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Horizontal transfer of the insect growth regulator pyriproxyfen to larval microcosms by gravid *Aedes albopictus* and *Ochlerotatus triseriatus* mosquitoes in the laboratory

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Abstract. The insect growth regulator (IGR) pyriproxyfen is highly active against mosquitoes (Diptera: Culicidae). Through continuous emersion of large larvae (instars 3–4) the concentration causing 50% inhibition of adult emergence (EI_{50}) was determined as 0.200 p.p.b. for *Aedes albopictus* (Skuse) and 3.5 to 7 times less for *Ochlerotatus triseriatus* (Say): IE_{50} 0.0288 p.p.b.

As a possible method of application to larval microscosms of these species that oviposit in water containers and phytotelmata, the horizontal transfer of pyriproxyfen to larval microcosms by adult mosquitoes was evaluated under laboratory conditions. Gravid females were forced to walk on surfaces treated with pyriproxyfen (tarsal contact exposure) and then allowed to oviposit in larval microcosms. Using replicate bioassay cages, each with an oviposition container, and a factorial experimental design, we assessed *Ae. albopictus* for the effects of (i) pyriproxyfen concentration (0.2, 0.3 and 0.4 mg/cm^2) contacted by gravid females, and (ii) the number of treated gravid females added to bioassay cages (one, three or five females/cage), on the mortality of larvae in oviposition containers. Only 0.2 mg/cm^2 treatment rate was tested on *Oc. triseriatus*.

A significant ($P < 0.05$) curvilinear response in inhibition of emergence (IE) was achieved on both species. Densities of one or three treated *Oc. triseriatus* females/cage yielded IE rates of only 21–27%, whereas five treated females/cage resulted in 70% inhibition. With *Ae. albopictus*, densities of three or five treated females/cage yielded 48–67% and 59–73% IE, respectively, whereas one treated female/cage gave only 4–30% inhibition.

Use of IGR-treated oviposition containers to achieve horizontal transfer of pyriproxyfen to mosquito oviposition sites could be a field management technique based on mosquito biology and behaviour. In binary choice tests with *Ae. albopictus*, horizontal transfer of pyriproxyfen from a container with a treated ovistrip (0.3 or 0.4 mg/cm^2) to an untreated microcosm resulted in 14–38% inhibition. In larval bioassays, pyriproxyfen activity declined markedly within 10 days. Forcibly exposing gravid female mosquitoes to pyriproxyfen-treated paper surface did not affect their fecundity. However, from the 1st to 2nd gonotrophic cycles the egg hatch rate declined by 30% ($P < 0.05$). Some variation of results could be due to interactions between females at the oviposition site, possibly causing disproportionate transfer of pyriproxyfen to larval microcosms. Comparative studies of the oviposition behaviour of each mosquito are warranted and would potentially provide information needed to improve the technique.

Key words. *Aedes albopictus*, *Ochlerotatus triseriatus*, adult mosquitoes, bioassay, emergence inhibition, IGR, insect growth regulator, metamorphosis, mosquito behaviour, mosquito control, mosquito immatures, oviposition, pyriproxyfen.

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Introduction

The container-inhabiting mosquitoes *Aedes albopictus* and *Ochlerotatus triseriatus* occur sympatrically in peridomestic urban and suburban habitats across the southeastern and midwestern United States (Moore *et al.*, 1990). Immature stages of these two species naturally occupy microcosms in tree-holes and other phytotelmata. Both species are nuisance pests as well as vectors of arboviruses. *Ochlerotatus triseriatus* is an established vector of La Crosse virus (Watts *et al.*, 1972), whereas *Ae. albopictus* is a competent experimental vector (Grimstad *et al.*, 1989), recently found naturally infected with La Crosse virus (Gerhardt *et al.*, 2001) and West Nile virus (Holick *et al.*, 2002), and well known as a dengue vector (Hawley, 1988).

