of the applied compound, the frequency of application, the concentration applied, the properties of the compound such as its solubility, and the dilution factor within media such as surface waters (Antonio *et al*., 2009). Therefore, in the environment, exposure to insecticides can be at both lethal and sublethal concentrations. Despite the potential of sublethal, chronic exposure to insecticides with respect to altering populations of mosquitoes (Antonio *et al*., 2009), most studies focus on the lethal effects of pesticides, primarily on adults (Paul *et al*., 2006; Pridgeon *et al*., 2008). Conversely, insights from studies of other aquatic insects suggest that sublethal concentrations of insecticides can affect the morphology, behaviour and physiology of insects (Desneux *et al*., 2007; Pestana *et al*., 2009a, b; Relyea, 2012; Woodley *et al*., 2015). These effects can potentially become magnified in the presence of other environmental stressors (Woodley *et al*., 2015). Similar effects are reported in mosquitoes of the genera *Culex* and *Aedes* for which larval exposure to pesticides such as malathion, atrazine and glyphosate alters rates of emergence, development time, longevity, body size, sex ratio and vector competence (Muturi & Alto, 2011; Muturi *et al*., 2011a, b; Bara *et al*., 2014).

Because mosquitoes do not provide parental care to offspring, natural selection should favour the ability of gravid females to select ideal aquatic habitats that increase the hatching of eggs and the survival of juveniles (Reiskind & Wilson, 2004). Oviposition by gravid females requires integration of complex biological, chemical and physical cues (Bentley & Day, 1989). Chemical contaminants can potentially disrupt this process by modifying the attractiveness of aquatic habitats (Kibuthu *et al*., 2016). Biologists who investigate ways of controlling vectors of insect-borne diseases are faced with the challenge of determining the effects of chemicals on the behaviour of mosquitoes as it relates to their ability to transmit pathogens (Ramirez *et al*., 2009).

Formulated pesticides are often sold as a mixture of active ingredients or may be tank-mixed by applicators. Ideally, insecticides with various modes of action are mixed on the assumption that they have complementary actions for killing target pests, thus making it more difficult for the target insects to develop resistance. When two insecticides are mixed, their effects can either be additive or they can interact to produce supra- or infra-additive effects on insects. Depending upon their physiologies and mechanism(s) of resistance, these effects can vary among species or strains of insects. Supra-additive effects are reported when combinations of plant extracts or synthetic insecticides are used to control populations of mosquitoes (Su, 1991; Thangam & Kathiresan, 1991). The use of combinations of insecticides to kill mosquitoes could permit the continued use of chemicals for which insects have already developed resistance. However, to the best of our knowledge, studies have not yet been conducted that aim to evaluate the joint action of sublethal concentrations of two commonly used synthetic, neurotoxic insecticides, comprising the OP chlorpyrifos and the NI imidacloprid, on the choice of oviposition site, larval development and emergence of adult mosquitoes.

The present study aims to determine whether chronic exposure of *C. pipiens* to sublethal concentrations of chlorpyrifos or imidacloprid individually or in combination can cause adverse effects on the duration of development, emergence of adults or the expression of five selected genes involved in the detoxification and resistance of insecticides. In addition, the present study investigates whether gravid females can sense either compound in solution and if this would affect oviposition. This is key with respect to determining whether proactive dosing of water bodies, particularly swamps, ponds and marshes, is a valid action for rendering the sites unsuitable to mosquitoes in rural communities, particularly in developing countries.

Materials and methods

Collection and rearing of C. pipiens

Rafts of eggs, larvae and pupae of *C. pipiens* were collected from a small water pool in the Quhafa region, Tanta City, Egypt. Methods for rearing have been described previously (El-Husseiny *et al*., 2014). Briefly, rafts of eggs were placed separately into white enamelled pans containing dechlorinated (DC) tap water until hatching. Larvae were reared at insectary conditions under an LD 16 : 8 h photocycle at $27 \pm 2\,^{\circ}C$ and 70–80% relative humidity. Larvae were fed tropical fish food. Pupae were placed into mosquito cages ($30 \times 30 \times 30$ cm) until eclosion into adults. Males were fed 10% sucrose solution. The colony was maintained using pigeon blood

for at least two generations before *C. pipiens* were used in experiments.

