

Dual-treatment autodissemination station with enhanced transfer of an insect growth regulator to mosquito oviposition sites

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Abstract

BACKGROUND: The Asian tiger mosquito, *Aedes albopictus* (Skuse), transmits important arboviral diseases and displaces native species. This peridomestic mosquito deposits eggs in natural and artificial containers. Container larval habitats tend to be cryptic and, therefore, difficult to reach by conventional insecticide treatments. We have developed an autodissemination station that transfers the insect growth regulator, pyriproxyfen, from the station to oviposition sites. Mosquitoes visiting the station become contaminated with an oil sticker followed by pyriproxyfen powder before exiting.

RESULTS: In a room (31 m³) bioassay a single station consistently achieved 100% *Ae. albopictus* pupal mortality against 10 oviposition containers. In a greenhouse (200 m³) assay with two stations and 12 oviposition cups, 91.7% of the cups had been contaminated as shown, and 57.1% pupal mortality was recorded. Pyriproxyfen transfer was also detected by visualizing mosquito 'tarsal prints' using a fluorescent dye.

CONCLUSIONS: The oil and pyriproxyfen powder dual-treatment station enhanced autodissemination efficacy by increasing toxicant attachment and retention on contaminated females. The autodissemination station offers a targeted, less environmentally damaging approach to manage cryptic container species.

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Keywords: *Aedes albopictus*; autodissemination; container mosquito; insect growth regulator; pyriproxyfen

1 INTRODUCTION

The highly invasive Asian tiger mosquito *Aedes albopictus* (Skuse) transmits several important arboviral diseases including dengue, yellow fever and chikungunya.¹ The mosquito is a tree-hole species that has adapted to urban containers generated by human activities.² The cryptic larval habitat preferred by this species is difficult to reach by traditional pesticide aerosol sprays, which is a key reason this species is difficult to manage using conventional means.³ Gravid *Ae. albopictus* females employ skip oviposition behavior in which eggs are distributed throughout multiple oviposition sites.⁴ This behavior was exploited to demonstrate that *Aedes aegypti* (L.) females contaminated at an artificial resting site with pyriproxyfen could transfer this insect growth regulator to oviposition sites, thereby inhibiting adult emergence.⁵ Pyriproxyfen acts as a juvenile hormone mimic and is exceptionally toxic to mosquito larvae with a 50% lethal concentration (LC₅₀) to *Ae. aegypti* and *Ae. albopictus* as low as 0.012 ppb.^{6,7}

Various efforts have been made to further develop the autodissemination concept since a study in Peru reported 42–98% *Ae. aegypti* emergence inhibition at a public cemetery using pots dusted with powdered pyriproxyfen.³ A modified sticky trap coated with 5% pyriproxyfen powder subsequently provided 40–70% mortality in a warehouse study in Italy.⁸

We previously described an autodissemination station constructed with biodegradable materials.⁶ There were, nevertheless,

limitations that tended to reduce effectiveness. The station's openings were large so not all visiting mosquitoes became contaminated. Contaminated mosquitoes could easily remove the powder formulation from their body parts by grooming because minimal sticker oil (10%) could be mixed with the powder due to caking. In subsequent small-room trials, 83% *Ae. albopictus* pupal mortality was obtained, indicating the need for further development.

An effective autodissemination station must: (1) be as or more attractive than competing oviposition containers in the same environment; (2) transfer the toxicant efficiently to the insect; (3) have formulations that resist insect grooming behaviors; and (4) have formulations that easily release the toxicant from the mosquito into the larval breeding site. Additional criteria such as longevity of efficacy and resistance to weather may be considered as well.

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To improve our previous station prototype,⁶ we designed a dual-treatment system that maintained the best features of the previous version such as biodegradability and auto-refilling, but with enhanced attraction, contamination and retention. A core feature for the new design was to force visiting mosquitoes to contact an oil sticker first, followed by a pyriproxyfen powder concentrate.

