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ORIGINAL PAPER

The use of *Aedes aegypti* larvae attractants to enhance the effectiveness of larvicides

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Abstract *Aedes aegypti* (L.) is an important dengue, chikungunya, and yellow fever vector. Immature stages of this species inhabit human-made containers placed in residential landscapes, and the application of larvicides inside containers that cannot be eliminated is still considered a priority in control programs. Larvicidal efficacy is influenced by several factors, including the formulation used, the water quality, and the susceptibility of larvae, among others. If an attractant can be incorporated into a slow-release larvicide formulation, it will be feasible to direct the larvae into the source of insecticide and thereby improving its efficacy. We studied the influence of 1-octen-3ol and 3-methylphenol on the rate of *Ae. aegypti* larvae mortality using the larvicides *Bacillus thuringiensis var. israelensis* (*Bti*), temephos, and spinosad. These chemicals were combined with the larvicides mixed with agar during the bioassays. Mortality was registered every 10 min, and a lethal time 50 (LT_{50}) was calculated. The inclusion of the *Ae. aegypti* larvae attractants with the larvicides into a solid agar matrix improved their efficiency obtaining a strong and marked reduction in the LT_{50} compared with the use of larvicides alone.

Keywords Mosquito · Larvae · Attractant · Insecticide

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Introduction

Aedes aegypti (L.) is a container-breeding mosquito that commonly inhabits urban and suburban areas throughout the world. They are diurnally active, highly anthropophilic, and a potential vector of the dengue, yellow fever, and chikungunya viruses to humans. Globally, there are 50 to 100 million dengue cases resulting in thousands of deaths annually (WHO 2006a). *Ae. aegypti* control is mainly directed against immature stages (education, source reduction, and larviciding) to reduce the production of new adult mosquitoes, with some efforts devoted to controlling adult mosquitoes using spatial sprays of adulticides during dengue outbreaks (Gratz 1999; Pilger et al. 2010).

Developmental stages of *Ae. aegypti* can be found in artificial containers and natural sites close to human dwellings (Barrera 1996; Scott et al. 2000). In such situations, the likely breeding sites can be treated with larvicides and oviposition repellents as components of the integrated approach to mosquito population management (Hwang et al. 1980; Curtis and Hill 1988; Xue et al. 2001; Tikar et al. 2014). However, not all breeding sites can be totally eliminated or made mosquito-proof, and it is difficult to involve all members in the community in a sustained clean-up campaign. In addition, neither adulticides nor larvicides are completely effective against *Ae. aegypti*. The development of novel, effective methods for the control of dengue vectors are therefore urgently needed, with particular emphasis on methods that are environmentally friendly, cost-effective, and suitable for integration into community-based control programs (Service 1992; Swaddiwudhipong et al. 1992; Chunsuttiwat and Wasakarawa 1994).

The behavior of the immature stages of holometabolous insects is mainly led by short-range orientation to food sources, feeding behavior, and defensive responses. The

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sensory requirements of the larvae are more limited than those of the adults, and this is reflected in the smaller number of integumental sensilla and the lower capabilities of certain larva sense organs (Xia 2008). Mosquito larvae manifest a number of behavioral responses towards different kinds of stimuli such as light, food, color, etc. (Merritt et al. 1992). Responses towards food sources are believed to be largely driven by olfactory chemosensory stimuli (Merritt et al. 1992) and have been studied in many mosquito species. *Aedes vexans* larvae, when placed in a dish with incompletely separated compartments, congregated in the compartments that contained pellets of fishmeal or wheat flour (Aly 1985). In addition, *Culex quinquefasciatus* larvae became concentrated and showed positive chemotaxis in regions of water containing casein hydrolysate or the amino acids phenylalanine, aspartic acid, and proline (Barber and Burnton 1983). Also, mosquito larvae accumulate in regions where there is food as the result of orthokinetic responses to soluble constituents diffusing from the food with the involvement of their olfactory systems (Merritt et al. 1992).