Insecticides

The OP insecticide chlorpyrifos and the NI insecticide imidacloprid were investigated. The two pesticides were chosen because they are the insecticides that are most widely used and most frequently detected globally and in Egypt (Al Naggar *et al*., 2015; Morrissey *et al*., 2015; Codling *et al*., 2016, 2018; El-Sofany *et al*., 2018). They were also selected to represent classes of neurotoxic insecticides (OPs and NIs) with different modes of action and different toxic potencies (Barron & Woodburn, 1995; Stoughton *et al*., 2008). The insecticides used in experiments were purchased from Sigma-Aldrich (Ontario, California) and were of technical grade (> 98% purity). Stock solutions (1000 mg mL^{-1}) of each insecticide were prepared in methanol (> 98% purity) and stored at −20 °C and then subsequent dilutions in water were made for each pesticide when required.

Determination of LC_{50}

The LC_{50} is the lethal concentration that results in 50% of a group of test organisms being killed within a specified period. It is a common assessment endpoint in bioassays used to measure acute toxicity of a compound. For *C. pipiens*, median lethal concentrations (LC_{50}) were calculated from five serially diluted concentrations of each insecticide that were tested against third-larval instar. Larvae ($n = 20$ per test) were placed into polyethylene terephthalate (PET) plastic disposable cups containing 150 mL of DC tap water fortified with either pesticide at five concentrations. The experiment was performed in triplicate for each concentration and repeated on three separate occasions (i.e. blocks in time). Mortalities of larvae were recorded after 24 h of exposure. Cups containing 150 mL of DC tap water spiked with 1 mL of methanol were used as a negative control. LC_{50} for each pesticide was estimated by use of the log-probit model using the LDP LINE software (http://www.ehabsoft.com/ldpline).

Exposure of C. pipiens to sublethal concentrations of chlorpyrifos and/or imidacloprid

Juvenile of *C. pipiens* were exposed to sublethal concentrations equivalent to 20% of LC_{50}, 10% of LC_{50} and 5% of LC_{50} of chlorpyrifos and imidacloprid, separately or combined. However, after chronic exposure to these sublethal concentrations and based on successful development and emergence of adult *C. pipiens*, sublethal concentrations of chlorpyrifos (20% of LC_{50}; 0.3 µg L^{-1}) and imidacloprid (5% of $LC_{50;}$ 6 µg L^{-1}) were chosen for testing.

Life table

Eggs laid by females in the laboratory colony were individually transferred to PET plastic disposable cups containing 200 mL of DC tap water spiked with a sublethal concentration of chlorpyrifos (0.3 µg L^{-1}) and imidacloprid (6 µg L^{-1}) and mixtures containing both sublethal concentrations. A cup containing 200 mL of tap water spiked with 1 mL of methanol was used as the solvent control. After hatching, cups were checked daily until pupation. Pupae were transferred using plastic droppers to small plastic cups filled with DC tap water and these cups were then transferred to wooden framed cages (30 × 30 × 30 cm) screened with a fine narrow mesh until the adults emerged. The onset of each life stage in each cup was recorded: hatching, second-instar larvae, third-instar larvae, fourth-instar larvae, pupae and adults. Each bioassay consisted of three replicate cages per treatment and was repeated on three separate occasions (i.e. blocks in time).

Gene expression

A quantitative real-time polymerase chain reaction (qRT-PCR) was performed to determine the effects of chronic exposure of *C. pipiens* to sublethal concentrations of each pesticide separately or combined on the expression level of five selected genes that are important for detoxification and resistance of insecticides (Gong *et al*., 2005; Wang *et al*., 2015). Samples of five to 10 third-instar larvae were collected during life table experiments and used for qRT-PCR. Total RNA was isolated from treated or control samples (0.03 g) by use of a RNeasy Lipid Tissue Mini Kit (Qiagen, Canada) in accordance with manufacturer's instructions. First-strand cDNA was synthesized using SensiFAST cDNA synthesis kit in accordance with manufacturer's instructions using a blend of random hexamer and anchored oligo (dT) 18 primers (Bioline, Singapore). qRT-PCR reactions were performed in a 25-µL reaction volume that contained 12.5 µL of SYBER Green PCR master mix, 2 µL of primer (50 nmol), 2 µL of template cDNA and 8.5 µL of deionized water, using a two-step cycling SensiFAST SYBR Lo-ROX kit in accordance with manufacturer's instructions (Bioline). The qRT-PCR primer sequences used in the present study are described in to Gong *et al.* (2005) and Wang *et al.* (2015), as shown in the Supporting information (Table S1). Beta actin was used as a housekeeping gene (Wang *et al*., 2015). The thermal cycling conditions were: 95 °C for 2 min, followed by 40 cycles 95 °C for 15 s, 52 °C for 30 s and 72 °C 30 s. In total, qRT-PCR was performed on RNA isolated from a total of three composite samples ($n = 30$) per treatment; one randomly selected from each set of treatments from each of the three bioassays (blocks). Fold changes in abundances of transcripts of detoxification genes of interest were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Oviposition

Choices for oviposition by gravid females of *C. pipiens* in the presence of cups of water and/or water containing sublethal concentrations of chlorpyrifos and imidacloprid, or a combination of both pesticides, were determined under laboratory conditions. Trials were initiated with 10 engorged females that were

Table 1. Susceptibility of third-instar larvae of *Culex pipiens* to chlorpyrifos and imidacloprid pesticides after 24 h of exposure.