2 MATERIALS AND METHODS

2.1 Station design

A dual-treatment autodissemination station was developed based on our earlier prototype⁶ with the same water reservoir but a new transfer chamber (Fig. 1A) constructed from a cardboard tube (100 × 155 mm). Gravid females attracted to the water enter the station from the top and cross into an inner funnel (mouth diameter 9.5 cm; height 8.0 cm). The bottom opening (10 mm diameter) in the funnel is a portal allowing mosquitoes to enter the transfer chamber. Here they encounter a mesh fabric barrier (39 apertures cm⁻²) that prevents access to the reservoir water and therefore egg depletion. The funnel is unidirectional, so females must exit the station by moving upward between the outer wall of the funnel and the inner wall of the transfer chamber. At the chamber top, exiting females must pass between two horizontal plates. Spacer blocks maintain a 6 mm gap between the transfer plate and the cover plate to force mosquitoes to exit by walking rather than flying. The upper or cover plate collects rainwater and dew which is directed to the reservoir chamber. The lower or transfer plate houses two concentric fabric bands (2.5 cm width, Fig. 1B). The inner band (Versi-DRY, Thermo Fisher Scientific, USA) is infused with an oil formulation (corn oil:Tween 80 1:1; 0.054 g cm⁻²) that acts as a sticker, whereas the outer band (Insul-Bright, Fabric Land NJ) is coated with a powder formulation of 35% pyriproxyfen (Esteem 35 WP Insect Growth Regulator, Valent Inc., USA, 0.015 g cm⁻²). A fluorescent tracer dye (Uvitex OB, Ciba Specialty Chemicals, Basel, Switzerland) was added to the powder formulation (0.1% w/w) so that residues could be visualized under long-wave ultraviolet light. The oil and powder bands are adjacent but are not in contact. Mosquitoes first pick up the oil sticker before contacting and becoming contaminated with the pyriproxyfen powder as they exit the chamber to search for oviposition sites. The station was dyed dark gray before a wax coating was applied. The water reservoir held 400 mL of oak leaf infusion.⁴

2.2 Mosquito colony and maintenance

Larvae for bioassays were obtained from an *Ae. albopictus* colony established from eggs collected in Mercer County, NJ, USA in 2008, and supplemented during 2009 and 2010. The colony was maintained at 26 ± 1 °C, at a relative humidity of 75% under an 16:8 h light/dark photoperiod. Adults were held in 2.7 m³ aluminum screen cages and supplied with 10% sucrose solution on cotton wicks. Restrained guinea pigs were used to blood-feed females (Rutgers University Animal Use Protocol #86–129) and subsequent eggs were collected on seed germination paper and stored at 26 ± 1 °C. Eggs were hatched as needed by flooding egg papers and the larvae were held in enamel trays with deionized water (1 L) and provisioned with Brewer's yeast (0.3 g) on alternate days. Third instars were used in all bioassays.

2.3 Bioassays

Bioassays for pyriproxyfen activity were conducted in plastic cups (450 mL) as described previously.⁶ Each cup received *Ae. albopictus*