Area-wide management of these and other container-inhabiting mosquitoes continues to be problematic. Host-seeking females are primarily day-active and lay desiccation-resistant eggs in man-made containers. Because adult emergence from container habitats is continuous, conventional adulticiding with space-sprays generally achieves inadequate and merely transient control of container-inhabiting mosquitoes such as *Ae. aegypti* (Focks *et al.*, 1987; Perich *et al.*, 1990; Newton & Reiter, 1992; Castle *et al.*, 1999). Application of larvicides to containers used as oviposition sites potentially accomplishes control for a longer period of time, but a house-to-house search is required to find and treat containers, which may not be feasible in large communities or in suburban woodland landscapes. In addition, treating larval production sites has produced insecticide resistant populations (Rawlins, 1998; Rawlins & Wan, 1995; Wirth & Georghiou, 1999).

Source reduction, achieved through the removal of containers, provided effective control of *Ae. aegypti* and *Ae. albopictus* in some urban areas of China (Kai-Lok *et al.*, 1972); however, the resurgence of dengue fever in areas of Asia, including China (Gubler, 1998), suggests that public cooperation in achieving source reduction has not been sustainable in the long term. This management technique has not been sustainable in large communities of Latin American countries (Lloyd *et al.*, 1992; Reiter & Gubler, 1997). In the U.S.A., control of *Ae. albopictus* and *Oc. triseriatus* through source reduction is difficult to accomplish because discarded containers are often hidden in peridomestic woodlands.

A novel method of control for container-inhabiting mosquitoes is suggested from laboratory research results reported by Itoh *et al.* (1994). They found that bloodfed females of *Ae. aegypti* that had been forced into contact with surfaces treated with the insect growth regulator (IGR) pyriproxyfen, transported sufficient amounts of the IGR to disrupt larval development in untreated oviposition sites. In related research, Itoh (1994) placed netting treated with pyriproxyfen and containers holding *Ae. aegypti* larvae in *Ae. aegypti*-infested houses in Thailand. Larvae in many of the sentinel containers failed to emerge, indicating that females transferred biologically active amounts of pyriproxyfen after contacting the IGR-treated netting.

Results of those experiments suggest that oviposition traps (ovitraps) could be used as delivery devices for biologically active materials, such as pyriproxyfen. After laying eggs on IGR-treated surfaces, females would search for and contaminate adjacent larval production sites as they deposit additional eggs. This control method has the potential to be effective and sustainable because it is based on the biology and oviposition behaviour of aedine mosquitoes.

Accordingly our objective was to determine if, after being exposed to pyriproxyfen-treated surfaces, ovipositing females of *Ae. albopictus* and *Oc. triseriatus* delivered biologically active amounts of the IGR to larval microcosms in the laboratory. Binary choice assays were conducted to determine if horizontal transfer of pyriproxyfen could be achieved between treated and untreated oviposition sites. Effects of pyriproxyfen on fecundity and egg hatchability were also investigated.

Materials and methods

Origin and maintenance of mosquito colonies

Laboratory colonies of *Ae. albopictus* and *Oc. triseriatus* were established from eggs collected in Raleigh, NC, U.S.A. in 1997, and larvae in Wilmington, NC, U.S.A. in 1998, respectively. Colonies were maintained at 26°C and a relative humidity of *c.* 75% under a photophase : scotophase of 16 : 8 h, with two 1-h periods of incandescent light at the beginning and end of the photophase to simulate dawn and dusk. Mosquito adults were kept in aluminium screen cages (cubic 30 cm: #1450 B, Bioquip Products, Santa Monica, CA, U.S.A.). Females were fed citrated pig blood through a natural membrane condom (Benzon & Apperson, 1987). Eggs were collected on seed germination paper (Anchor Paper Co., Hudson, WI, U.S.A.) of a light brown colour (Steinley *et al.*, 1991), placed in zip-lock plastic bags and held in sealed polypropylene containers. When mosquitoes were needed, eggs were hatched by immersion and the larvae reared as described by Trexler *et al.* (1998). Larvae were fed a 2 : 1 (w : w) mixture of liver powder : baker's yeast (ICN Biochemicals, Aurora, OH, U.S.A.) on a standardized schedule (Gerberg *et al.*, 1994). Larvae were reared under optimal conditions so that late instars and adults would be of uniform size.