Pesticide	*n*	LC_{20} (95% CI)(μg L^{-1})	LC_{50} (95% CI)(μg L^{-1})	LC_{90} (95% CI)(μg L^{-1})	Slope (mean ± SE)
Chlorpyrifos	360	0.11 (0.02–0.36)	1.5 (0.5–3.3)	158.9 (56.5–766.6)	0.6 ± 0.08
Imidacloprid	360	40.89 (6.5–65.4)	117.2 (32.7–214.6)	581.8 (354.2–2589.5)	1.8 ± 0.17

[a]*n*, number of larvae tested; CI, confidence interval.

Table 2. Developmental time and adult emergence in days (mean ± SD) of *Culex pipiens* exposed to sublethal concentrations of chlorpyrifos and imidacloprid, as well as a mixture of both separately.

		Larval development (d)				
Treatment	Hatching (days)[a]	Second-instar larvae	Third-instar larvae	Fouth-instar larvae	Pupation (days)	Adult emergence (days)[b]
Control	2.0 ± 0.0	7.25 ± 0.75^{A}	9.25 ± 0.75^{AB}	13.25 ± 0.75^{A}	21.5 ± 0.5^{A}	25.5 ± 0.5^{A}
Chlorpyrifos (20% of LC_{50})	2.0 ± 0.0	4 ± 0.0^{B}	7.25 ± 0.48^{A}	9.75 ± 0.85^{A}	22.25 ± 0.48^{A}	26.5 ± 2.47^{A}
Imidacloprid (5% of LC_{50})	2.0 ± 0.0	5.75 ± 0.75^{AB}	12.25 ± 1.25^{B}	21 ± 0.0^{B}	Failed	Failed[d]
Binary mixture[c]	2.0 ± 0.0	5 ± 0.0^{B}	11.5 ± 0.5^{B}	19.25 ± 1.75^{B}	29 ± 1.35^{B}	Failed

[a]Exposure to different treatment started at zero days.

[b]The time of adult emergence was compared using Student's *t*-test; similar letters denote no significant difference, $P < 0.05$).

[c]Mixture of both chlorpyrifos (20% of LC_{50}) and imidacloprid (5% of LC_{50}).

[d]Failed in eclosion.

Different uppercase letters denote significant differences in development time (days) for each stage compared with the control (one-way analysis of variance with Tukey's post-hoc test, $P < 0.05$).

placed into wooden-framed cage (30 × 30 × 30 cm) containing three plastic cups (4 × 4 × 4 cm). Of the three PET plastic cups, all contained 30 mL of DC water, although two cups were fortified with either (20% LC_{50}; 0.3 μg L^{-1}) or (5% LC_{50}; 6 μg L^{-1}) of either chlorpyrifos or imidacloprid. An additional experiment was conducted in which the control cup was replaced with water fortified with a mixture of 20% of LC_{50} or 5% of LC_{50} for chlorpyrifos and imidacloprid, respectively. Both experiments were repeated in triplicate. Mortality (%) of gravid females either on the surface of water in cups or on the bottom of the cage was observed daily. Numbers of egg rafts deposited relative to surviving females in each cup were recorded. The study was carried out in strict accordance with the recommendations of the Guide for the Care of Use of Laboratory Animals of the National Institute of Health (National Research Council, 2010), with approval from the Committee of the Faculty of Science (permit no. IACUC-SCI-TU-0068).

Statistical analysis

The results were analyzed using PRISM, version 5.00 (GraphPad Software, La Jolla, California). Normality of results was assessed by use of the Kolomogrov– Smirnov test and homogeneity of variance was determined with Levene's test. If necessary, data were $\log_{10}$-transformed to better approximate normality and homogeneity of variance. The developmental time of each stage in the life table, the abundance of transcripts of genes involved in detoxification and resistance, and the time required for adult emergence were assessed by one-way analysis of variance followed by Tukey's post-hoc test and Student's *t*-test, respectively. An alpha level of 0.05 was used for all tests.

