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The efficacy of pyriproxyfen-treated resting boxes on the reproductivity of *Aedes aegypti* (Diptera: Culicidae) in the laboratory

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Abstract. Pyrethroid resistance is a problem for controlling the dengue vector *Aedes aegypti* worldwide. One strategy to cope with resistance is to use another insecticide with a different mode of action. Pyriproxyfen (PPF), an insect growth regulator, is normally used at very low concentrations for controlling the immature stages of mosquitoes. At high concentrations, it has a reproductive effect on exposed female mosquitoes. In this study, we demonstrated by using CDC bottle and cone bioassays that tarsal contact with 333 mg AI PPF/m^2 for 1 min was sufficient to cause over 95% emergence inhibition (EI) in the progeny of exposed *Ae. aegypti* females. Exposure for 5 min completely inhibited fecundity. As *Ae. aegypti* adult mosquitoes are generally drawn towards darker areas, we evaluated the efficacy of a resting box (35x35x55 cm) treated with PPF aimed at disrupting reproductivity of free-flying mosquitoes in the laboratory. We found that the resting box led to 94% EI of exposed females, either before or after blood feeding. The resting box was also attractive for male mosquitoes. Exposed males could transfer sufficient PPF to virgin females via copulation to cause about 90% EI. Additionally, PPF-exposed gravid females from the treated resting box were able to disseminate sufficient PPF to small larvae-containing cups to reduce adult emergence by 50%. Based on 10 min exposure, the residual effect of PPF-treated resting boxes (over 80% EI) was observed over a 4 month-period. PPF-treated resting boxes may potentially be useful in dengue vector control programs, however further evaluation under natural field conditions are needed.

INTRODUCTION

Aedes aegypti is the main vector of viral diseases including dengue fever, dengue hemorrhagic fever, Zika and chikungunya which are important public health problems in many tropical and subtropical countries. The World Health Organization (WHO) estimates that 50–100 million dengue infections occur every year and almost half the world's population is living in countries where dengue is endemic (WHO, 2018). In Thailand, the Ministry of Public Health reported 47,847 cases in 2017 (73.13 per 100,000), including 58 deaths (Ministry of Public Health, 2018). Since there are no effective vaccines or specific treatments for dengue, control of disease transmission is primarily based on vector control, including insecticide applications and management of mosquito larval habitats (WHO, 2012).

Pyrethroids have been widely used for controlling *Ae. aegypti* populations due to their low toxicity to humans and mammals

but high toxicity to insects (WHO, 2009). However, *Ae. aegypti* populations resistant to pyrethroids and other classes of insecticides have been found throughout Thailand and other countries (Somboon *et al.*, 2003; Yanola *et al.*, 2011; WHO; 2012; Stenhouse *et al.*, 2013). The main mechanisms of pyrethroid resistance in *Ae. aegypti* are commonly known to be metabolic detoxification, through enzymes such as the cytochrome P450s, and knockdown resistance (Smith *et al.*, 2016). Knockdown resistance (*kdr*), which is an important mechanism of cross resistance to pyrethroids and DDT, is associated with single or multiple mutations in the genes encoding voltage-gated sodium channel proteins (Hemingway *et al.*, 2004). In *Ae. aegypti*, a valine to glycine/isoleucine transversion at position 1016 within domain II of the VGSC (V1016G/V1016I), a serine-to-proline mutation at position 989 in domain II (S989P), and a phenylalanine to cysteine substitution at position 1534 within domain II (F1534C) are associated with varying degrees of resistance to pyrethroids in Asia and/or Latin America (Brengues *et al.*, 2003; Harris *et al.*, 2010; Yanola *et al.*, 2011; Stenhouse *et al.*, 2013; Plernsub *et al.*, 2016a; Amelia-Yap *et al.*, 2018). A dramatic increase in resistance relative to increased activity of detoxification enzymes and increased frequency of *kdr* alleles could hamper the efficacy of vector control using pyrethroids (Marcombe *et al.*, 2009; Vera-Maloof *et al.*, 2015; Deming *et al.*, 2016; Plernsub *et al.*, 2016b; Sayano *et al.*, 2016). Therefore, the application of alternative chemicals that are not overcome by these mechanisms is one strategy for managing resistant vector populations (WHO, 2012).

Pyriproxyfen (PPF), an insect growth regulator analog, is one of the most promising products currently available, PPF can affect an insect by direct contact or ingestion. It has low mammalian toxicity and environmental impact and it is recommended by WHO for vector control (WHO, 2001). For mosquito control, PPF is normally applied at low concentrations (about 20-50 ppb) in a variety of aquatic habitat types including those utilized by *Ae. aegypti* and *Ae. albopictus* (Chavasse *et al.*, 1995; Yapabandara *et al.*, 2001; Sihuincha *et al.*, 2005; Vythilingam *et al.*, 2005). The morphogenetic effect of PPF is primarily seen during larval-pupal transformation. Therefore, death occurs at the pupal stage and adult mosquitoes fail to emerge. Moreover, at high dosages, tarsal contact with a PPF-treated substrate affects fecundity and leads to sterility of adult female mosquitoes, including *Ae. aegypti*, *Ae. albopictus*, as well as various *Anopheles* and *Culex* species (Itoh *et al.*, 1994; Ohashi *et al.*, 2012; Harris *et al.*, 2013; Mbare *et al.*, 2013; Koama *et al.*, 2015). Several researchers have developed PPF-treated 'dissemination stations' to contaminate gravid female mosquitoes and subsequently transfer PPF to larvae in water-holding containers at concentrations that affect adult emergence, in a process known as 'autodissemination' (Itoh *et al.*, 1994; Devine *et al.*, 2009; Caputo *et al.*, 2012; Gaugler *et al.*, 2011; Suman *et al.*, 2014; Abad-Franch *et al.*, 2015; Unlu *et al.*, 2017). Venereal transfer of PPF from contaminated males to virgin females has also been observed (Gaugler *et al.*, 2011; Mains *et al.*, 2015). However, most PPF-treated devices that have been developed thus far are modified from ovitraps, and are therefore rarely attractive to newly blood-fed or non-blood-fed female and male mosquitoes.