Test chemical

Technical grade (97.3%) pyriproxyfen (2-[1-methyl-2-(4-phenoxyphenoxy)ethoxy]pyridine), from MGK Chemical Co (Minneapolis, MN, U.S.A.), was used for all experiments.

Determination of larval susceptibility to pyriproxyfen

IGR activity against larvae of the two species was determined so that effects of pyriproxyfen on larval development and adult emergence in subsequent experiments (*vide infra*)

could be interpreted in terms of the amount of chemical transferred.

The F_{6-7} and F_3 generations of *Ae. albopictus* and *Oc. triseriatus*, respectively, were used in experiments. Bioassay methods used were similar to those of Itoh *et al.* (1994) and Ali *et al.* (1995). Susceptibility to pyriproxyfen was determined separately for each species. Stock solutions of pyriproxyfen were prepared in acetone. To achieve the desired final concentration of pyriproxyfen in bioassays, 0.5 ml of the appropriate acetone solution was added to 100 ml of 1-day-old tapwater in a 250-ml glass beaker that contained 20 late 3rd/early 4th-stadium larvae. IGR susceptibility of each mosquito species was bioassayed using six concentrations of pyriproxyfen that inhibited emergence (EI) between >0 to <100%. Each bioassay was replicated five times on different dates. Five control beakers treated with 0.5 ml of acetone and containing 20 larvae were run with each bioassay replicate. Food was provided each day on a per larva basis (Gerberg *et al.*, 1994). After the IGR was applied, beakers were placed in a fume hood and covered with Parafilm M® (Fisher Scientific, Pittsburgh, PA, U.S.A.).

Pupae were removed from each beaker on a daily basis and placed in labelled shell glass vials plugged with cotton to prevent adults from escaping. Daily counts of dead mosquitoes and emerged adults for each beaker and vial were recorded. Each test was terminated when all larvae and pupae died or adults had emerged.

Horizontal transfer of pyriproxyfen to larval microcosms by treated females

Gravid females were deliberately exposed by tarsal contact with pyriproxyfen-treated surfaces to determine if mosquitoes, after walking on the treated paper, could transport amounts sufficient to disrupt the development of larvae held in oviposition containers. Methods modified from Itoh *et al.* (1994) were used. Assays were performed separately for *Ae. albopictus* and *Oc. triseriatus*. Each combination of treatment rate and number of IGR-exposed females was replicated 12 times. Technical grade pyriproxyfen was diluted with a 50 : 1 mixture of acetone : corn oil and applied to disks (diameter 9 cm) of seed germination paper. Itoh *et al.* (1994) determined effects of pyriproxyfen at an application rate of 0.1 mg active ingredient per cm^2 (mg a.i./cm^2). In our initial experiments involving *Ae. albopictus*, we doubled this treatment rate, evaluating pyriproxyfen at a treatment rate of 0.2 mg/cm^2. Subsequently, based on results of these experiments, application rates of 0.3 and 0.4 mg a.i./cm^2 were evaluated. For *Oc. triseriatus*, pyriproxyfen was tested at one rate (0.2 mg a.i./cm^2). After acetone–oil solutions of pyriproxyfen were applied, paper discs were air dried for 20 min in a fume hood and inserted into shell glass vials (2.1 × 7 cm). The treated disks were hydrated by inverting the vials and placing them in a beaker of distilled water. The paper was saturated with water because in subsequent experiments, IGR-treated oviposition papers were to be placed in water-filled containers. Control disks of seed germination paper were treated with 1 ml of the acetone–oil mixture, air-dried and hydrated as described for the IGR-treated disks.