The aquatic larval habitats inherently represent a confined and, therefore, a more easily targeted site for mosquito control strategies. Although a lot of efforts have been put into developing novel repellents and attractants for adult mosquitoes with low toxicity to non-target organisms, very few similar studies have been done on larvae. The available larvicides focus on high efficiency of killing and low toxicity towards other organisms. Furthermore, if coupled with larval attractants, larvicides may have an improved chance to kill their larvae targets.

Since larvicides for *Ae.aegypti* control are mainly used in drinking water, not all the known compounds can be used. World Health Organization (WHO) allows only temephos, *Bacillus thuringiensis var. israelensis* (*Bti*), spinosad, and some IGRs such as methoprene, pyriproxyfen, and novaluron (WHO 2007a, b, 2008, 2009, 2010). In addition, some synthetic pyrethroids are very effective but care must be taken when used as larvicides due to their toxicity to aquatic non-target organisms (WHO 2006b). Besides, the use of larvicides is limited by issues such as the emergence of resistance, already known for temephos in almost Latin-American countries including Argentina (Majori et al. 1986; Coosemans and Carnevale 1995; Braga et al. 2004; Ocampo et al. 2011). The efficacy of larvicides relies on several factors including the formulation, water quality, and the susceptibility of the targeted larvae (Walker and Lynch 2007; Harburguer et al. 2009). If a slow-release formulation could be modified by adding a strong attractant, it would be possible to increase the larval density proximate to insecticides and thereby greatly enhance their effectiveness.

In this study, we combine the study of chemical attractants of well-known *Ae. aegypti* larvae combined with larvicides in order to increase their selectivity and efficiency. The aim of this work is to contribute to innovation in control strategies by using more selective modes of action, lower risk to non-target organisms, and lower environmental impact tools.

Materials and methods

Insecticides

A commercial formulation of *Bti* was used Larvicidal Mosquito Dunks® by Summit Chemical Co., 10 % *p*/*p*, 7000 international toxic units/mg. Technical-grade spinosad was provided by Dow AgroSciences. Technical-grade temephos (97.6 %) was provided by Supelco Analytical Bellefonte, PA, USA.

Chemicals

1-Octen-3-ol (98 %) and 3-methylphenol (>97 %) were purchased from Sigma-Aldrich (St. Louis, USA). Acetone (>99.8 %) was purchased from Merck (Germany). Ethanol absolute (99.5 %) was purchased from Sintorgan S.A. (Argentina). Agar-agar was purchased from Parafarm® (Saporiti, Argentina).

Biological material

A susceptible strain of *Ae. aegypti* (CIPEIN) was used. This strain, originated from the Rockefeller strain in Venezuela, had been kept in the laboratory since 1996, reared at 25 ±2 °C under L:D 12:12 h according to previous reports from the laboratory (Seccacini et al. 2006). For this study, 100 late third-instar or early fourth-instar larvae were used. These larvae were washed with dechlorinated water and kept without food for 2 h at 27 °C.

Combination of insecticide plus attractant

Odorant stock solutions were prepared by dissolving a specific amount of the odorants in a preheated 5 % agar-agar solution. The concentrations used in this study were 10^{-03} and 0.1 mg/ml of 1-octen-3-ol and 3-methylphenol. These compounds and concentrations were chosen as they evoke attractant responses in *Ae. aegypti* larvae (Gonzalez et al. 2015).

For larvicidal bioassays, 2 g of the *Bti* commercial briquette were weighted out. Spinosad (40 mg/ml) and temephos (1 mg/ml) solutions were prepared in acetone and ethanol, respectively. Whatman filter papers no. 2 (4.25 cm diameter) were impregnated with 0.5 ml of the stock solution of each larvicide. Acetone and ethanol were allowed to evaporate for 24 h.