Results

Determination of LC_{50}

The potency of a pesticide is an expression of how toxic it is. Based on the survival of third-instar larvae of *C. pipiens* 24 h post-treatment, chlorpyrifos ($LC_{50} = 1.5$ μg L^{-1}) was more potent (killed more mosquitoes) than imidacloprid ($LC_{50} = 117.2$ μg L^{-1}) (Table 1). Based on a comparison of LC_{20s} (sublethal concentrations) values of chlorpyrifos ($LC_{20} = 0.19$ μg L^{-1}) and imidacloprid ($LC_{20} = 40.89$ μg L^{-1}) against *C. pipiens* larvae, chlorpyrifos was 372-fold more potent than imidacloprid (Table 2).

Life table

Developmental time and emergence rate of adult *C. pipiens* exposed to sublethal concentrations of chlorpyrifos (20% of LC_{50}) and imidacloprid (5% of LC_{50}) or a binary mixture of both pesticides resulted in adverse effects (Table 2). Eggs hatched after 2 days exposure in all treatments and in controls. Duration until first ecdysis was significantly less in individuals exposed to only chlorpyrifos (4 days) and the binary mixture (5 days) compared with the control (7.25 ± 0.75 days) ($F = 6.66$; d.f. = 3, $P = 0.006$). There were variations in time required for second ecdysis between treatments, although this was not significantly different compared with the control (Table 2).

Fourth-instar larvae appeared significantly later in cups containing imidacloprid (21 ± 0.0 days) and the binary mixture (19.25 ± 3.5 days) compared with that in the control (13.25 days) or chlorpyrifos (9.75 days) ($F = 25.12$; d.f. = 3, $P = 0.0001$). The time required for pupation was significantly longer in the

binary mixture (29 ± 2.7 days) compared with that in the control (21.5 ± 1.0 days) or chlorpyrifos alone (22.25 ± 1.0 days) ($F = 22.14$; d.f. $= 2$, $P = 0.0003$). Third-instar larvae in imidacloprid failed to successfully pass the third ecdysis. Adults of *C. pipiens* in both imidacloprid and the binary mixture remained attached to their puparium by their appendages and failed to completely emerge and thus died. No significant difference in time required for adult emergence was observed when exposed to chlorpyrifos (26.5 ± 4.9 days) compared with unexposed (control) individuals (25.5 ± 1.0 days) ($P = 0.82$) (Table 2).

Gene expression

Expression of genes important in detoxification of insecticides were significantly different in third-instar larvae instar of *C. pipiens* chronically exposed to sublethal concentrations of chlorpyrifos and/or imidacloprid, separately or combined, compared with unexposed larvae (control) [chemotrypsin-1: $F = 17.46$; d.f. $= 3$, $P = 0.0007$; theta glutathione *S*-transferase (GST): $F = 4.82$; d.f. $= 3$, $P = 0.033$; cytochrome P450 (CYP6F1): $F = 5.77$; d.f. $= 3$, $P = 0.021$; CYP *b*: 22.16; d.f. $= 3$, $P = 0.0006$] (Figs 1 and 2). By contrast, relative to the control, the expression of cytochrome p450c was not significantly affected in third-instar larvae exposed to either insecticide, individually or in combination ($F = 2.73$; d.f. $= 3$, $P = 0.11$). Expression of chymotrypsin-1 in larvae of *C. pipiens* exposed to chlorpyrifos, imidacloprid or their binary mixture was down-regulated by 296-, 71- and 35-fold relative to control larvae. However, expression of GST RNA was 66-fold greater in *C. pipiens* larvae exposed to the binary mixture compared with the control (Fig. 1).

Chronic exposure of *C. pipiens* larvae to sublethal concentrations of imidacloprid resulted in down-regulated expression of CYP6F1 by 13.5-fold relative to that of the control. Expression of detoxifying encoding gene CYP 450 B in larvae of *C. pipiens* was 3.5- and 6.4-fold greater than the control in larvae exposed to sublethal concentrations of imidacloprid, alone or in combination with chlorpyrifos, respectively (Fig. 2).