Females of each mosquito species, aged 7 days, were blood-fed on a human forearm and placed in a holding cage for 4 days to allow the mosquitoes to digest the bloodmeals and develop their eggs (Trexler *et al.*, 1997). During this period, a sucrose solution (10%) was supplied *ad libitum*. Laboratory colonies of both mosquito species are virus-free. The protocol that involved blood-feeding mosquitoes on a human was approved by the Institutional Review Board at North Carolina State University (Human Use Protocol IRB# 1388). After the holding period, mosquitoes of each species were transferred in groups of one, three or five gravid females to separate exposure vials which were immediately sealed with a square of cheese-cloth. Females were exposed to pyriproxyfen-treated paper for 1 h at ~26°C, then groups of one, three or five females were transferred into individual clear Plexiglas® cages (cubic 30 cm) placed on three shelves (six cages per shelf) held in a metal frame. Bioassay cages were fitted with disposable plastic sleeves, which were changed after each use. Between bioassays, cages were thoroughly washed with 95% ethanol. Lighting was provided by two 35-watt fluorescent tubes placed over each shelf and a 25-watt incandescent bulb placed at one end of the frame. Lights were operated to provide a photophase : scotophase of 16 : 8 h, with a 1 h period of incandescent light at the beginning and end of the photophase.

In each experiment, there were 18 treatment cages arranged in a randomized block design, with three blocks (= shelves) of two cages for each number of females per cage in the experiment. For each experimental replicate, six control cages, each containing five gravid females, were maintained in a separate room, but under equivalent conditions of temperature and photoperiod. Before being transferred into control cages, females were held in vials containing seed germination treated with an acetone–oil solution for a 1 h period. A 250-ml glass beaker containing an oviposition strip (3 × 10 cm) of seed germination paper treated with the acetone–oil solution and 20 conspecific late 3rd/early 4th stadium larvae in 100 ml of 1-day-old tapwater was placed in each treatment and control cage just prior to the transfer of females. Control and treatment beakers were wrapped with black construction paper and placed on top of black paper to darken the containers, which enhanced their attractiveness as oviposition sites (Wilton, 1968). The water level in beakers was adjusted to 100 ml and food was added daily on a per larva basis (Gerberg *et al.*, 1994) until experiments ended. After 48 h, females were aspirated from the cages and dissected to determine if they contained retained eggs. If a female retained eggs, that replicate was deleted from the data set. Immediately after dissections were completed, oviposition strips were removed and eggs on the strips and surface of the water were counted. Beakers containing larvae were placed in a fume hood and covered with Parafilm®. Mosquitoes

mortality and emergence were recorded daily, as described above for the larval bioassays. Treatment rates used for each species were evaluated twice on different dates.

Horizontal transfer of pyriproxyfen between a treated ovitrap and an untreated ovitrap

These experiments were conducted to determine if females, after laying eggs in an oviposition site containing pyriproxyfen-treated oviposition papers, could deposit sufficient amounts of pyriproxyfen to disrupt the development of larvae in an adjacent oviposition container. For *Ae. albopictus*, three slightly different binary choice bioassays were employed; however, in all bioassays, each beaker contained 20 conspecific late 3rd stadium larvae in 100 ml of 1-day-old tapwater and an oviposition strip that was treated with pyriproxyfen or the acetone–oil solution. In the first experiment, both beakers were wrapped with black construction paper but the bottoms were not covered. In the second experiment, one beaker (containing an IGR-treated oviposition paper) was wrapped with and set on black construction paper and the second beaker (containing an acetone–oil-treated oviposition paper) was wrapped with and set on white construction paper. In the third experiment, both beakers were wrapped in and set on black construction paper. Pyriproxyfen treatment rates of 0.3 and 0.4 mg/cm^2 were used in the 1st and 2nd–3rd experiments, respectively. After a pair of beakers was placed in bioassay cages, five gravid females were transferred into each of the 18 bioassay cages. A 10% sucrose solution was provided continuously in each cage during experimentation. Six control cages, configured identically to the treatment cages, were placed in a separate room. However, each of the two control beakers contained an oviposition strip treated with acetone–corn oil solution. Beakers were placed randomly on opposite sides of each cage directly adjacent to the walls. After the 48-h oviposition period, mosquitoes were processed and data recorded as described in the previous experiments.

Residual effectiveness of pyriproxyfen transferred by treated females

The residual activity of pyriproxyfen deposited in oviposition containers was determined by introducing pre-treated gravid *Ae. albopictus* females, after they had been forcibly exposed to the IGR 0.3 mg a.i./cm^2, to each of 18 bioassay cages at densities of one, three or five females/cage. Each cage contained a beaker with 20 conspecific 3rd stadium larvae and an oviposition strip. When larvae in all replicate cages had pupated and transferred to labelled vials, and any larvae that hatched from eggs laid on the surface of the water were removed, 20 conspecific 1st stadium larvae were added to each beaker. Subsequently, mosquitoes were handled and mortality and emergence data recorded as previously described.