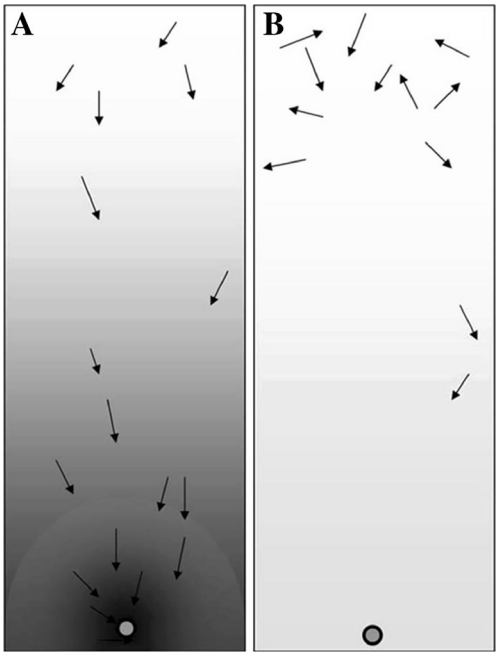

Fig. 1 Schematic diagram showing the setup of the larvicidal bioassay. **a** Larvicide plus attractant. **b** Larvicide alone. The *arrows* represent mosquito larvae

Larvicidal bioassay

The larvicidal activity of the selected insecticides in combination with the larval attractants was evaluated on *Ae. aegypti* larvae according to the following method. Plastic containers (27 × 37 × 9 cm) with 3 l of dechlorinated water maintained at 27 °C were placed next to each other under the same light conditions. Both the insecticide (either filter papers impregnated or briquette) and the odorant (in agar) to be tested were bound, with insect pins (Bioquip N°6), at one end of the container and maintained completely submerged. A concentration gradient of the odorant in the water was allowed to be formed for 30 min. One hundred late third-instar and/or early fourth-instar *Ae. aegypti* larvae, carefully washed, were released at the opposite side of the odorant plus insecticide (Fig. 1). Larvae mortality was recorded every 10 min for 6 h starting 20 min after the larvae were incorporated to the container. The trial included a control with only insecticide and no odorant, and a negative control (with odorant but without insecticide). The time required for 50 % of the population in the container to die (LT_{50}) was calculated by the Litchfield and Wilcoxon (1949) method.

Statistical analysis

The median lethal time (LT_{50}) with 95 % confidence interval (CI) was obtained by means of PoloPlus 2.0 software (LeOra Software Company, Petaluma, CA) and was expressed in minutes.

Results

Table 1 shows the results of LT_{50} for *Bti* in the presence of different concentrations of the odorant 3-methylphenol. With increasing concentrations of 3-methylphenol, we found that the time required for 50 % mortality decreases from 100 to 88.9 min; however, this difference was not significant. The best performance was obtained for *Bti* plus 0.1 mg/ml 3-methylphenol. The confidence intervals at 95 % indicate that LT_{50} for the control (153 min), using *Bti* alone, was significantly different from LT_{50} in all the concentrations.

Table 2 shows the results of LT_{50} of *Bti* combined with different concentrations of 1-octen-3ol. Again, the larvicide that was combined with the odorant resulted more effective than when used alone. Both concentrations tested showed statistically different efficacy than the control. A slightly better larvicidal effect was obtained with 10^{-03} mg/ml of 1-octen-3ol (76.1 min) than for 0.1 mg/ml (80.5 min); however, this difference was not statistically different. Those concentrations of attractants that were the most effective in reducing the LT_{50} were selected to test their efficacy with the other two insecticides.

