Permethrin and malathion LD\textsubscript{90} values for Culex quinquefasciatus vary with topical application site

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Abstract. Prior research in multiple insect species has demonstrated that insecticide-induced mortality varies according to the body region exposed on the insect. This variation has been demonstrated in Culex quinquefasciatus Say (Diptera: Culicidae), but has not been quantified using dose–response curves. Applications of technical permethrin or malathion to one of three body regions on Cx. quinquefasciatus resulted in dose–response curves that were not equivalent to one another. The generated LD\textsubscript{90} values and curves for each body region were compared with previously reported LD values for analogous sites in several mosquito species, specifically the mesothorax. Based on the present results, the permethrin and malathion LD\textsubscript{50} and LD\textsubscript{90} concentrations required for droplets impinging on the abdomen and mesothorax of Cx. quinquefasciatus when applied through ground-based spray systems utilized by mosquito control programmes were calculated.

Key words. autotomization, dose–response curve, droplet, sub-lethal effect.

Introduction

Insecticides are utilized by mosquito control and abatement programmes to control mosquito populations. Prior to commercial use, these insecticides must be evaluated on target organisms and non-target organisms in order for their effectiveness and their impacts on the surrounding environment to be properly evaluated. One method commonly used to evaluate the effects of an insecticide on an organism is a dose–response bioassay.

Bioassays are divided into two major categories: qualitative and quantitative (Busvine, 1971). Qualitative bioassays record the physical effects associated with exposure to a compound, such as mortality, without consideration of concentration, as epitomized by use of a discriminating dose. Quantitative bioassays record the physical effects associated with exposure to a compound at known compound concentrations.

Dose–response bioassays are quantitative bioassays and are performed for a number of reasons, including to assess: (a) the sub-lethal and lethal effects of an insecticide; (b) its potency and efficacy, and (c) resistance and cross-resistance to common insecticides [World Health Organization (WHO), 2009]. Dose–response bioassays assess known doses of insecticide that will result in mortality and should evaluate several doses that elicit mortality ranging between 10 and 99% (Robertson \textit{et al.}, 1984). In order to evaluate the toxicity of a potential insecticide, a dose–response curve was designed to determine the lethal dose (LD) at which a proportion of the targeted organisms are killed. The importance of these dose–response bioassays is that their use forwards understanding of the effects associated with specific amounts of a compound on an organism, such as toxicity effects.

Several bioassay techniques have been utilized to evaluate insecticide toxicity; often a technique is chosen based on the organism of interest and method of delivery or application of the insecticide. These include direct application to the insect, such as is used in a topical bioassay, exposure to surfaces...
treated with a residual insecticide, as used in bottle, cone and insecticide-impregnated paper bioassays, and exposure to the vapour phase of insecticides for fumigant bioassay. Of these bioassay techniques, topical application bioassays are distinct in that they deliver a known insecticide dose directly to the exoskeleton of the organism, thereby ensuring the exposure of the organism to a specific amount of insecticide (Busvine, 1971).

The insecticide LD₉₀ value for malathion was reported to be 10 ng per mosquito in Ochlerotatus taeniorhynchus (= Aedes taeniorhynchus) (Wiedemann) (Diptera: Culicidae), but in Stomoxys calcitrans (= Aedes sollicitans) (Walker) (Diptera: Culicidae) LD₉₀ values ranged between 5.1 ng and 60 ng per mosquito (Weidhaas et al., 1970; Khoo & Sutherland, 1983).

Several other mosquito species have been evaluated for permethrin toxicity, with findings showing LD₉₀ values in the range of 0.12–6.90 ng/mg of mosquito (Pridgeon et al., 2008). However, these lethal dose values were generated by applying insecticide diluted in acetone to the scutum of the mosquito thorax as defined by Harbach & Knight (1980). Numerous studies have been conducted that compare insecticide applications on one body region with those on another (Fisher, 1952; Rai & Roan, 1959; Ahmed & Gardiner, 1967; Keiser et al., 1971; Scott et al., 1986); however, only in the study by Aldridge et al. (2016) has the difference in mortality between topical insecticide applications to different body regions in a mosquito been assessed using a topical bioassay.