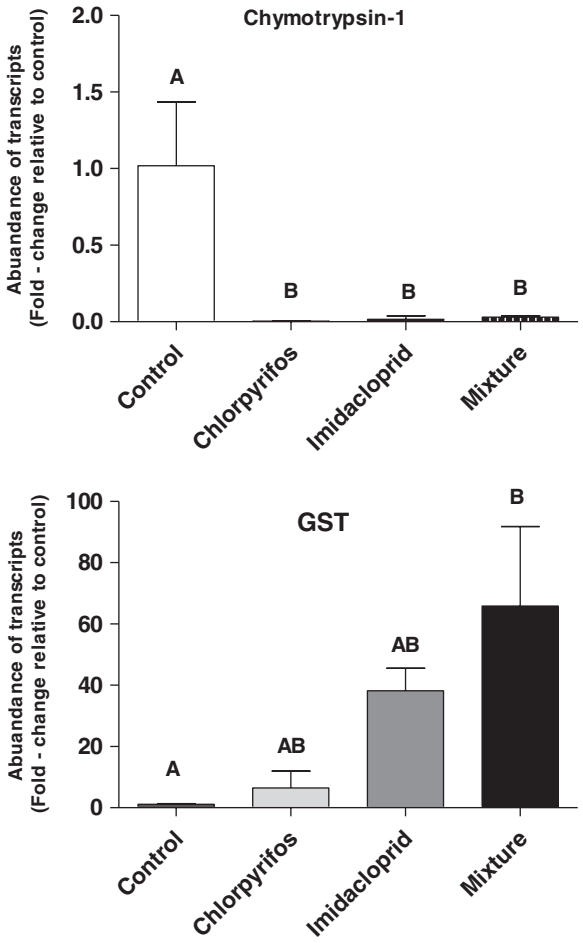

Fig. 1. Fold-change in abundances of transcripts of genes involved in pesticide detoxification in third-larval instar of *Culex pipiens* chronically exposed to sublethal concentrations of chlorpyrifos (20% of LC_{50}), imidacloprid (5% of LC_{50}) individually or in combination. Bars represent the mean ± SEM concentration of three samples. Different uppercase letters denote significant differences among treatments (one-way analysis of variance with Tukey's post-hoc test, $P < 0.05$). GST, glutathione *S*-transferase.

Choice of place of oviposition

No eggs were observed in water containing either of the insecticides, either individually or in combination (Table 3). Oviposition occurred only in the first experiment where gravid females laid their eggs in the control water cups. Of the surviving females (53.33%) in the first experiment, egg rafts observed in the control matched the number of survivors, indicating 100% laying in the control. No eggs were laid during the second experiment, during which there was no water that did not contain one or both of the insecticides (Table 4). The mean mortality of gravid *C. pipiens* that tried to oviposit in imidacloprid but failed was 20.00% in the first experiment and 26.67% in the second experiment. No mortality of *C. pipiens* exposed to chlorpyrifos occurred in either experiment. However, in the second experiment where cups of water containing a binary mixture of both insecticides were provided, the mean mortality in these cups was 20.00% (Table 4). The mean percentage of gravid females that failed to lay eggs and died at the bottom of the experimental cage was 26.67% in the first experiment, although it was 53.33% in the second experiment, where no clean water was provided.

Discussion

The extensive use of vector control insecticides raises concern over the development of resistance of targeted insects to insecticides and any adverse effects on nontarget wildlife and humans. Therefore, there is an urgent need for new tactics and approaches regarding the use of pesticides for the control of mosquitoes.

In the present study, chlorpyrifos is observed to be more potent than imidacloprid against larvae of *C. pipiens*, which is consistent with the results for several insecticides tested