Table 3 shows the results of LT_{50} of spinosad in the presence of both odorants. As the results found for *Bti*, when the larvicide was combined with the odorants, it resulted more effective than using the larvicide alone. No significant

Table 1 LT_{50} of *Ae. aegypti* larvae for *Bti* plus 3-methylphenol at different concentrations

Treatment	*n*	Slope (SE)	LT_{50} (95 % CI) [min]
Control (odorant)	4	–	>360[a]
Control (*Bti*)	4	6.015 (0.185)	153.1a (145.1–163.3)
10^{-03} mg/ml 3-methylphenol + *Bti*	4	5.818 (0.150)	100.0b (94.9–105.4)
0.1 mg/ml 3-methylphenol + *Bti*	4	6.295 (0.153)	88.9b (81.5–96.3)

Numbers followed by the same letter are not significantly different from each other based on non-overlap of confidence limits. $P < 0.05$

CI confidence interval

[a] No mortality was recorded in the untreated control at the end of the assay (6 h)

Table 2 LT_{50} of *Ae. aegypti* larvae for *Bti* plus 1-octen-3ol at different concentrations

Treatment	*n*	Slope (SE)	LT_{50} (95 % CI) [min]
Control (odorant)	3	–	>360[a]
Control (*Bti*)	3	4.999 (0.172)	115.5a (107.1–126.3)
10^{-03} mg/ml 1-octen-3ol + *Bti*	3	5.147 (0.161)	76.1b (64.3–87.5)
0.1 mg/ml 1-octen-3ol + *Bti*	3	5.740 (0.179)	80.5b (71.4–89.7)

Numbers followed by the same letter are not significantly different from each other based on non-overlap of confidence limits. $P < 0.05$

CI confidence interval

[a] No mortality was recorded in the untreated control at the end of the assay (6 h)

differences were found between the odorant used, and LT_{50} values were higher than when we used *Bti*.

Finally, when the larvicide temephos was combined with the odorants, the LT_{50} were significantly lower than when used alone (Table 4). Furthermore, LT_{50} with 1-octen-3ol (122.4 min) was significantly lower than with 3-methylphenol (146.6 min). LT_{50} values for the combination of temephos plus the odorant were similar to those for spinosad but higher than the ones for *Bti*.

Discussion

In this work, we studied the lethal time of larval mortality 50 (LT_{50}) by combining known larval attractants with larvicides of conventional use in order to determine whether the attractant effect would enhance the larvicidal effect.

When *Bti* was used in combination with the attractants 1-octen-3ol or 3-methylphenol, a significant reduction in the LT_{50} was observed with respect to the control of *Bti* alone. The best performances were achieved for concentrations of 10^{-03} and 0.1 mg/ml of 1-octen-3ol and 3-methylphenol, respectively.

Bti is a spore-forming bacterium that produces a proteinaceous incrustation in a crystalliferous body during sporulation. The crystal consists of a least four protein protoxins. Upon digestion by an insect having a sufficiently high midgut pH, the dissolved protoxins are enzymatically converted to the toxins (Federici and Wu 1994; Patil et al. 2012). If the larva is a mosquito or other susceptible dipteran species, the toxins attach to receptors in the midgut membrane and initiate a process ending in cell lysis. An individual insect dies when a sufficient amount of toxin is ingested and activated (Skovmand et al. 1998). Ultimately, the mortality in a population of susceptible larvae is dependent upon the quality and quantity of toxin ingested. Due to this fact, by combining *Bti* with a larval attractant, the time to run into the larvicide is reduced and the intake accelerated, producing an overall reduction in the LT_{50}.

Results of our work show that when spinosad was used in combination with the larval attractants, also a significant reduction in the LT_{50} was observed. Spinosad is highly active by both contact and ingestion to numerous pests in the orders Lepidoptera, Diptera, Thysanoptera, Coleoptera, Orthoptera, Hymenoptera, and others (Bret et al. 1997; Kovendan et al. 2012). It affects the insect nervous system at unique sites on the nicotinic acetylcholine and gamma-aminobutyric acid (GABA) receptors (Hertlein et al. 2010). Field studies suggest that rates of spinosad used nowadays will need to be increased in response to habitats with very high levels of liquid or solid sewage such as cisterns (Cetin et al. 2005) or street drains (Sadanandane et al. 2009). This observed reduction in spinosad's larvicidal efficacy could be due to adsorption, soil

Table 3 LT_{50} of *Ae. aegypti* larvae for spinosad plus 3-methylphenol and 1-octen-3ol at 0.1 and 10^{-03} mg/ml, respectively