Effects of pyriproxyfen on egg hatch from 1st and 2nd gonotrophic cycles

Single gravid females of *Ae. albopictus*, forcibly treated with pyriproxyfen (0.3 mg a.i./cm^2), or the acetone–corn oil control treatment, were released into cages that were configured for single-beaker bioassays. At the end of the 48-h oviposition period, oviposition containers were collected and the eggs deposited on surface of the water and on each ovistrip were counted. Ovistrips were placed on moist paper towels and stored in sealed, plastic containers under conditions of long day length (LD 16 : 8 h) at 26°C to allow eggs to complete embryonation.

Three days after oviposition strips were collected, females were allowed to take a second bloodmeal. On the fourth day after mosquitoes had fed, a fresh oviposition container was placed in each cage. After 48 h, females were removed, dissected and discarded from the trial if retained eggs were found. Second gonotrophic cycle eggs deposited in each container and on each ovistrip were counted, and the strips stored as previously described.

Oviposition strips for each gonotrophic cycle were stored for 10 days under the conditions described above; subsequently, eggs were carefully removed from each strip and placed in individual watch glasses containing 3 ml of a hatching solution (Novak & Shroyer, 1978) consisting of a 0.25% solution of nutrient broth (Difco Laboratories, Detroit, MI, U.S.A.) in 1-day-old tapwater. After 48 h, counts of hatched eggs in each watch glass were taken.

Analyses of data

For larval bioassays, pyriproxyfen susceptibility data for each species was subjected to log-dose, probit-mortality analyses (PROC PROBIT: SAS, 1989) so that concentrations that inhibited adult emergence by 50% (IE_{50}) and 95% (IE_{95}) could be estimated. Prior to these analyses, the percentage inhibition of emergence of adults in treated containers was corrected for mortality in control containers (Abbott, 1925). Results of forced-contact experiments were analysed by a two-way (*Oc. triseriatus*) or three-way (*Ae. albopictus*) ANOVA (PROC GLM: SAS, 1989) to determine if inhibition of emergence varied significantly between trials, the number of females per bioassay cage or application rates. A visual examination of a scatter plot of predicted values against residuals (Draper & Smith, 1981) revealed that the residuals were normally distributed about a mean of zero, indicating that the error variance was stable and that the data would not have to be transformed prior to analysis. Single degree of freedom tests (Neter *et al.*, 1996) were performed across the levels of each independent variable to determine if linear or quadratic effects were achieved in the percentage mortality of mosquitoes.

Amounts of pyriproxyfen transferred by gravid females were calculated from the percentage inhibition of emergence in larval bioassays using the slope and intercept coefficients from log dose-probit mortality analyses.

The residual toxicity of pyriproxyfen was evaluated by examining changes in the mortality levels of a 2nd larval cohort that was added to forced-contact oviposition container when the 1st larval cohort had died or completed development. To determine if pyriproxyfen treatments reduced fecundity or egg viability, a data set of differences between the two gonotrophic cycles was created for the number of eggs laid or percentage egg hatch for each female that completed both cycles. This data set was used to generate a t statistic (PROC MEANS: SAS, 1989).

Differences between microcosm configurations for the levels of mosquito mortality achieved in the untreated container in binary choice assays were tested for significance by calculating probability of difference values in least significant difference tests for least square means (LSM) mortality levels (SAS, 1989) under the hypothesis H_0: $LSM_{(l)} = LSM_{(j)}$. The levels of mosquito mortality in the untreated larval microcosm may be directly related to the amount of time gravid females spent in oviposition sites. Reasonably, the number of eggs laid may reflect the residence time of gravid females inside larval microcosms. Egg densities in treated and untreated containers were compared to determine if levels of mortality achieved in the untreated larval containers were associated with the residence time of gravid females in microcosms. A data set of differences between each paired treated and untreated microcosms was created for the number of eggs laid. This data set was used to generate a t statistic, (SAS, 1989).