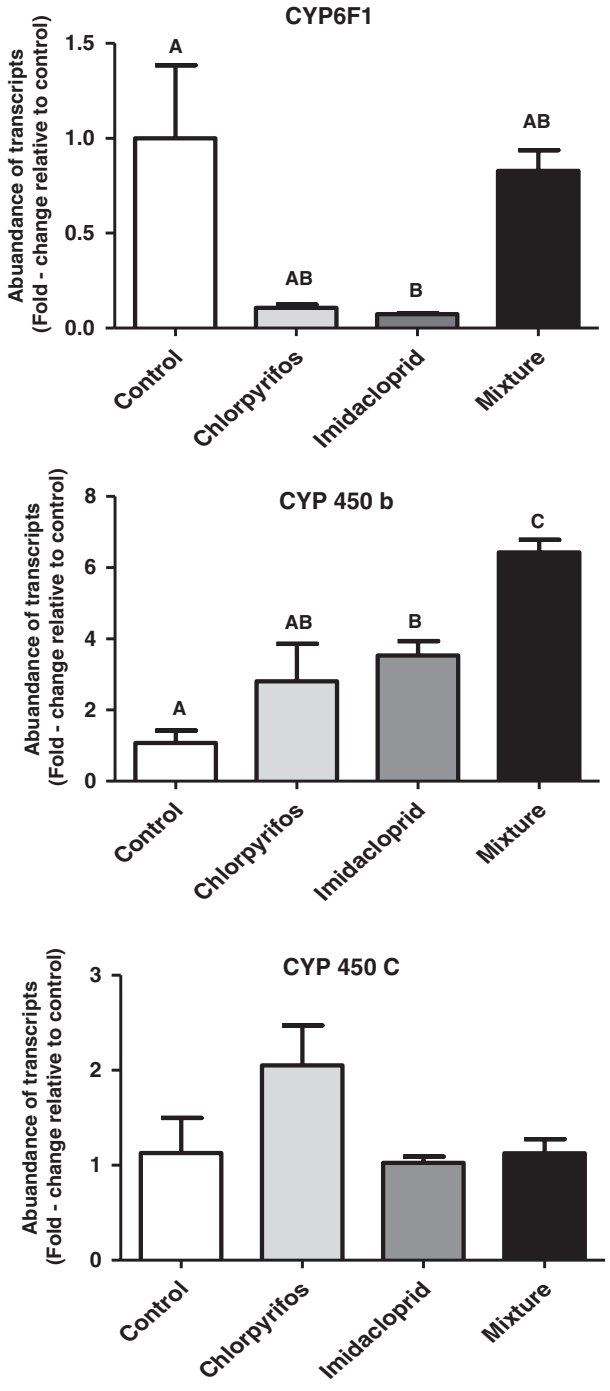

Fig. 2. Fold-change in abundances of transcripts of cytochrome P450 genes involved in pesticide detoxification in third-larval instar of *Culex pipiens* chronically exposed to sublethal concentrations of chlorpyrifos (20% of LC_{50}) and imidacloprid (5% of LC_{50}), individually or combined. Bars represent the mean ± SEM concentration of three samples. Different uppercase letters denote significant differences among treatments (one-way analysis of variance with Tukey's post-hoc test, $P < 0.05$).

previously, including the carbamate propoxur; the OPs fenitrothion, diazinon and chlorpyrifos; the synthetic pyrethroids permethrin and sumicidin; and the insect growth regulators 1-4-trifluoromethoxyphenyl and chlorfluazuron, for which chlorpyrifos is more potent to a susceptible strain of *C. pipiens* (Salama *et al*., 2002).

The observation that both development time and time to emergence of adult *C. pipiens* are affected by exposure to sublethal concentrations of chlorpyrifos or imidacloprid, or a binary mixture of both insecticides, is consistent with the results of other studies. For example, the time required for pupation and emergence of adult *C. quinquefasciatus* is significantly longer after exposure to malathion, methoprene, propoxur or resmethrin (Robert & Olson, 1989). Similarly, exposure of *Anopheles arabiensis* and *C. quinquefasciatus* to ammonium sulphate and diammonium phosphate increases the duration of the developmental time, whereas rates of emergence of *An. arabiensis* are significantly decreased after exposure to cypermethrin (Kibuthu *et al*., 2016).

In the present study, the gene chymotrypsin-1 is the most significantly down-regulated gene in all susceptible larvae exposed to sublethal concentrations of chlorpyrifos and/or imidacloprid. Similar findings are reported by Vellichirammal *et al.* (2015) who identify three transcripts coding for chymotrypsin-like serine proteases that are down-regulated after exposure to sublethal concentrations of Cry1F protoxin in the susceptible strain of corn borer (*Ostrinia nubilalis*). By contrast, overexpression of chymotrypsin is detected in strains of *C. pipiens pallens* mosquitoes resistant to the pyrethroid deltamethrin (Wu *et al*., 2004) or the OP parathion (Wang *et al*., 2015) insecticides. Chymotrypsin functions in the digestive systems of many organisms, including insects, and aids the metabolism of endogenous and xenobiotic peptide compounds. It is hypothesized that chymotrypsin can sequester or degrade pesticides (Wang *et al*., 2015); however, its down-regulation in response to sublethal concentrations of different pesticides in the present study and protoxins in the previous study of Vellichirammal *et al.* (2015) might indicate its lesser role in xenobiotic detoxification. However, the responses are mainly dependent on the life stage exposed, the duration of exposure and the concentrations of pesticides used, and therefore further studies are required to adequately clarify its role in detoxification.