Artificial resting boxes (usually black or red in color) of various sizes and shapes have been used to collect mosquitoes that naturally prefer to rest in shade or protected locations (Service, 1993). Edman *et al.* (1998) revealed that resting boxes were very attractive to both sexes of *Ae. aegypti* and the devices have proved to be useful for quickly sampling *Ae. aegypti* populations. They also speculated that treatment of resting boxes with a non-repellent pyrethroid deployed in a community would prevent or suppress transmission of dengue. Since pyrethroid resistance is already widespread among *Ae. aegypti* populations, it is worthwhile to evaluate PPF which has different mode of action and lacks repellency (Sihuincha *et al.*, 2005). In the

current study, we developed a resting box sprayed with PPF and demonstrated in the laboratory that this device led to significant reductions in fecundity among free-flying *Ae. aegypti* females. We also demonstrated that the treated resting box can act as a dissemination station. Additionally, it is also attractive to *Ae. aegypti* males that can subsequently transfer PPF to females via copulation and thus disrupt female reproductivity. The results from this study provide important information on the use of PPF, which could be implemented in dengue control programs in the future.

MATERIALS AND METHODS

Mosquito strains

Subcolonies of three laboratory strains of *Ae. aegypti* (PMD, PMD-R and UPK-R) were used in this study. The PMD and PMD-R originated from Ban Pang Mai Daeng, Mae Tang District, Chiang Mai Province, Thailand (Prapanthadara *et al.*, 2002). The PMD strain is pyrethroid susceptible but resistant to DDT. No *kdr* alleles (G1016, P989, C1534) have been observed in this strain. The PMD-R strain, harboring S989+V1016+C1534 homozygous alleles, is resistant to DDT and permethrin, but susceptible to deltamethrin. The UPK-R *Ae. aegypti* strain was established by deltamethrin selection of wild *Ae. aegypti* collected in Chiang Mai city in 2006 (Plernsub *et al.*, 2016a), and has been maintained in the laboratory since then. This strain harbors P989+G1016+F1534 homozygous alleles and is resistant to DDT, permethrin and deltamethrin. It is slightly resistant to malathion (Choovattanapakorn *et al.*, 2017). Pyrethroid resistance in the PMD-R and UPK-R strains is conferred mainly by *kdr*, whereas mixed function oxidases play about a 30% role (Somwang *et al.*, 2011; Choovattanapakorn *et al.*, 2017).

Mosquitoes were maintained in an insectary at 25±2°C, 70±10% relative humidity and a photo-period of 12:12 h (light:dark). The adults were maintained in 30x30x30 cm^3 cages provided with a 10% sucrose solution and 10% v/v multivitamin syrup soaked onto cotton wool which was changed twice a week. A rabbit biscuit (Top Rabbit™, Thailand) was used as larval food throughout the study. The rearing of mosquitoes followed our routine procedures as previously described (Stenhouse *et al.*, 2013). Bloodmeals were provided using an artificial membrane feeding method with defibrinated cow blood (Finlayson *et al.*, 2015).

Effective dose of PPF on material surfaces

CDC bottle bioassay

The CDC bottle bioassay (CDC, 2017) was used, with minor modifications (Sihuincha *et al.*, 2005), to screen for an effective dose of PPF that could reduce >80% reproductivity of pyrethroid susceptible (PMD) and resistant (PMD-R and UPK-R) *Ae. aegypti* females after exposures of no more than 10 min. Our preliminary study revealed that glass bottles (250 ml Duran with an inner surface of approximately 300 cm^2) coated with 1 mg AI and 5 mg AI PPF/bottle (about 33 and 166 mg AI/m^2, respectively) did not give satisfactory results. Therefore, in the current study, we used 10 mg AI PPF/bottle (about 333 mg AI/m^2), which is close to the dose used for coating nettings (about 350 mg AI/m^2) (Ohba *et al.*, 2013; Ngufor *et al.*, 2014). The PPF stock solution was prepared by adding 630 mg of technical grade PPF (Sumitomo, Japan) to 12.5 ml of acetone (Merck Ltd., Thailand). For coating, 200 µl of PPF stock solution was poured into each bottle. An additional 1 ml of acetone was added to the bottle to facilitate uniform coating of the bottles. The bottles were capped and rotated in all planes to ensure they were evenly coated. Caps were then removed to allow the acetone to fully evaporate, while continuing to rotate the bottles. A control bottle was coated with acetone only. The coated bottles were wrapped with aluminum foil and kept in a dark place until use.