In the present study, LD₉₀ values for permethrin and malathion were determined by bioassay using topical application at three body regions in Culex quinquefasciatus: (a) the mesothoracic spiracle; (b) the metathoracic leg tarsi, and (c) the sternites of apical segments of the abdomen. These three body regions were selected for evaluation because they have, respectively: (a) the greatest sensitivity; (b) the least sensitivity, and (c) a historical precedent to insecticide exposure in Cx. quinquefasciatus as identified in Aldridge et al. (2016). The LD₉₀ values were compared with previously reported dose responses using the mesothoracic spiracular area. Optimal droplet diameters were calculated based upon LD₉₀ values. The roles of the new target body regions assessed in this study in future insecticide toxicity evaluations are discussed.

Materials and methods

_Culex quinquefasciatus_ were obtained from colonies maintained at the U.S. Department of Agriculture—Agricultural Research Service (USDA-ARS) Center for Medical, Agricultural and Veterinary Entomology (CMAVE), in Gainesville (FL, U.S.A.) since 1995 (Vrzal et al., 2010). Mosquitoes were reared following the protocols published by Gerberg et al. (1994). For the present study, following eclosion, adult mosquitoes were segregated into temporal cohorts in separate 30 × 30 × 30-cm screened cages by date of emergence. Only female mosquitoes that emerged on the second day following the emergence of the initial cohort were used in this study. Adult mosquitoes were provided with access to 10% sucrose solution ad libitum.

Several hundred 3-day-old, non-blood-fed, female _Cx. quinquefasciatus_ were aspirated and anaesthetized with carbon dioxide. Sedated mosquitoes were removed and placed in a filter paper-lined glass Petri dish placed on a 4 °C chill table (Model 1431; BioQuip Products, Inc., Rancho Dominguez, CA, U.S.A.) for at least 5 min to complete sedation prior to manipulation. Using forceps, mosquitoes were manipulated into position on a glass slide on the chill table. Technical-grade insecticide was used for topical application of treatments. Insecticide active ingredients evaluated were permethrin (98.5%; AMVAC Chemical Corp., Newport Beach, CA, U.S.A.) and malathion (97.2%; Sigma-Aldrich Corp., St. Louis, MO, U.S.A.). Rapeseed methyl ester (RME) (96.5%; UCY Energy Group, Altfor, Germany) was used as the control and as the diluent for the technical-grade insecticides. Multiple diluents were tested for both insecticide solvency and health interaction with _Cx. quinquefasciatus_ before RME was selected as a result of its ability to dissolve both the technical insecticides, cause low control mortality, and express a longer evaporation time, which is necessary to support the much smaller volume droplet used. The technical-grade insecticide concentrations applied to the mosquitoes were diluted in volume to deposit a droplet containing either 0.8, 1.6, 3.1, 4.7, 5.7, 6.8, 14, 9.8, 11.7, 23.4 ng of permethrin, or 0.8, 1.6, 3.2, 4.8, 5.8, 6.9, 8.3, 10.0, 12.0, 23.9 ng of malathion for a given insecticide dose. A droplet of RME or a droplet of RME-diluted technical-grade insecticide was applied to a distinct body region of a mosquito using a 7000 series 0.50-μL glass syringe (Harvard Apparatus, Inc., Cambridge, MA, U.S.A.), inserted into a 50-step dosage dispenser (Model PB600-1; Harvard Apparatus, Inc.). This arrangement, coupled with the dilution of technical-grade insecticides with RME, facilitated the consistent delivery of a 267.3-μm diameter droplet dispensed by each step from the dosage dispenser.

_A priori_ experiments using multiple diluents were tested to identify one diluent that would not contribute to the mortality or morbidity of the mosquito in the absence of the insecticide, and that could serve as a solvent for both technical insecticides. The solvent RME proved an effective solvent for technical-grade insecticides. Unfortunately, RME dissolved the plastic surface of the bioassay chamber and caused complications that were addressed by adding a paper liner insert to the bioassay cup.

A total of 945 _Cx. quinquefasciatus_, consisting of 45 control and 900 insecticide-treated mosquitoes, were tested for mortality using the two technical-grade insecticides each applied individually to one of three body regions. These sites included the abdomen, characterized as expressing high insecticide sensitivity, the leg, reported by Aldridge et al. (2016) as expressing low sensitivity, and the mesothorax, a commonly assessed body region that allows for comparison across prior studies involving topical bioassays. Doses were selected empirically through pilot studies to provide valid dose–mortality curves.