The major superfamilies of genes implicated in the metabolism of insecticides by insects are the GSTs, cytochrome P450s and carboxyl/cholinesterases (Shen *et al*., 2003; Enayati *et al*., 2005; Hardstone, 2009; Wang *et al*., 2015). In the present study, theta GST is overexpressed by 66-fold only in *C. pipiens* larvae exposed to the binary mixture of insecticides compared with the control. Similarly, in a comparison of a parathion-resistant strain and a susceptible strain of *Culex* mosquitoes, GST is up-regulated by 19-fold (Wang *et al*., 2015). Up-regulation of the expression of GST is associated with resistance to all major classes of insecticides (Enayati *et al*., 2005), although it is not clear why theta GST is overexpressed in *C. pipiens* during the present study and not overexpressed in a parathion-susceptible strain (Wang *et al*., 2015). This might be because of the effect of sublethal concentrations and/or interaction of chlorpyrifos and imidacloprid. Several studies

Table 3. Mortality (%) and oviposition choice of gravid females of mosquitoes (*Culex pipiens*) exposed to sublethal concentrations of chlorpyrifos or imidacloprid pesticides under laboratory conditions.

Mortality rate (%)					
Chlorpyrifos(20% of LC_{50})	Imidacloprid (5% of LC_{50})	Control(Water)	On bottom of the cage	Survival rate (%)	Percentage of egg rafts (only on water)
0.00 ± 0.00	20.00 ± 8.16	0.00 ± 0.00	26.67 ± 7.20	53.33 ± 2.72	100[a] ± 0.00

[a]Number of egg rafts deposited relative to survival rate percentage (i.e. all survived and oviposit their egg rafts on water surface).

Table 4. Mortality (%) and oviposition choice of gravid females of mosquitoes (*Culex pipiens*) exposed to sublethal concentrations of either chlorpyrifos or/and imidacloprid or combined under laboratory conditions.

Mortality rate (%)					
Chlorpyrifos (20% of LC_{50})	Imidacloprid (5% of LC_{50})	Binary mixture[a]	On bottom of the cage	Survival rate (%)	Percentage of egg rafts
0.00 ± 0.00	26.67 ± 7.20	20.00 ± 9.43	53.33 ± 2.72	0.00 ± 0.00	0.00 ± 0.00

[a]Binary mixture of (20% of LC_{50} + 5% of LC_{50}) of chlorpyrifos and imidacloprid, respectively.

report adaptive responses subsequent to exposure to sublethal concentrations of chemical pesticides (Zanuncio *et al*., 2005; Deng *et al*., 2016).

Mosquitoes have a large repertoire of cytochrome P450s (> 100 genes) (David *et al*., 2013). By pinpointing the key enzymes associated with insecticide resistance, we can begin to develop new tools for implementing control interventions and perhaps design more targeted approaches with less unintentional environmental damage (David *et al*., 2013). In the present study, cytochrome P450s genes quantified in the third-instar larvae of *C. pipiens* exhibit differential expression in responses to pesticides. CYP6F1 is significantly down-regulated by 13.5-fold in *C. pipiens* larvae exposed to imidacloprid compared with the control, and CYP *b* is significantly overexpressed by 3.5- and 6.4-fold in larvae exposed to imidacloprid and the binary mixture treated larvae, respectively. In fourth-instar larvae of the deltamethrin-resistant strain, CYP6F1 gene is expressed to a greater extent than in the susceptible strain of *C. pipiens pallens* (Gong *et al*., 2005). Thus, if the function of CYP6F1 is involved in xenobiotic metabolism, overexpression of the gene in fourth-instar larvae might be indicative of the adaptation of larvae to metabolize xenobiotics. However, its down-regulation in the present study in response to lower concentrations of imidacloprid might indicate a lower capability of the larvae with respect to metabolizing the neonicotinoid pesticide.

Cytochrome *b* is a component of respiratory chain complex III, also known as bc1 complex in the mitochondria of eukaryotes, and it participates in electron transport and the generation of ATP (Esposti *et al*., 1993). Although, during the present study, cytochrome *b* is up-regulated in larvae exposed to imidacloprid and the binary mixture, it is also over-expressed by 2.1-fold in parathion-resistant mosquitoes (Wang *et al*., 2015). Overexpression of this gene in third-instar larvae in the present study might be a result of the effect of sublethal concentrations of imidacloprid alone or in combination with chlorpyrifos that induce an adaptive response in which mitochondrial respiration increases, thus consuming more energy to metabolize more insecticide. Moreover, the family of cytochrome P450 genes is large and is involved in a wide variety of metabolic functions and so its expression might vary among insecticides and be dose-dependent, with similar levels of up-regulation of CYP genes being reported in pesticide-susceptible strains of moths and beetles (Bautista *et al*., 2007; Liang *et al*., 2015).