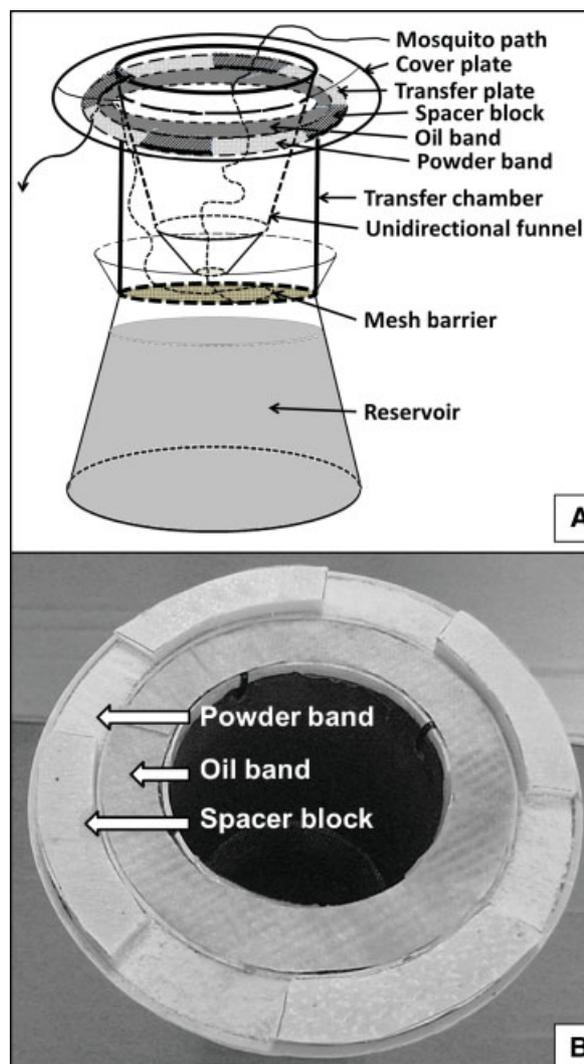


Figure 1. Schematic drawings showing (A) dual-treatment autodissemination station and (B) top view of transfer plate: concentric oil- and powder-impregnated fabric bands mounted on a single plate.

larvae (20) and water (200 mL). Larvae were introduced into the cups and the lid replaced. Food (30 mg yeast L⁻¹) and water lost due to evaporation were provided twice a week. Incomplete emergence or adult with attached exuvia were recorded as pupal death.

2.4 Attraction

The dual-treatment prototype was tested for gravid female attraction in a small cage (2.3 m³) against the earlier prototype in a choice experiment. Station exits were obstructed to trap visiting females. The earlier version was modified by inserting a unidirectional funnel between the transfer chamber (mesh removed) and reservoir to trap mosquitoes. Twenty gravid females were released into the cage for 24 h and the number of mosquitoes per station was counted. The experiment was repeated three times with three replicates each.

2.5 Attachment and retention

Pyriproxyfen attachment and retention were determined by allowing gravid females (15) to exit from the station and into a holding cage (2.2 m³). Females were then individually collected

via an aspirator after 0, 2, 4, 8 and 24 h and transferred to a lidded cup (450 mL) with water (250 mL). The cup was gently shaken so that the mosquito contacted the water surface. A 50 mL water sample was then withdrawn for residue analysis with the remaining 200 mL of water used to assays for insecticidal activity (as described in Section 2.3). The residue samples were stored in a brown glass bottle at -20°C and shipped to Golden Pacific Laboratories (CA, USA) on dry ice. The samples thawed, diluted 1 : 1 with acetonitrile and an aliquot was passed through a $0.45\ \mu\text{m}$ filter. Analysis was conducted using an AB Sciex API 5000 mass spectrometer monitoring both primary and confirmatory ion pairs. Each set of samples contained a water blank, as well as control samples fortified at the limit of quantification and at a higher level to bracket expected sample concentrations. The experiment was repeated three times with three replicates each.

2.6 Serial transfer

The probability for multiple container contamination from a single contaminated female (i.e. serial contamination) was examined. Gravid females (5) were transferred into a station and permitted to exit into a cage ($1\ \text{m}^3$) containing oviposition cups (5, 10 or 15) with water (200 mL). The mosquitoes were allowed 5 days for oviposition. The cups were then assayed for pyriproxyfen activity as described in Section 2.3. There were two replicates for each treatment and the experiment was repeated twice.

2.7 Room efficacy

Efficacy trials were conducted in a small room measuring 2.1 m in width, 5.7 m in length and 2.6 m in height ($31\ \text{m}^3$). An autodissemination station was placed in the room center and 10 sentinel oviposition cups (450 mL, exterior painted black) containing 250 mL of tap water were positioned equidistant and 30 cm from the walls. Fifty gravid females were introduced for a 5-day test at $26\text{--}28^{\circ}\text{C}$, $60\text{--}85\%$ RH and 16 : 8 h light/dark photoperiod. The cups then were tested for pyriproxyfen transfer by larval bioassay as described in Section 2.3. Ten additional untreated cups were included as controls. Pupal mortality was analyzed against their respective controls with a *t*-test ($P < 0.05$). The experiment was repeated twice.