Each of the insecticide treatments (permethrin and malathion) were evaluated in 450 mosquitoes, wherein at least 15 mosquitoes were tested for each insecticide treatment–dilution–body region combination. Experiments were replicated across three broods in which at least two mosquitoes from each brood were assessed for each insecticide treatment–dilution–body region combination.

After the insecticide or the RME-only droplet had been applied to a body region, mosquitoes were placed individually into 29.6-mL plastic cups lined with a tissue paper insert covered by
nylon tulle with 1-mm² openings that was secured by a silicone rubber O-ring. A cotton ball saturated with 10% sucrose solution was placed on the tulle allowing ad libitum access. During the experiment, mosquitoes were held in an incubator at 25 °C, 60% relative humidity, under an LD 12:12 h cycle.

Post-treatment morbidity and mortality assessments were performed visually and recorded at 24 ± 1 h after topical application. Morbidity was defined as any mosquito that exhibited outward signs of physiological distress or unusual behaviours, such as uncontrolled flying (e.g. flying upside down) and difficulty maintaining balance. Mortality was defined as any mosquito that was incapable of standing or that remained unresponsive when probed. Behaviours associated with morbidity were recorded as they may be indications of sub-lethal exposure and possibly be isolated among similarly exposed individuals (Sutherland et al., 1967; Aldridge et al., 2016). Sub-lethal behaviours were behaviours or effects that were identified as being linked with dosages of insecticide that did not cause mortality (Sutherland et al., 1967; Aldridge et al., 2016).

Mosquito mortality associated with the three body regions on the mosquito was assessed as a binary response (alive or dead), with morbidity and mortality assessments pooled together. Bioassay data from 24-h assessments were analysed using the statistical package SAS Version 9.2 (SAS Institute, Inc., Cary, NC, U.S.A.) to fit a generalized linear model based on a binomial distribution with a logit link.

The model had the following form: \( \text{logit}(y/n) = \mu + \text{brood} + \text{insecticide} + \text{region} + \text{insecticide}^{*}\text{region} + e \), where \( y/n \) is the binary response of mortality and morbidity for an insect and \( n=1 \) is the total number of insects per container (i.e. cup); brood is a blocking factor of experimental replication; insecticide is the insecticide treatment; region is the body region; insecticide*region is the interaction, and \( e \) is the residual term. Insecticide was considered a quantitative variable and generated slopes were compared between treatments. The lethal dose curves across the three body regions were calculated to determine those body regions that were most and least sensitive, and to estimate the LD_{50} values for each of the insecticide and body region combinations. Fitted logistic regressions were plotted following the back-transformed equation (inverse link). The LD_{50} values for body regions and insecticides were considered as significantly different \( (P < 0.05) \) if the 95% confidence intervals (CIs) did not overlap. Additional mosquito rearing, experimental and evaluation protocols are described in Aldridge et al. (2016).

Results

Observations on the spread of the insecticide–RME combination, or RME following droplet placement were similar across all insecticide treatments and similar to the observations in Aldridge et al. (2016). The abdomen and mesothorax demonstrated high mortality to both the tested insecticides, whereas the leg expressed low mortality. The LD_{50} values for permethrin applied to the abdomen and mesothorax were 4.5 ng (95% CI 3.3–5.5 ng) \((n = 150)\) and 5.5 ng (95% CI 4.5–6.0 ng) \((n = 150)\), respectively. The LD_{90} values for permethrin applied to the abdomen and mesothorax were 11.1 ng (95% CI 9.5–14.2 ng) and 7.4 ng (95% CI 6.3–9.4 ng), respectively. The LD_{50} values for malathion applied to the abdomen and mesothorax were 9.7 ng (95% CI 8.2–12.5 ng) \((n = 150)\) and 8.7 ng (95% CI 6.3–10.5 ng) \((n = 150)\), respectively. A lethal dose value above 10% could not be calculated for the leg with permethrin or malathion at the doses tested because of low levels of mortality.

The dose–response curve for permethrin (Fig. 1) shows that the mortality associated with application to the abdomen did not overlap the curve generated for application to the mesothorax above 30% mortality. The dose–response curve for malathion (Fig. 2) demonstrates that the mortality associated with application to the abdomen overlapped the curve generated for application to the mesothorax above 40% mortality. The curves generated when either malathion or permethrin was applied to the leg did not overlap those for application to the other body regions assessed.