For bioassay, groups of ten mosquitoes (5-7 days old) consisting of blood-fed females (24 h post feeding) and non-blood-fed females were exposed to the treated

bottles for 1, 3, 5 and 10 min. Three replicates were performed. Control mosquitoes were exposed to acetone-treated bottles for 10 min. The blood-fed females were transferred individually to 16 oz cups lined with filter paper, hereafter called ovicups. They were reared for four days for egg maturation. Thereafter, to each ovicup, 70 ml of hay infused water (10 g of dry grass submerged in 1 liter of water for one week, and diluted with 10 parts of water before use) were added since this medium encourages oviposition of *Ae. aegypti* (Reiter *et al.*, 1991). The non-blood-fed group were transferred to a cage, and provided with a blood meal 24 h after exposure. They were then removed and transferred to ovicups and held for four days for egg maturation. After that the hay infused water (70 ml) was added and held for 2 days for oviposition. The filter papers with eggs were air-dried for 3-4 days and returned to the same ovicups for egg hatching. The hatched larvae were reared in these cups until the emergence of adults. The following parameters including oviposition rate (number of oviposited female per total females), number of eggs laid, hatching rate, pupation rate and adult emergence rate were determined in this and other experiments. The percentage of emergence inhibition (EI) was calculated according to the following formula: EI (%) = 100 – (number of emergence from treated mosquitoes/number of emergence from control) x 100.

Cone bioassay on treated material

This experiment aimed at evaluating the efficacy of PPF treated surfaces to reduce the reproductivity of *Ae. aegypti* females (PMD strain) before and after a blood meal, using a simple spraying method. Cone bioassays were performed according to WHO protocol (WHO, 2006; 2013). Black polypropylene sheets (35×25 cm or 875 cm^2), after rubbing with sandpaper, were sprayed with the PPF technical grade dissolved in acetone at a dosage of 333 mg AI/m^2 using a foggy sprayer (1 liter) (Moong Pattana PCL., Thailand). An amount of 166 ml acetone is sufficient to cover one m^2 area. The control polypropylene sheet was sprayed with acetone only. The sprayed sheets were allowed to dry and stored in a dark place until use.

The effect of PPF exposure on *Ae. aegypti* females was evaluated with six different blood feeding conditions, i.e. 1) immediately after blood meal, 2) 24 h after blood meal, 3) immediately before blood meal, 4) 24 h before blood meal, 5) four days after blood meal, and 6) four days before blood meal. For the bioassays, the plastic cones were attached to the black polypropylene sheets which were placed vertically. Ten females with or without blood feeding as above were released into each of the plastic cones and left for 10 min. For blood-fed groups, exposure was performed immediately (1), 24 h (2), and four days (5) after a blood meal. For non-blood-fed groups, exposure was performed immediately (3), 24 h (4) and four days (6) before a blood meal. Control groups with similar blood feeding conditions were exposed to acetone-treated polypropylene sheets. All groups were allowed to lay eggs in the ovicups as mentioned earlier. The experiment was performed in triplicate (total 30 females each group).

PPF-treated resting box

The objective of this experiment was to evaluate the efficacy of PPF-treated resting box to reduce the fecundity of free flying *Ae. aegypti* females (PMD strain) before and after a blood meal. Resting boxes were constructed from brown cardboard cartons (35×35×55 cm) (Figure 1). The inner surface was lined with black polypropylene sheets. Before being assembled, the inner side of the sheets were rubbed with sandpaper and sprayed with PPF technical grade solution (333 mg AI/m^2) using the foggy sprayer as above. For the untreated box, the sheets were sprayed with acetone only.

The experiment was carried out in a room (20 m^2 and 3 m high) in which two nets (1.3×1.86×1.46 m) were set. The PPF-treated box was placed in one net and the acetone-treated box, as a control, in the

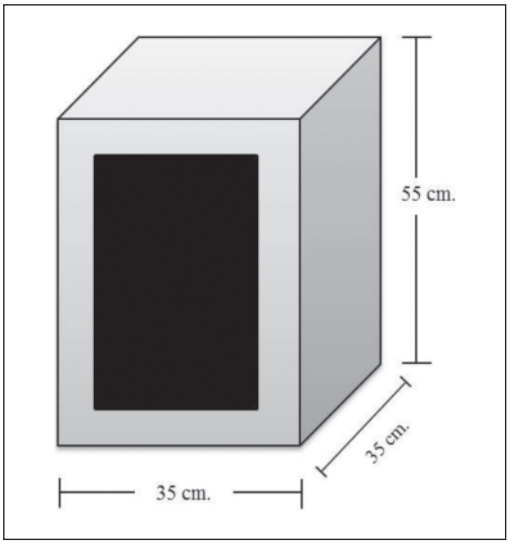

Figure 1. PPF-treated resting box.

other. The room was maintained at ambient temperature (about 27-32°C) with a 12 h photo period. One group of 30 newly blood-fed females were released into each of the nets. The mosquitoes were provided with a 10% sucrose and 10% v/v multivitamin syrup soaked onto cotton wool. After 24 h, all mosquitoes found inside and outside the resting box were removed and transferred individually to ovicups containing hay infused water and held until oviposition. For the non-blood-fed group, the mosquitoes were released into the net enclosing a treated resting box and held for 24 h. Thereafter, they were removed and provided with a blood meal and transferred individually to ovicups as above. Special care was taken during the experiment to prevent contamination by using dedicated equipment, e.g. aspirators, oviposition cups, trays, pipettes. This experiment was performed in triplicate.

Males as vehicles to transfer PPF to *Ae. aegypti* females

An experiment was devised to determine if the PPF-treated resting box is attractive to free-flying *Ae. aegypti* males by which PPF-exposed males would be able to transfer an effective dose to females via copulation. The experiment was conducted in the net enclosing a PPF-treated resting box as above. At 12:00 a.m., 30 *Ae. aegypti* males (5-7 days old, PMD strain) were released into the net and provided with a 10% sucrose and 10% v/v multivitamin syrup soaked onto cotton wool. After 24 h, the resting box was removed while the males were retained inside the net. Since it is not possible to know how many males were actually exposed to PPF, we determined the sterility and fecundity of females after allowing to mate with the males compared with the control.