2.8 Greenhouse efficacy

Efficacy trials were conducted in a greenhouse with two bays ($100\ \text{m}^3$ each) connected by an open doorway and window. A mix of ornamental plants covered more than half of the $5 \times 8\ \text{m}$ floor space in each bay to add complexity and sheltered microhabitats. One station was positioned in the center of each bay with six sentinel ovi-cups (450 mL, exterior painted black) each containing 250 mL water were positioned equidistant and 30 cm from the walls. One hundred gravid females (2 days post blood feeding) were released into each bay and provisioned with 10% sugar solution. The cups were assayed for pyriproxyfen activity as described in Section 2.3 after the 5-day experiment. The photoperiod was controlled at 16 : 8 h light/dark.

2.9 Data analysis

Treatment mortality was corrected for control mortality using Abbott's formula.⁹ Percent pupal mortality was calculated for larval bioassay of pyriproxyfen, i.e. attachment and retention, serial transfer test, large room and greenhouse experiments. Comparison of pyriproxyfen attachment and retention was

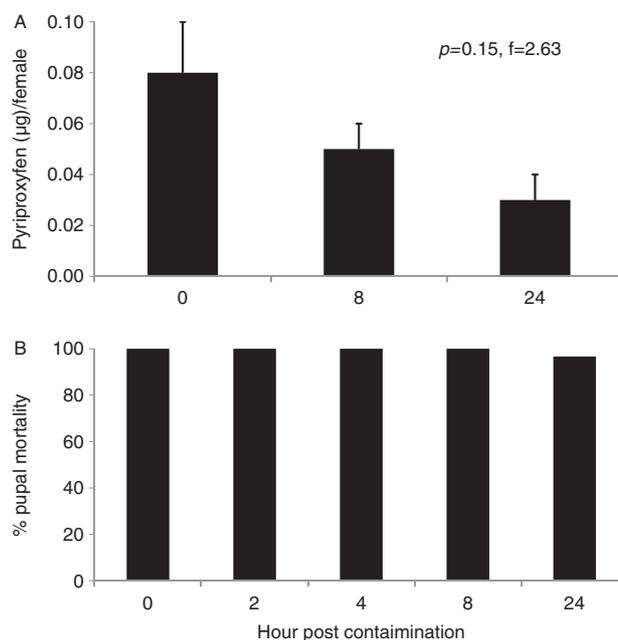


Figure 2. (A) Attachment and retention of pyriproxyfen (μg) from individual gravid *Aedes albopictus* females 0, 8 and 24 h after exiting from the autodissemination station. (B) Mean pupal mortality of *Aedes albopictus* from individual gravid females 0, 2, 4, 8 and 24 h post contamination.

determined with one-way analysis of variance (ANOVA) using Fisher's least significant difference (LSD) method at $P < 0.05$. All values are expressed as mean \pm standard error (SE).

3 RESULTS

3.1 Attraction

The dual-treatment autodissemination station was nearly three times more attractive to gravid females than our earlier prototype at $73.28 \pm 16.47\%$ versus $26.72 \pm 6.92\%$ entering in a choice experiment.

3.2 Attachment and retention

Residue analysis revealed that individual *Ae. albopictus* adult females were contaminated on average with $0.08 \pm 0.02\ \mu\text{g}$ of pyriproxyfen immediately after exiting from the station. The amount of attached active ingredient decreased with time, as mosquitoes contaminated with 0.05 and $0.03 \pm 0.01\ \mu\text{g}$ pyriproxyfen after 8 and 24 h (Fig 2A). Despite these pyriproxyfen losses of 37.5 and 62.5% respectively, bioassays showed females transferred sufficient toxicant to oviposition cups to induce 100 and $96.0 \pm 1.7\%$ emergence inhibition (Fig. 2B). Tarsal 'prints' of gravid mosquitoes on the cup surface as visualized with ultraviolet light demonstrated physical transfer of pyriproxyfen from the station to the female tarsal segments as well as residues on the inner surface of oviposition cups from visiting females in a room assay (Fig. 3).