Results and discussion

Larval susceptibility to pyriproxyfen

The IGR was very active against both mosquito species: EI_{50} and EI_{95} values for inhibition of *Ae. albopictus* emergence were 0.200 p.p.b. and 0.668 p.p.b., respectively (Table 1). *Ochlerotatus triseriatus* was 3.5–7 times more susceptible to the IGR, manifesting an EI_{50} of 0.0288 p.p.b. and EI_{95} of 0.179 p.p.b. (Table 1). The shallow slopes of the log dose-probit mortality lines for pyriproxyfen bioassays (Table 1) indicated that larvae of both mosquitoes exhibited heterogeneous responses to the IGR. In a comparable study, Ali *et al.* (1995) reported an IE_{50} for *Ae. albopictus* of 0.11 p.p.b., which is similar to the pyriproxyfen susceptibility of the strain of *Ae. albopictus* used in our experiments. Comparative susceptibly data for pyriproxyfen are not available for *Oc. triseriatus* as this mosquito has not been tested previously against this IGR.

Our bioassays used only late instars of each mosquito species. In natural settings, breeding sites may contain a mixture of instars. Whether pyriproxyfen is differentially toxic to *Ae. albopictus* and *Oc. triseriatus* instars is unknown, but relevant to our study and therefore worthy of further research.

Horizontal transfer of pyriproxyfen to larval microcosms by treated females

In the ANOVA, a significant variation ($P < 0.05$) in percentage inhibition of emergence was observed between trials for *Ae. albopictus* (Table 2), but not for *Oc. triseriatus* (Table 3). Likewise, the degree to which the number of females added per cage inhibited emergence also varied significantly ($P < 0.05$) between trials, but only for *Ae. albopictus* (Tables 2 and 3). Transfer of pyriproxyfen that disrupted larval or pupal development was significantly ($P < 0.01$) related to the amount of IGR on surfaces that females were forced in contact with (Table 2) and the number of treated females added to each cage (Tables 2 and 3). For *Ae. albopictus*, the number of females transferred to cages in combination with pyriproxyfen treatment rates only exerted additive effects on the percentage inhibition of adult emergence.

Aedes albopictus exhibited a strong curvilinear response (linear effect: $F = 61.96$; d.f. = 1,17; $P = 0.0001$; quadratic effect: $F = 7.21$; d.f. = 1,17; $P = 0.0091$) in percentage inhibition of emergence over the three population densities of pyriproxyfen-treated females tested (Fig. 1). In contrast, adult emergence was inhibited in a linear fashion (linear effect: $F = 9.79$; d.f. = 1,17; $P = 0.0026$; quadratic effect: $F = 1.85$; d.f. = 1,17; $P = 0.18$) over the three concentrations of pyriproxyfen that gravid females were forced to contact. For *Oc. triseriatus*, a significant curvilinear response (linear effect: $F = 28.85$, d.f. = 1,5; $P = 0.0001$; quadratic effect: $F = 5.97$; d.f. = 1.5; $P = 0.023$) in mosquito mortality was exhibited also over the three densities of IGR-treated females evaluated (Figs 1 and 2).

Estimated amounts of pyriproxyfen transferred to larval microcosms by gravid *Ae. albopictus* varied depending on the density of females per cage and concentration of pyriproxyfen that females were forced in contact with (Table 4). Mean amounts ranged from a low of 0.0057 µg at one female per cage and an exposure rate of 0.2 mg/cm^2 to a high of 0.52 µg at five females per cage and an exposure rate of 0.4 mg/cm^2 (Table 4). Only one treatment rate (0.2 mg/cm^2) was used in force contact experiments involving *Oc. triseriatus*. When ovipositing, average amounts of pyriproxyfen transferred by gravid mosquitoes ranged from 0.0013 to 0.0088 µg at one and five females per cage, respectively (Table 4).

Table 1. Effects of pyriproxyfen on the development of 4th instars of *Aedes albopictus* and *Ochlerotatus triseriatus*.