Gravid mosquitoes lay eggs on the surface of water selected for conditions that are suitable for the development of larvae (Mayhew, 2001; Scheirs & De Bruyn, 2002). To make such selections, they use physical and chemical cues to search for locations that are suitable for oviposition (Benzon & Apperson, 1988; Bentley & Day, 1989). In the present study, female *Culex* lay eggs only in cups filled with clean water. No mortality occurs in chlorpyrifos-treated cups, which clearly indicates that sublethal concentrations of chlorpyrifos have a repellent effect on gravid females of *C. pipiens*, preventing them from attempting to lay eggs. Similar results are observed for emulsifiable concentrate formulations of chlorpyrifos against *Aedes triseriatus* (Say) ovipositing females (Mather & DeFoliart, 1983). Consequently, the results of the present study suggest that chlorpyrifos could be used as a repellent against *C. pipiens* even at lower concentrations (20% of LC_{50}).

Imidacloprid does not deter oviposition, although it is toxic to gravid female mosquitoes and, consequently, female mosquitoes attempt to oviposit but die in cups containing imidacloprid before laying eggs. The same responsiveness of unsuccessful oviposition of gravid female mosquitoes to the binary mixture is likely a result of the imidacloprid alone. Deterrence of oviposition of female mosquitoes by insecticides is reported previously for *Anopheles stephensi* Liston, *Aedes aegypti* and *C. quinquefasciatus* (Say) after exposure to the pyrethroids cypermethrin, fenvalarate, decamethrin and permethrin, although at a concentration of LC_{99} of the first-larval instar of target mosquito species (Verma, 1986). This is similar to the results obtained from a study in *C. pipiens* after exposure to diflubenzuron and temephos at a concentration equivalent to half of the standard recommended rate of application (Akiner & Eksi, 2015). Consequently, it is suggested that the binary mixture of chlorpyrifos and imidacloprid at sublethal concentrations (20% of LC_{50} and 5% of LC_{50}, respectively), as employed during the present study, could be used as a repellent of oviposition by *C. pipiens*.

Pesticides are currently regulated individually but are often present in aquatic systems as mixtures of toxicants and little information exists regarding the toxicological interactions involving insecticide mixtures that are routinely detected at low concentrations in biomonitoring (LeBlanc *et al*., 2012). Therefore, it is hypothesized that combined effects of chlorpyrifos and imidacloprid at sublethal concentrations might exhibit greater toxicity against *C. pipiens* mosquitoes via synergistic or additive actions compared with each pesticide alone, particularly both insecticides that are neurotoxins. However, the genes quantified in third-instar larvae of *C. pipiens* in the present study exhibit differential expression in their responses to individual pesticides or to the binary mixture of both pesticides. Based on life table and oviposition choice data, it is difficult to anticipate or make conclusions regarding the type of interaction in the binary mixture. However, it appears that the main adverse effects observed after exposure to the binary mixture are likely caused by imidacloprid. In this context, the OP–imidacloprid mixtures (chlorpyrifos–imidacloprid and imidacloprid–dimethoate) exhibit opposing effects (synergism and antagonism, respectively) against *Chironomus dilutus* larvae. These results could be related to a combination of OP water solubility and cytochrome P450 enzymes (CYP), which are active in metabolizing toxicants in many animals, including insects (LeBlanc *et al*., 2012).

In conclusion, exposure to either insecticide individually or in combination leads to complete inhibition of oviposition by gravid *C. pipiens* females. Additionally, chronic exposures of juvenile *C. pipiens* to sublethal concentrations of chlorpyrifos or imidacloprid has significant adverse effects on development, pupation, adult emergence. Although some studies show that exposure of mosquitoes to sublethal concentrations of insecticides may magnify their response to other chemical insecticides/cross-resistance, the use of combinations of sublethal concentrations of insecticides to kill mosquitoes could permit the continued use of chemicals for which insects have already developed resistance. Finally, the findings of the present study clearly support the action of a proactive dosing of water bodies (ponds, marshes and swamps), particularly those formed as a result of heavy rains or flooding, with sublethal concentrations of chlorpyrifos and/or imidacloprid insecticides to control mosquitoes in rural communities, especially in developing countries and in wetlands in general. More studies are required to confirm the potential methodological application of these insecticides and the use of combinations of sublethal concentrations of other pesticides to avoid the development of resistance.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Sequences of primers used for quantification of abundances of transcripts of genes involved in detoxification of pesticides in mosquitoes (*Culex pipiens*).

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