3.3 Serial transfer

All sentinel containers exhibited pyriproxyfen transfer from the five gravid mosquitoes regardless of the mosquito: container ratio, as the lowest pupal mortality recorded from a single cup was 55% compared with 0% in the controls. That is, even when five contaminated females were presented with 15 cups, a challenge

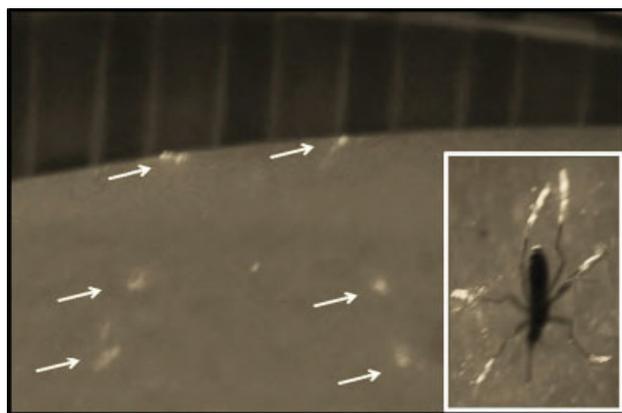


Figure 3. Six tarsal prints (arrows) as visualized under ultraviolet light show residues of pyriproxyfen transfer from a visiting female to the inner surface of a sentinel oviposition cup in a room assay. (Insert) Pyriproxyfen transfer from an autodissemination station to female *Aedes albopictus*.

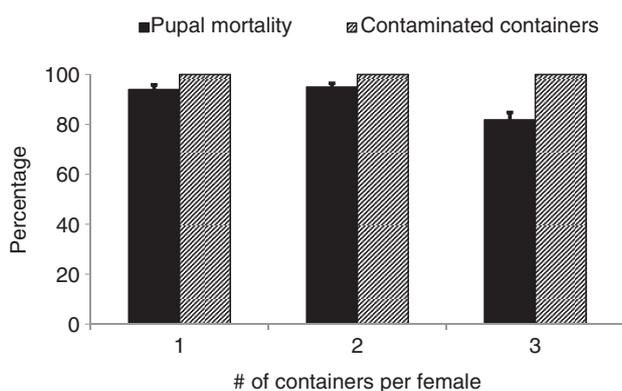


Figure 4. Evidence for the serial transfer of pyriproxyfen from contaminated *Aedes albopictus* gravid females to sentinel oviposition cups at female : cup ratios of 1 : 1, 1 : 2 or 1 : 3 in small-cage experiments.

of three cups per female, they were able to transfer highly toxic concentrations to every cup. Pupal mortality in each container was 94.5 ± 1.87 , 95.2 ± 1.49 and $81.8 \pm 2.97\%$ for the mosquito container ratio of 1 : 1, 1 : 2 and 1 : 3 respectively (Fig. 4).

3.4 Room efficacy

In small-room trials, the station delivered sufficient insect growth regulator to cause 100% pupal mortality in all 10 of the sentinel oviposition cups per station challenged. No control mortality was recorded.

3.5 Greenhouse efficacy

In the greenhouse trial, bioassays showed that mosquitoes had transferred pyriproxyfen from the autodissemination stations to 11 of the 12 (91.7%) sentinel oviposition cups. Pupal mortality in the cups ranged from 10 to 100% and no control mortality was recorded. Overall pupal mortality was $57.1 \pm 11.4\%$, with $50.8 \pm 14.4\%$ in the first bay and $64.3 \pm 18.6\%$ in the second bay (Fig. 5). Temperature ranged from 22 to 30 °C and relative humidity from 45 to 70% over the experiment.