Species	*n*	EI_{50} (p.p.b.)	(95% fiducial limits)	EI_{95} (p.p.b.)	(95% fiducial limits)	Slope	SE
Ae. albopictus	480	0.200	(0.168, 0.229)	0.668	(0.547, 0.902)	3.14	0.38
Oc. triseriatus	600	0.0288	(0.0237, 0.0343)	0.179	(0.134, 0.262)	2.07	0.19

Table 2. Results of ANOVA for effects of pyriproxyfen treatment rate (0.2, 0.3 and 0.4 mg/cm^2) and number of females released in bioassay cages (one, three and five females per cage) on development of *Aedes albopictus* larvae in forced-contact experiments.

Source	d.f.	F	$P > F$
Trial	1	4.38	0.040
Rate	2	5.70	0.0051
Rate * trial	2	2.44	0.095
No. females	2	34.83	<0.0001
No. females * trial	2	3.40	0.0391
Rate * no. females	4	0.26	0.901
Rate * no. females * trial	4	0.28	0.890
Error	69		

The shape of the response curves for *Ae. albopictus* and *Oc. triseriatus* were opposite (Figs 1 and 2). Percentage mortality was significantly lower ($P < 0.01$) at a density of one female per cage and increased to higher and equivalent levels of mortality at three and five gravid *Ae. albopictus* females per cage (Fig. 1). In contrast, the lowest mortality of *Oc. triseriatus* was achieved at the two lower densities of females per cage and increased to a significantly higher level ($P < 0.01$) at five females per cage (Fig. 2).

Itoh *et al.* (1994) reported results of laboratory experiments in which blood-fed *Ae. aegypti* were forced to contact surfaces coated with an oil-based solution of pyriproxyfen (0.1 mg/cm^2). They found a direct relationship between inhibition of adult emergence and the numbers of days before or after blood-feeding mosquitoes were added to cages containing bioassay larvae. Treated females that were transferred 4 days before bloodmeals achieved an approximate 20% inhibition in emergence, whereas females that were gravid transferred sufficient pyriproxyfen to completely inhibited adult emergence of bioassay larvae. In our experiment, 100% mortality was achieved consistently when a treated female died on the surface of the water; otherwise, when all treated females survived complete mortality of sentinel larvae was occasionally achieved. The greater effects reported for experiments of Itoh and coworkers likely resulted from the higher susceptibility of *Ae. aegypti* to pyriproxyfen. Larval LC_{50} results (Itoh *et al.*, 1994) indicate that *Ae. aegypti* was approximately four times more susceptible to pyriproxyfen than *Ae. albopictus* used in our experiments. In contrast to the differential effects of population density of treated females on percentage mortality

Table 3. Results of ANOVA for effects of number of females released in bioassay cages (one, three and five females per cage) on development of *Aedes triseriatus* larvae in pyriproxyfen (0.2 mg/cm^2) force-contact experiments.

Source	d.f.	F	$P > F$
Trial	1	0.04	0.841
No. females	2	16.74	<0.0001
No. females * trial	2	0.24	0.789
Error	23		