For permethrin, sub-lethal effects observed were defined as effects not observed in the control assay, such as disorientation, leg autotomization and incessant grooming behaviour to the abdomen, and were recorded at doses that ranged across the entire spectrum tested. Sub-lethal effects on mosquitoes surviving 24 h following permethrin exposure are presented in Fig. 3, which shows that up to 76% of mosquitoes in a treatment exposed at 10 ng expressed an effect. Malathion generated sub-lethal effects in two instances, once from application of a droplet containing 5.8 ng, and once from application of a droplet containing 23.4 ng, impacting only 7% of mosquitoes tested with malathion at the doses mentioned \((n = 15)\).

Discussion

Deposition of insecticide droplets on mosquito body regions has been little studied, and the subsequent mortality associated with
Body regions after 24h (of droplets of malathion (ng) diluted in rapeseed methyl ester to distinct body region as 11.11ng per mosquito, respectively (Fisher, 1952). Similar results from Fisher (1952) and Rai & Roan (1959) suggest that as an insecticide is applied closer to central nervous tissue (i.e. the brain and thoracic ganglia), the mortality rate can be expected to increase. This hypothesis was supported by Scott et al. (1986) after topical application of cypermethrin and allethrin in Blattella germanica L. (Blattodea: Blattellidae) elicited significantly increased mortality at similar levels when applied to the tarsus vs. the pronotum. However, in Scott et al. (1986), the effects of the insecticide differed between applications to the tip of the abdomen vs. the thorax, which distinguishes their results from those of the present study, in which applications of insecticide to these two regions generated similar mortality responses.

Pridgeon et al. (2008) reported that the LD95 value of permethrin, when applied to the mesothorax of 7-day-old Cx. quinquefasciatus, was 13.9ng per mosquito, as calculated from the reported average mosquito weight. The present study, which utilized mosquitoes from the same colony, resulted in LD90 values of 7.4ng per mosquito when permethrin was topically applied to the mesothorax and 11.1ng per mosquito when it was applied to the abdomen in 3-day-old Cx. quinquefasciatus. The LD90 values for these regions differed in their 95% CIs. The higher LD value for the mesothorax generated by Pridgeon et al. (2008) may be attributable to several factors, primarily the calculation of an LD95 rather than an LD90 value. Additional factors include the age of the mosquitoes tested, the use of acetone rather than RME as a solvent, and the spread associated with the droplet volume deposited as a result of the different volumes applied. Furthermore, the results in this study indicate no overlap in 95% CIs for LD90 values of permethrin when applied to the thorax and abdomen. These results do not match the findings of Aldridge et al. (2016), which indicate no significant difference between effects of permethrin applied to the abdomen and thorax, respectively. However, the earlier study (Aldridge et al., 2016) qualitatively assessed the difference between body regions by utilizing a formulated product containing PBO, using BVA-13 oil as the carrier, and using mosquitoes that ranged in age from 3 to 5 days, whereas the current study quantitatively assessed the difference between body regions by utilizing technical permethrin without PBO, using RME as a carrier, and applying droplets to 3-day-old mosquitoes.

Gilotra et al. (1972) found that the LD95 of malathion, when applied subcutaneously to several strains of Cx. quinquefasciatus, ranged between 29 and 117ng per mosquito. In St. sollicitans collected from Louisiana and Texas, malathion induced LD90 values of 5.7 and 5.1ng per mosquito, respectively, following topical application to the thorax (Khoo & Sutherland, 1983). Conversely, in St. sollicitans collected from New Jersey, topically applied malathion demonstrated LD95 values that ranged between 13 and 60ng per mosquito (Khoo & Sutherland, 1983). Evaluation of these insecticides as commercial formulations in field populations of mosquitoes would be likely to provide slightly different mortality responses. Thus, conclusions drawn from comparisons between field-collected
specimens should be interpreted with care. Weidhaas et al. (1970) recorded an \( LD_{100} \) value for malathion of 10 ng per mosquito when the insecticide was applied to the thorax of *Oc. taeniorhynus* (Wiedemann) from Florida, which is comparable to the \( LD_{90} \) values for malathion applied to the mesothorax (8.67 ng per mosquito) and abdomen (9.69 ng per mosquito) obtained in the current study using 3-day-old *Cx. quinquefasciatus*. The \( LD_{90} \) values and CIs associated with exposure to malathion in these regions in the current study did not differ from one another.