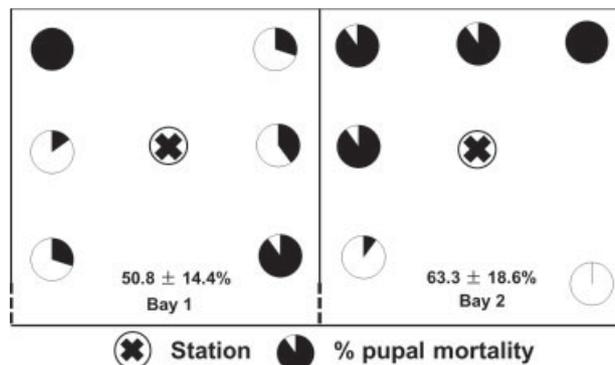


Figure 5. *Aedes albopictus* pupal mortality in a greenhouse efficacy experiment testing two autodissemination stations and twelve sentinel oviposition cups.

4 DISCUSSION

The dual-treatment station design maintained key features of the previous design including biodegradability, auto-refilling and low maintenance, while improving attraction, attachment, retention and efficacy.

The new design with a top opening for mosquito entry made this station more attractive to gravid mosquitoes than the previous design with side openings. Further improvement can be made by developing an oviposition attractant better than oak leaf infusion. Gravid females of *Ae. albopictus* show preferences to certain chemical cues associated with different plant leaf infusions and bacterial activities.^{10,11} The plant-derived oviposition pheromone luring gravid mosquitoes of *Culex* species has been described and synthesized.^{12,13} The chemical *n*-heneicosane extracted from conspecific larval conditioned water also showed activity for attracting gravid *Ae. aegypti* (L).¹⁴ Stations baited with an effective oviposition pheromone would compete with competing oviposition sites for visits from gravid females and thereby enhance contamination of larval habitats.

Enhanced pyriproxyfen attachment and retention were achieved by forcing gravid mosquitoes to walk across the dual-treatment system. The narrow gap at the station top precludes mosquitoes from exiting the station by flying. Here they are treated with the oil sticker followed by the pyriproxyfen powder formulation. This resolved a key limitation of the previous version in which only 10% oil could be applied to the powder formulation due to caking. By separating the two formulations so they are now presented to visiting mosquitoes sequentially, caking is no longer an issue and loading is enhanced. Moreover, even 24 h post contamination, sufficient pyriproxyfen persisted on individual mosquitoes to cause nearly complete *Ae. albopictus* pupal mortality.

Enhanced performance was demonstrated with the new station design in the small-room experiment. The new station design delivered complete pupal mortality in all 10 oviposition cups per station challenged. By contrast, 81% pupal mortality was obtained in tests with the earlier station and only four cups per station were challenged.⁴ Testing the same number of gravid female *Ae. aegypti* in a cage one-quarter smaller (31 vs. 8 m³) and with three oviposition cups, Devine *et al* reported $69 \pm 9.0\%$ pupal mortality.³ The maximum number of containers one station might contaminate with 50 gravid females was not tested in our study. Our serial transfer experiment, however, revealed that one gravid female was capable of contaminating at least three containers

with toxic levels of pyriproxyfen. The successful demonstration of multiple larval habitats becoming contaminated following a station visit by a single mosquito amplifies the potential impact of autodissemination as a container mosquito management tactic.