Treatment	*n*	Slope (SE)	LT_{50} (95 % CI) [min]
Control (odorant)	3	–	>360[a]
Control (spinosad)	3	4.955 (0.255)	224.5a (212.3–240.1)
0.1 mg/ml 3-methylphenol + spinosad	3	3.693 (0.115)	120.5b (117.3–123.9)
10^{-03} mg/ml 1-octen-3ol + spinosad	3	3.308 (0.108)	126.6b (122.8–130.7)

Numbers followed by the same letter are not significantly different from each other based on non-overlap of confidence limits. $P < 0.05$

CI confidence interval

[a] No mortality was recorded in the untreated control at the end of the assay (6 h)

Table 4 LT_{50} of *Ae. aegypti* larvae for temephos plus 3-methylphenol and 1-octen-3ol at 0.1 and 10^{-03} mg/ml, respectively

Treatment	*n*	Slope (SE)	LT_{50} (95 % CI) [min]
Control (odorant)	4	–	>360[a]
Control (temephos)	4	5.666 (0.137)	251.9a (245.5–259.0)
0.1 mg/ml 3-methylphenol + temephos	4	5.357 (0.096)	146.6b (142.4–150.8)
10^{-03} mg/ml 1-octen-3ol + temephos	4	5.828 (0.105)	122.4c (117.6–127.2)

Numbers followed by the same letter are not significantly different from each other based on non-overlap of confidence limits. $P < 0.05$

CI confidence interval

[a] No mortality was recorded in the untreated control at the end of the assay (6 h)

microbial degradation, decreased ingestion by larvae, or all—although adsorption is the likelier explanation given the much longer half-lives involved in microbial degradation (Saunders and Bret 1997). Incorporating a larval attractant to a formulation of spinosad, as proposed in our work, could improve its effectiveness in habitats with high levels of sewage since ingestion by mosquito larvae would occur before the larvicide is adsorbed and its efficacy reduced.

Also, when temephos was used in combination with larval attractants, a significant reduction in the LT_{50} was observed. Temephos is a soluble organophosphate (OP) that has been widely used for mosquito larvae control for up to 50 years. It has a neurotoxic mode of action which inhibits acetylcholinesterase and is still widely used in mosquito control programs (Ang and Satwant 2001; Tikar et al. 2009).

The use of push-pull tactics fits within the emerging view that vector control strategies should be expanded beyond insecticide-dependent methods (Cook et al. 2006). Combining the mechanisms of attraction and repellency has the potential to result in a synergistic effect (Thomas et al. 2012). By 'pushing' mosquitoes away from certain places using repellents, one could stimulate their movement towards other places where they are 'pulled' into traps baited with attractive cues (Menger et al. 2014). From the results obtained in this study, it gives rise to the possibility of applying the push-pull tactic for controlling mosquito larvae, expelling them from their shelters with a repellent, and directing them into traps with attractants combined with a lethal agent. Although it is known that *Ae. aegypti* larvae inhabits confined containers, some evidence has been found that they also can be located in shallow water bodies (Chadee et al. 1998) and therefore this kind of tactics could be applied in such cases. The results obtained in this work for *Ae. aegypti* could be extended to mosquitoes that live in large water bodies, such as *Anopheles* or *Culex*, considering that larvae attractants for *C. pipiens quinquefasciatus* and *Anopheles gambiae* have already been identified (Barber and Burnton 1983; Xia 2008).

Behavioral and toxicological evidence presented here shows that larval attractants decrease the lethal times of the three larvicides tested. The best results were obtained with *Bti*, due possibly to the fact that we used a commercial briquette with inert ingredients that could increase its bioavailability unlike with temephos and spinosad wherein technical grade was used.

The use of a slow-release formulation with the addition of a strong attraction may increase larval density near the insecticide area and thereby enhancing its effectiveness and skipping or reducing problems of lack of persistence associated with the effect of UV radiation, temperature, and microbial degradation.

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