The \( LD_{90} \) values for permethrin identified in this experiment, if applied as a formulated concentration matching the permethrin concentration in Aqualuer 20-20° (Value Garden Supply, St Joseph, MO, U.S.A.), would produce droplets of 22.8 \( \mu \)m and 26.1 \( \mu \)m in diameter for the mesothorax and abdomen, respectively. The malathion \( LD_{90} \) values identified in this experiment, if applied as a formulated concentration matching the malathion concentration in Fyfanon® ULV (FMC Corp., Philadelphia, PA, U.S.A.), would produce droplets of 23.8 \( \mu \)m and 24.7 \( \mu \)m in diameter for the mesothorax and abdomen, respectively. The droplet sizes estimated from this experiment are similar to the optimal droplet size (25.0 \( \mu \)m) Weidhaas et al. (1970) determined to kill *Oc. taeniorhynus*. By contrast, in the present study little mortality was recorded when malathion or permethrin was applied to the legs and neither an \( LD_{90} \) nor a droplet size was calculated because the amount of insecticide needed would have exceeded guidelines for droplet size used in ground-based ultra-low-volume (ULV) sprayers set forth by the WHO (2006).

Sub-lethal effects were recorded from applications of permethrin (Fig. 3). As documented previously by Rudolfs (1930) and MacNay (1939), it is speculated that leg autotomization may be a form of insecticide resistance in the form of sequestration and elimination that prevents mosquitoes from absorbing a lethal dose through their legs in a manner similar to that concluded by Moore & Tabashnik (1989) in investigations of the diamond-back moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae). Alternatively, leg autotomization may be an artefact of a natural response to the grasping of a leg by a predator, a snare (i.e. a web) or a sticky secretion; further research is needed in regard to this speculation.

Sub-lethal doses of permethrin (\( LD_{25} \)) calculated by Cohnstaedt & Allan (2011) demonstrated that, when applied to the thorax, permethrin caused disruption of flight direction and orientation in *Cx. quinquefasciatus*. The present study did not assess flight behaviour, direction or orientation, but did record sub-lethal effects, as illustrated in Fig. 3. Additionally, sub-lethal effects, specifically the incidence of leg autotomization, increased as the concentration of permethrin increased. It is suspected that leg autotomization was not prevalent in applications of malathion because malathion must be absorbed and metabolized into malafoxon before it can act upon the nerve cell (Fukuto & Sims, 1971). Once absorbed, it moves from the leg into the thorax, where it is bioactivated, possibly compromising any sequestration of malathion by autotomy.

An observed spread of RME and RME + insecticide was noted. The present authors speculate that the diffusion of lethal or sub-lethal doses of insecticide could be exploited as a control measure. The targeting of specific regions of the insect exoskeleton, such as sensory organs, may disrupt a range of behaviours. For example, disruption of the sensory organs on the tip of the mosquito proboscis by exploitation of mosquito grooming behaviour, in addition to diffusion of the insecticide across the cuticle, may disrupt blood feeding in female mosquitoes and is therefore deserving of investigation.

In summary, the results presented herein quantify the potency of permethrin and malathion when applied to distinct mosquito body regions previously identified by Aldridge et al. (2016). The gaps in mortality between effects at treated body regions at specific insecticide concentrations illustrate the benefits of delivering insecticide to the abdomen and thorax compared with the leg. Areas generated between the dose–effect curves for each insecticide and body region illustrate the potential mortality range and therefore may allow for the generation of more effective insecticide efficacy models for the specific insecticide (Figs 1 and 2). Furthermore, insecticide droplet deposition, characterized to predominantly impinge on the legs and wings of mosquitoes, rarely on the thorax, and occasionally on the abdomen and proboscis/antennae (Lofgren et al., 1973; Cooperband et al., 2010), in conjunction with the data from the present study, provides a better understanding of the interaction of inherent toxicity, product placement and expected field application insecticide efficacy. An important result of this study is that the reported dose–effect curves produced estimated \( LD_{90} \) values that can be used to generate droplet diameter ranges for insecticide concentrations across various body regions. Finally, sub-lethal effects were reported from various body regions and were comparable with previously reported experiments focusing on sub-lethal concentrations (Fig. 3). However, the paucity of information on the influence of insecticide droplet deposition on the mosquito body and the resulting mosquito mortality or morbidity provides avenues for future investigation.

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