Thirty non-blood-fed, virgin females (5-7 days old, separated at the pupal stage) were subsequently released into the cage and allowed to mate for 2 days. Thereafter, the females were removed, provided with a blood meal and transferred individually into ovicups. Four days post blood feeding, hay infused water was added and females were allowed to lay eggs for two days. The filter papers with eggs were removed, air-dried for 4-5 days and then submerged into the hay infused water in the same cup. Three replicates were conducted for treatment and control groups.

Autodissemination of PPF exposed females to clean breeding site

This experiment aimed at determining the ability of PPF-exposed females to transfer the chemical into clean breeding sites and affect the development of existing *Ae. aegypti* larvae and pupae. Twenty newly blood-fed females were released in the net enclosing a PPF-treated resting box as above. Four days later, five ovicups, each containing 100 ml of hay infused water and 10 third instar larvae and 5 pupae, were introduced into the net, one at each corner and one in the middle, and provided with the larval food. The ovicups were left for 3 days and then removed from the net for further rearing in the insectary. The development of larvae, pupation and adult emergence in the cups with and without laid eggs were recorded. The results were compared against a control group exposed to an acetone-treated resting box. Three replicates were conducted.

Residual effect of PPF-treated resting box

This study was meant to determine how long the PPF-treated box would continue to significantly affect the reproductivity of *Ae. aegypti* females. For bioassay, three plastic cones were randomly attached to the inner surface of the PPF-treated resting box. Ten newly blood-fed females were released into each of the plastic cones and held for 10 min. The control mosquitoes were exposed to an acetone-treated box. After exposure, the mosquitoes were removed and allowed to lay eggs individually in ovicups. The bioassays were performed every month for 6 months after treatment of resting box.

Data analysis

Data were analyzed by using SPSS version 22.0. Pearson's chi-squared test or Fisher's exact test was used for comparing oviposition rates. Student's t-test and ANOVA were performed for comparing mean values of hatching larvae, pupation and adult emergence. Data were log transformed (n+1) to normalize the distribution and equalize the standard deviations before statistical analysis.

RESULTS

Effective dose of PPF on material surfaces

The effects of PPF exposure (10 mg AI/bottle), 24 h before and after a blood meal, on the reproductivity of the pyrethroid susceptible strain (PMD) and resistant strains (PMD-R and UPK-R), using CDC bottle bioassays, are presented in Table 1. Mortalities were very low or non-existent in the various treatment and control groups. Compared with the control groups, the oviposition rates were not significantly different in both of the resistant strains, regardless of blood-feeding. However, oviposition was statistically different in the susceptible strain only at 10 min exposure, but not at 1, 3 and 5 min exposure. Significant reductions in mean numbers of eggs laid were observed in all strains, however, the effect was more pronounced in the susceptible strain. There was about 85% reduction after 1 min exposure in the susceptible strain compared with 10-25% reductions in the resistant strains. Further effects were observed in egg hatchability and adult emergence: in all strains a slightly greater effect was observed when exposure took place after a blood meal as this condition required shorter exposure time to completely inhibit egg hatching. Exposure for only 1 min resulted in over 95% emergence inhibition (EI, adult emerging per number of eggs laid) in all strains. Exposure for 5 min completely inhibited the hatchability of eggs in all strains.

Cone bioassay on treated material

The results of cone bioassays (333 mg AI/m^2 and 10 min exposure) with the pyrethroid susceptible strain (PMD) for different blood feeding conditions are presented in Table 2. Although the oviposition rates were not significantly different in all conditions, the mean numbers of laid eggs and hatching rates were all significantly reduced when compared against the control groups. Complete inhibition of adult emergence (100% EI) was observed when the females were exposed to PPF within 24 h before or after blood meal, mostly due to low numbers of egg laying and failure of egg hatching (treatments 1-4). Dissection of the ovaries of these females (n = 10) revealed that the ovarian follicles were in immature stages with some retaining eggs (Figure 2). Exposure 4 days after blood feeding (treatment 5) and 4 days before blood feeding (treatment 6) resulted in about 71 and 33% EI, respectively.

PPF-treated resting box

The effects of the PPF-treated resting box (333 mg AI/m^2) on free-flying mosquitoes are presented in Table 3. All females blood-fed before or after releasing into the net laid eggs, but the mean numbers of eggs in both groups were reduced by 65% compared to the control groups. The hatching rates of deposited eggs were reduced by 85% and 89%, respectively. Consequently, the adult emergent rates were 5.8 and 2.3% (94.2 and 97.7% EI),

Table 1. Reproductivity of blood-fed and non-blood-fed *Ae. aegypti* females after exposure to PPF 10 mg AI/bottle under different blood feeding conditions. Different letters in the same column indicate significantly differences ($P < 0.05$)