Our greenhouse trial showed considerably lower efficacy than the room assays. The greenhouse was more challenging as this environment was far more complex with plants covering more than one-half of the floor space. Sentinel oviposition cups showing low or no pupal mortality tended to be at the south side of the greenhouse exposed to direct sunlight, reduced humidity due to the heating system mounted nearby, and human disturbance from maintaining the plants. These cups were located in habitat less favorable to gravid mosquitoes because *Ae. albopictus* prefers shaded areas with high humidity for resting and oviposition.^{15–18} Patchy mosquito mortality was previously observed in autodissemination and was attributed to the frequency of visiting mosquitoes.⁸ Our results support this explanation by showing that greater mortality was associated with cups located at the shady area possessing higher humidity and therefore the most preferred environment for mosquitoes to visit, rest and oviposit. Thus autodissemination efficacy is dependent upon station placement in microenvironments favored by gravid mosquitoes.

Oviposition site selection is a challenging undertaking for females that lack parental care. The greenhouse offered variation for *Ae. albopictus* in site selection with temperature, moisture and light gradients. This variation is reflected in the pupal mortality recorded from each sentinel oviposition cup. That is, cups sited in greenhouse areas with unfavorable oviposition microenvironments likely showed low pupal mortality due to a low number of visits and therefore limited opportunities for pyriproxyfen transfer. We submit that the moderate mortality recorded from the greenhouse trial underestimates this trial's true efficacy. Females detect and respond to variation in oviposition site quality, which determines transfer probability. Cups receiving frequent visits, and therefore by definition sited in the most favorable microenvironments for *Ae. albopictus* visits would contribute the most biting mosquitoes to the next generation. Cups sited in less favorable microenvironments receive fewer visits and therefore contribute minimally to the overall population. In short, autodissemination impacts the more preferred oviposition sites due to a greater frequency of visits.

The most encouraging result from the greenhouse study was that nearly all of the cups had been contaminated. The most disappointing result was that contamination resulted in incomplete pupal mortality in most cups. We determined that, under laboratory conditions, females exiting the station are able to transfer sufficient insect growth regulator to kill 100% of the mosquitoes tested even a full day subsequently. Under greenhouse conditions, where mosquitoes have more opportunities for flight, and come into contact with plants and attendant moisture from watering or dew, active ingredient may be reduced. We suggest that further improvements in formulation are required to increase insecticide loading and retention. An oil sticker formulation containing active ingredient would substantially increase overall pyriproxyfen loading and also would be more difficult to remove via grooming behaviors. We did not add pyriproxyfen to the oil because crystallization was frequently observed; this is an obstacle we are now targeting. Further, the powder we tested contained 35% pyriproxyfen so there is an opportunity to increase active ingredient concentration, as well to develop particle sizes that are optimal for mosquito loading and retention. Smaller

particles are picked up by insects more readily and are more difficult to remove by mechanical means,¹⁹ yet release effectively in water due to their high surface area to volume ratio. Further, the commercial pyriproxyfen formulation we tested was a hydroscopic wetttable powder, and therefore was susceptible to caking at the high humidity sometimes experienced outside the laboratory. Developing custom formulations possessing enhanced properties, particularly with increased pyriproxyfen, will have to consider whether adult longevity²⁰ is negatively impacted, which would reduce autodissemination opportunities.

Autodissemination against insect pests other than mosquitoes, such as house flies, has shown early promise.²¹ Target insect behavior and breeding habitat will vary according to species, requiring station modifications, attractants and formulations. For example, the exit gap in the current station design which forces the small *Ae. albopictus* adults to make effective contact with the insecticide formulations, would need adjustment according to the size of the selected insect.

Our dual-treatment autodissemination station provides increased female attraction, enhanced attachment and retention. Although the device has delivered encouraging results in room and greenhouse tests further improvements are needed, particularly in formulation technology, before autodissemination could be available as a new tool to supplement conventional mosquito control. This technology would exploit the largely untapped potential of an insecticide extensively used in agriculture but which has seen little use against mosquitoes. It offers special promise to advance pest management practices in sensitive urban areas afflicted by container mosquitoes.

ACKNOWLEDGEMENTS

We thank L. McCuiston for laboratory assistance. This work was supported in part by a grant from the Deployed War-Fighter Protection (DWFP) Research Program, funded by the US Department of Defense through the Armed Forces Pest Management Board (AFPMB).

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