Mosquito strains	PPF exposure	Exposure time (min)	Oviposited females/ total (%)	Number of eggs (mean±sd)	Number of hatching larvae (mean±sd)	Number of pupae (mean±sd)	Number of emerged adults (mean±sd)
PMD	After blood meal	Control	30/30 (100.0)^{a}	46.9±8.1^{a}	46.9±8.1^{a}	46.8±8.1^{a}	46.8±8.1^{a}
		1	26/30 (86.7)^{a}	6.5±5.2^{b}	2.6±2.8^{b}	1.1±1.2^{b}	0.7±1.3^{b}
		3	27/30 (90.0)^{a}	6.9±5.1^{b}	0^{b}	–	–
		5	25/30 (83.3)^{a}	4.7±4.0^{b}	0^{b}	–	–
		10	20/30 (66.7)^{b}	3.8±4.2^{b}	0^{b}	–	–
	Before blood meal	Control	30/30 (100.0)^{a}	50.6±9.9^{a}	50.5±9.9^{a}	50.5±9.9^{a}	50.4±9.9^{a}
		1	24/30 (80.0)^{a}	6.9±6.5^{b}	3.6±2.7^{b}	1.7±1.8^{b}	0.6±1.1^{b}
		3	22/30 (73.3)^{a}	6.7±6.0^{b}	2.3±3.0^{bc}	1.0±2.1^{b}	0.5±0.7^{b}
		5	26/30 (86.7)^{a}	5.5±4.5^{bc}	0^{c}	–	–
		10	13/30 (43.3)^{b}	1.3±2.3^{c}	0^{c}	–	–
PMD-R	After blood meal	Control	30/30 (100.0)^{a}	38.6±7.6^{a}	38.5±8.5^{a}	38.4±8.5^{a}	38.4±8.6^{a}
		1	30/30 (100.0)^{a}	33.0±4.6^{b}	10.7±7.4^{b}	3.6±2.6^{b}	1.3±1.9^{b}
		3	30/30 (100.0)^{a}	26.9±6.5^{c}	0^{c}	–	–
		5	30/30 (100.0)^{a}	22.0±6.6^{d}	0^{c}	–	–
		10	30/30 (100.0)^{a}	19.5±4.6^{d}	0^{c}	–	–
	Before blood meal	Control	30/30 (100.0)^{a}	38.8±8.1^{a}	38.7±8.2^{a}	38.7±8.3^{a}	38.7±8.3^{a}
		1	30/30 (100.0)^{a}	34.9±7.7^{ab}	11.5±7.5^{b}	3.9±2.3^{b}	1.2±2.1^{b}
		3	30/30 (100.0)^{a}	32.8±6.8^{b}	0^{c}	–	–
		5	30/30 (100.0)^{a}	25.6±7.9^{c}	0^{c}	–	–
		10	30/30 (100.0)^{a}	18.8±5.5^{d}	0^{c}	–	–
UPK-R	After blood meal	Control	30/30 (100.0)^{a}	41.6±10.3^{a}	41.5±10.2^{a}	41.4±10.3^{a}	41.4±10.3^{a}
		1	30/30 (100.0)^{a}	31.3±6.7^{b}	9.3±5.8^{b}	5.7±3.5^{b}	2.1±2.9^{b}
		3	30/30 (100.0)^{a}	32.1±5.8^{b}	0^{c}	–	–
		5	30/30 (100.0)^{a}	26.1±3.7^{c}	0^{c}	–	–
		10	30/30 (100.0)^{a}	24.7±3.8^{c}	0^{c}	–	–
	Before blood meal	Control	30/30 (100.0)^{a}	44.0±9.1^{a}	43.9±9.2^{a}	43.8±9.2^{a}	43.7±9.3^{a}
		1	30/30 (100.0)^{a}	34.6±4.6^{b}	11.8±6.0^{b}	3.9±2.7^{b}	1.4±2.1^{b}
		3	30/30 (100.0)^{a}	33.9±3.7^{b}	6.3±4.5^{c}	2.4±1.9^{b}	2.1±1.4^{b}
		5	30/30 (100.0)^{a}	23.6±3.7^{c}	0^{d}	–	–
		10	30/30 (100.0)^{a}	21.4±2.8^{c}	0^{d}	–	–

respectively. Dissection of unhatched eggs from the blood-fed groups (n = 707) revealed that 94% were embryonated, whereas those from the non-blood-fed groups (blood-fed after exposure) (n = 688) showed 47% embryonation.

Males as vehicles to transfer PPF to *Ae. aegypti* females

Table 4 presents the reproductivity of females after being allowed to copulate with the male mosquitoes previously confined in the net with a PPF treated resting box (333 mg AI/m^2). All females in the treatment and control groups laid eggs, but each female in the former group laid significantly lower number of eggs, indicating that they were all contacted with PPF-exposed males. Significant reductions in mean numbers of eggs laid, hatched larvae, pupae and emergent adults were observed in the treated group. The adult emergence rate was about 11% (89% EI). Examination of the spermathecae of all females revealed the presence of spermatozoa, indicating that the females were copulated.

Table 2. Reproductivity of *Ae. aegypti* (PMD) females with different blood feeding conditions and exposure of PPF (333 mg AI/m^2 for 10 min) using cone bioassays. In each treatment, different letters in the same columns indicate significant differences ($P < 0.05$)

Treatment	PPF exposure	Group	Oviposited female/total (%)	Number of eggs (mean±sd)	Number of hatched larvae (mean±sd)	Number of pupae (mean±sd)	Number of emerged adults (mean±sd)
1	Immediately after blood meal	control	30/30 (100.0)[a]	105.3±14.9[a]	105.3±14.9[a]	105.1±14.8	105.0±14.8
		treatment	27/30 (90.0)[a]	17.6±7.6[b]	0[b]	–	–
2	24 hr after blood meal	control	30/30 (100.0)[a]	71.0±11.1[a]	70.9± 11.2[a]	70.8±11.3	70.7±11.3
		treatment	26/30 (86.7)[a]	14.6±7.6[b]	0[b]	–	–
3	Immediately before blood meal	control	30/30 (100.0)[a]	100.1±9.0[a]	99.9±8.9[a]	99.9±8.9[a]	99.8±8.9[a]
		treatment	24/30 (80.0)[b]	15.6±10.7[b]	2.6±5.3[b]	1.0±2.0[b]	0[b]
4	24 hr before blood meal	control	30/30 (100.0)[a]	64.9±6.3[a]	64.7±6.4[a]	64.6±6.5	64.6±6.5
		treatment	30/30 (100.0)[a]	25.5±7.0[b]	0[b]	–	–
5	4 days after blood meal	control	30/30 (100.0)[a]	94.0±12.7[a]	99.9± 8.9[a]	99.9±8.9[a]	91.9±14.5[a]
		treatment	30/30 (100.0)[a]	30.8±7.9[b]	29 ± 8.7[b]	27.2±9.0[b]	27.0±8.9[b]
6	4 days before blood meal	control	30/30 (100.0)[a]	98.9±8.5[a]	98.8±8.7[a]	98.7±8.8[a]	98.6±9.0[a]
		treatment	30/30 (100.0)[a]	67.3±5.9[b]	67±5.8[b]	66.7±5.9[b]	66.4±6.1[b]

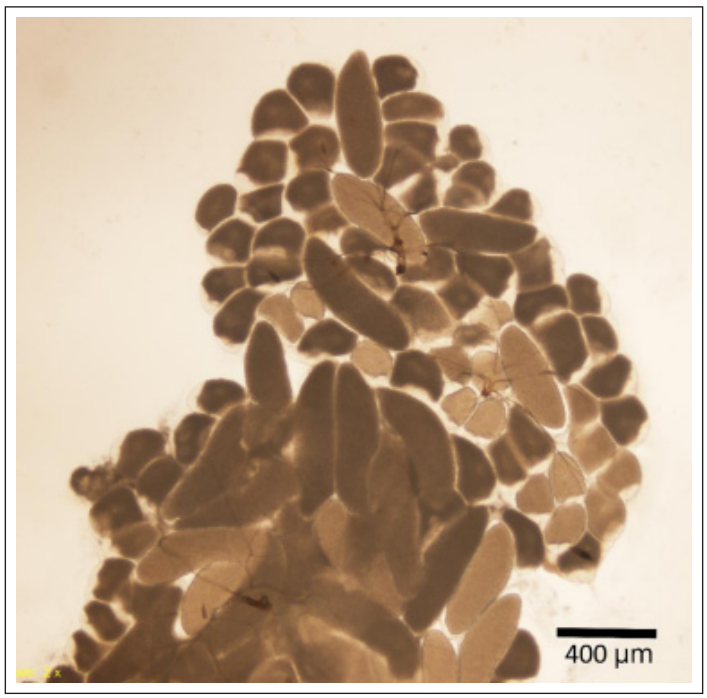

Figure 2. Dissected ovaries of blood-fed *Ae. aegypti*, seven days after PPF exposure, showing immaturity of egg follicles and retained eggs.

Table 3. Reproductivity of *Ae. aegypti* (PMD), blood-fed and non-blood-fed groups, released into net enclosure with PPF-treated resting box

	Blood-fed females			Non blood-fed females		
	Control	Treatment	*P*	Control	Treatment	*P*
Number of oviposited females/total (%)	30/30 (100.0)	30/30 (100.0)	–	30/30 (100.0)	30/30 (100.0)	–
Number of egg laid (mean±sd)	52.4±4.4	18.3±6.5	< 0.001	44.7±4.1	15.8±7.6	< 0.001
Number of hatched larvae (mean±sd)	50.8±4.5	7.3±4.6	< 0.001	44.7±4.0	5.0±3.8	< 0.001
Number of pupae (mean±sd)	50.3±4.5	4.0±2.3	< 0.001	44.4±4.0	1.9±1.8	< 0.001
Number of emerged adults (mean±sd)	50.0±4.5	2.9±1.9	< 0.001	44.2±4.0	1.0±1.0	< 0.001

Table 4. Reproductivity of *Ae. aegypti* females (PMD) after copulation with PPF contaminated males

	Control	Treatment	*P*
Number of oviposited females/total (%)	30/30 (100)	30/30 (100)	–
Number of eggs laid (mean±sd)	47.0±4.2	25.8±5.8	< 0.001
Number of hatched larvae (mean±sd)	46.7±4.2	16.6±5.5	< 0.001
Number of pupae (mean±sd)	46.4±4.3	10.0±4.4	< 0.001
Number of emerged adults (mean±sd)	45.5±3.5	5.0±2.8	< 0.001

Table 5. Mortality of *Ae. aegypti* larvae and pupae in the ovicups after the oviposition of PPF-exposed *Ae. aegypti* females. Three experiments were performed. In each experiment, different letters in the same rows indicate significant differences ($P < 0.05$)

	Experiment 1		Experiment 2		Experiment 3	
	Control	Treatment	Control	Treatment	Control	Treatment
Number of eggs laid[1] (mean±sd)	240.8±8.3[a]	230.0±8.4[a]	244.4±10.7[a]	216.6±10.8[b]	246.4±9.9[a]	233.6±7.7[a]
Dead larvae/total (%)[2]	0/50 (0)[a]	22/50 (44)[b]	0/50 (0)[a]	24/50 (48)[b]	0/50 (0)[a]	25/50 (50)[b]
Adult emergence (%)	50/50 (100)[a]	28/50 (56)[b]	50/50 (100)[a]	26/50 (52)[b]	50/50 (100)[a]	25/50 (50)[b]
Dead pupae/total (%)[2]	0/25 (0)[a]	0/25 (0)[a]	0/25 (0)[a]	0/25 (0)[a]	0/25 (0)[a]	0/25 (0)[a]
Adult emergence (%)	25/25 (100)[a]	25/25 (100)[a]	25/25 (100)[a]	25/25 (100)[a]	25/25 (100)[a]	25/25 (100)[a]

[1]Eggs in ovicups laid by blood-fed *Ae. aegypti* females.
[2]Larvae and pupae that were introduced into the ovicups.

Table 6. Residual effects of PPF-treated resting box (333 mg AI/m^2 and 10 min exposure) of blood-fed *Ae. aegypti* females (n = 30 for each group) over 6-month period

Month	Group	Eggs		% Hatching	% Pupation	% Adult emergence	% Emergence inhibition
		Total	Average				
0	Control	2130	71.00	99.80	99.80	99.60	
	Treatment	438	14.00	0.00	0.00	0.00	100
1	Control	1397	46.60	99.36	98.85	97.28	
	Treatment	1151	38.40	41.70	17.98	2.00	97.94
2	Control	1379	45.97	99.64	98.98	98.69	
	Treatment	935	31.17	47.70	18.93	4.28	95.66
3	Control	1352	45.07	99.48	99.11	98.67	
	Treatment	815	27.17	46.50	26.13	7.48	92.42
4	Control	1768	58.93	99.55	99.26	98.64	
	Treatment	998	33.27	52.20	39.98	19.74	79.99
5	Control	1770	59.00	99.32	98.36	97.51	
	Treatment	1514	50.47	73.45	52.91	31.70	67.49
6	Control	1518	50.60	99.47	98.68	98.29	
	Treatment	1289	42.97	88.91	68.66	55.55	43.83

Autodissemination of PPF exposed females to clean breeding site

All of the ovicups, containing larvae and pupae, placed inside the net were visited by the gravid females as indicated by the eggs deposited. In the three experiments, the mean numbers of eggs found in the ovicups were not significantly different, except in experiment 2 in which the number of eggs found was slightly lower than that observed for the control group (P = 0.03) (Table 5). About half of the introduced larvae in the ovicups died during pupation, but those that successfully developed to the pupal stage emerged to adults. The adult emergence rates ranged from 50-56% (44-50% EI). There was no effect on the introduced pupae.

Residual effect of PPF-treated resting box

The results of the cone bioassays revealed that the mean numbers of eggs laid per female in the treatment groups were lowest (about 80% reduction) when PPF was newly sprayed, and maintained about 10-30% lower than the control groups during the 6-month period (Table 6). The hatching rates were zero at the beginning of the test, rose to about 50% during the first four months and gradually increased to about 90% at month 6. Similar trends were observed in the pupation and adult emergence rates. Eighty percent of EI were maintained up to four months.

DISCUSSION

Although PPF is generally applied at very low concentrations (about 20-50 ppb) into breeding sites to inhibit emergence of adult mosquitoes, exposure of adult females to high concentrations through tarsal contact can affect egg production and egg hatchability. The efficacy of PPF depends on concentration, exposure time and the resistance status of the target insects. Sihuincha *et al.* (2005) showed that exposure for 30, 60 and 120 min in PPF-bottles (0.1 mg AI/bottle or 3 mg AI/m^2) reduced the hatchability of eggs laid by exposed *Ae. aegypti* females by 70-90%. In nature, however, *Ae. aegypti* adults, especially males and non-blood-fed females, may rest on a surface only briefly. Therefore, in this study, we evaluated the efficacy of high concentrations of PPF (333 mg AI/m^2) under short exposure times (1-10 min). The PPF concentration used in this study is slightly lower than the dose of 1% PPF (350 mg AI/m^2) normally used for coating nets, including commercially available treated nets, e.g. Olyset Duo® (Sumitomo) (Ohba *et al.*, 2013; Ngufor *et al.*, 2014; Jaffer *et al.*, 2015; Koama *et al.*, 2015; Tiono *et al.*, 2015). Higher doses of PPF ($\geq$ 1000 mg AI/m^2) have also been evaluated (Itoh *et al.*, 1994; Dell-Chism & Apperson, 2003), however, such doses may be too high and not economical for large scale use.

In our CDC bottle bioassays, exposure for only one minute at 333 mg AI/m^2 significantly affected the fecundity and egg hatchability of exposed *Ae. aegypti* relative to controls. The pyrethroid susceptible strain (PMD) was more affected than the resistant strains (PMD-R and UPK-R). This may be attributed to the increased activity of mixed function oxidases in the resistant strains (Somwang *et al.*, 2011; Choovattanapakorn *et al.*, 2017). A recent study demonstrated that a subset of P450 enzymes can metabolize PPF in *An. gambiae* (Yunta *et al.*, 2016). Resistance to PPF has also been reported in some agricultural insects (Zhang *et al.*, 1998; Horowitz *et al.*, 1999), and P450s and glutathione-s-transferases appear to be involved in PPF detoxification (Ma *et al.*, 2010; Karatolos *et al.*, 2012). However, only low levels of PPF resistance were found amongst *Ae. aegypti* and *Ae. albopictus* collected in Malaysia (Lau *et al.*, 2015). In the current study, exposure for 5 minutes was sufficient to completely inhibit egg hatchability in the pyrethroid-resistant strains, indicating that the effects of PPF exposure are not minimized by *kdr* alleles in these strains.

Exposure to PPF resulted in abnormal development of the ovarian follicles and retention of eggs in the ovaries. Similar results have also been observed in *An. arabiensis* and *An. gambiae* (Harris *et al.*, 2013; Koama *et al.*, 2015). PPF mimics juvenile hormone which is known to regulate insect ovarian development. A decline in juvenile hormone levels during or up to 36 hours after blood feeding is necessary for normal egg development of *Ae. aegypti* (Shapiro *et al.*, 1986). Therefore, persistence of juvenile hormone levels would interfere with egg development.

The interval between insecticide exposure and blood-feeding is one of the key determinants of PPF efficacy. Our cone bioassays of PPF-treated polypropylene boards indicated that exposure within 24 h before or after a blood meal, not only largely reduced the number of eggs deposited, but also strongly inhibited egg hatchability (Table 2). Exposure four days after a blood meal resulted in an approximately 70% reduction of fecundity. By contrast, exposure four days before blood meal, which was about eight days from contact to oviposition, resulted in the lowest efficacy (about 32% reduction of fecundity). These results imply that the efficacy of PPF was reduced with time after exposure. Itoh *et al.* (1994) reported that the amount of PPF found in exposed females gradually reduced over time and was not detectable after day 7. Losses may be due to a combination of events including flight, aspiration, post-capture holding and detoxifying enzymes. PPF more effectively disrupts embryonic development when mosquitoes are treated at an early

stage of ovarian development, i.e. within 24 h before and after blood-feeding. Similar results have been observed in other studies (Itoh *et al.*, 1994; Ohashi *et al.*, 2012; Jaffer *et al.*, 2015). Harris *et al.* (2013) reported that female *An. arabiensis* mosquitoes that blood-fed one day prior to PPF exposure (3 mg AI/m^2 for 30 min) produced no viable offspring, however there was no effect if they blood-fed one or three days after PPF exposure, or three days before PPF exposure. They also reported that mosquitoes were sterile for the first gonotrophic cycle but able to lay eggs in subsequent cycles. Koama *et al.* (2015) observed that exposure of *An. gambiae* to PPF treated nets (350 mg AI/m^2) for 3 minutes, either 4 days before or after blood-feeding, resulted in significant reductions in fecundity. In addition, a significant reduction in fecundity was still observed after the second and third blood meals, suggesting that this effect was irreversible. These differences are probably due to differences in mosquito species/strains, insecticide resistance status, bioassays, as well as doses and formulations of PPF.

The effect of PPF on hatching inhibition, or ovicidal activity, is dose dependent and is attributed to disruption of the hormonal balance regulating embryonic development (Suman *et al.*, 2013). Deposited eggs may receive PPF during their development in the ovaries and from PPF dissolved in the water transferred by ovipositing females. Newly deposited eggs of *Ae. aegypti* are more susceptible to PPF than fully embryonated eggs since chorionic membranes are more permeable in newly deposited eggs (Suman *et al.*, 2013). The current study also confirms that PPF has no effect on the pupae, as reported by previous studies (reviewed by Kono *et al.*, 1997).

Since PPF has no repellency effect on mosquitoes and is highly effective for several months, PPF-treated resting boxes could be useful for controlling pyrethroid-resistant *Ae. aegypti*. They are relatively simple and do not require any sophisticated tools. Ponlawat *et al.* (2013) developed a collapsible resting device coated with pulverized PPF (50 mg AI/m^2). The results showed that this device was attractive to *Ae. aegypti* and suppressed the mosquito population after it was introduced into a village in Thailand. However, they found that PPF powder was not suitable for surface treatment as the granules accumulate on the bottom over time, and suggested that a more soluble formulation should be used instead. In the current study, the bioassay results of PPF-sprayed broads and PPF-coated CDC bottles were in agreement indicating that our spraying method is reliable. In addition, it is simple and inexpensive so that spraying could be repeated easily. The use of PPF dissolved in a solvent such as acetone is effective, but the residual effect of PPF on different kinds of surface materials requires further studies. Other solvents, such as isopropyl alcohol, may also be used (Ohashi *et al.*, 2012). PPF is light-sensitive and this property may be disadvantageous for outdoor residual spraying applications.

Under our laboratory conditions, the PPF-treated resting box was very attractive to *Ae. aegypti*. More importantly, unlike PPF-treated ovitraps, the resting box was attractive to both female and male mosquitoes. Contact with PPF directly affects the exposed females and disrupts oviposition and fertility. In addition, this device could also contaminate male mosquitoes and help to transfer PPF not only to virgin females, but also mated females due to multiple mating behavior which occurs in *Ae. aegypti* as well as *Ae. albopictus* (Boyer *et al.*, 2012; Helinski *et al.*, 2012). Exposure to PPF appears to have no effect on mating ability of exposed males. Thus the PPF-treated resting box can act as a dissemination station. In recent studies, male mosquitoes were treated with PPF manually before releasing (Gaugler *et al.*, 2011; Mains *et al.*, 2015). Our device represents a 'passive' controlling approach, which does not require much attention and continuously affects mosquitoes over time. It is designed for use indoors or under roof around the house. Retreatment using our

spraying method is very simple and may be applied every four months to maintain high efficacy. The portable nature of the resting boxes and their low cost per unit imply that they could be deployed in high numbers and in a wide variety of properties, with minimal disruption to local residents. Further evaluation under natural field conditions is needed as there are several factors involved including the presence of other dark objects that can compete with resting boxes. Further modifications might be necessary to improve the efficacy of treated resting boxes. Residual application of PPF is not limited to resting boxes; it may be applied to dark surfaces inside and around houses, including key breeding containers (such as used tires). Further studies are warranted to determine the suitability of domestic applications of PPE for vector control.

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