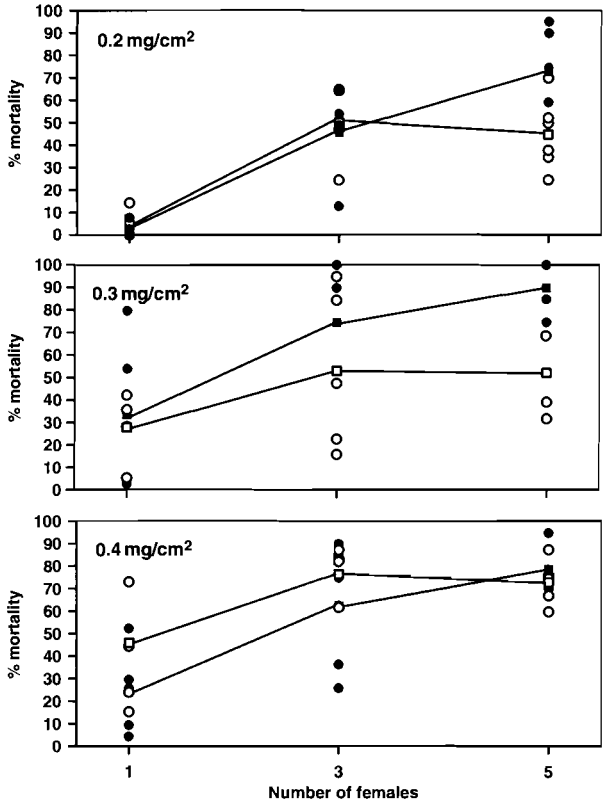

Fig. 1. Mosquito mortality (inhibition of emergence) achieved in larval microcosms through horizontal movement of pyriproxyfen by gravid *Aedes albopictus* that were forced to contact pyriproxyfen-treated paper: 0.2, 0.3 or 0.4 mg a.i./cm^2. Squares = means, circles = data points for two replicates differentiated by black and white symbols. Solid lines represent mean levels of mortality for each trial.

found in our experiments, Itoh and coworkers reported equivalent effects were achieved at one, three or five *Ae. aegypti* per cage. Again, these dissimilar results suggest that *Ae. aegypti* was relatively more susceptible to pyriproxyfen.

In our study, contrasting results for mosquito mortality achieved across the three population densities of females was likely due to differences in the oviposition behaviour of the two mosquito species. *Ochlerotatus triseriatus* exhibits crepuscular oviposition behaviour, whereas *Ae. albopictus* initiates oviposition at midday and terminates egg laying at dusk (Trexler *et al.*, 1997). With a comparatively narrower window for egg laying, interactions at the oviposition site at the higher two densities of *Oc. triseriatus* females would lead to a decrease in residence time inside the oviposition container, resulting in comparatively smaller amounts of pyriproxyfen being transferred. In each bioassay, some eggs of *Ae. albopictus* were consistently found on the surface of the water of larval microcosms whereas eggs of *Oc. triseriatus* were only laid on the oviposition strip. These results suggest that *Ae. albopictus* spent comparatively more time resting or ovipositing on the surface of the water, which could account for the greater percentage mortality

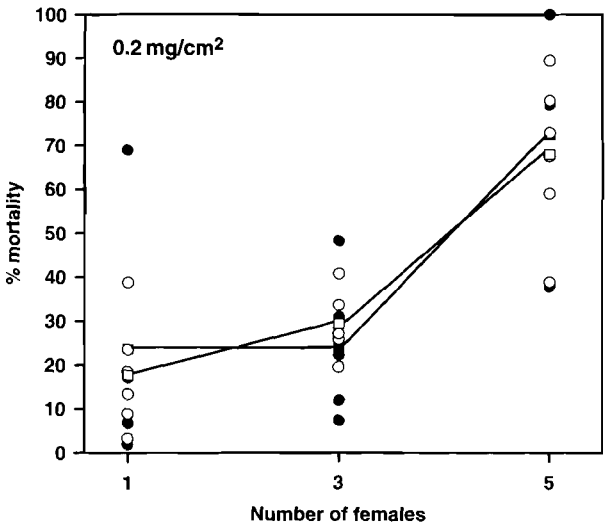

Fig. 2. Mosquito mortality (inhibition of emergence) achieved in larval microcosms through horizontal movement of pyriproxyfen by gravid *Ochlerotatus triseriatus* that were forced to contact pyriproxyfen-treated paper, 0.2 mg a.i./cm^2. Squares = means, circles = data points for two replicates differentiated by black and white symbols. Solid lines represent mean levels of mortality for each trial.

achieved at the higher two densities of females per cage. Behaviours that comprise the oviposition process for *Ae. albopictus* and *Oc. triseriatus* would have to be quantified to confirm our hypotheses. In this regard, the oviposition behaviour of both mosquitoes merits additional research.

Horizontal transfer of pyriproxyfen between a treated oviposition site and untreated larval microcosm

Binary choice assays were conducted to determine if untreated gravid *Ae. albopictus* could transfer biologically active amounts of pyriproxyfen from larval microcosms containing IGR-treated ovistrips to microcosms containing untreated ovistrips and larvae. The type of oviposition site/larval microcosm used in each of the three trials was slightly altered after each trial was conducted. We attempted to increase the amount of pyriproxyfen transferred by manipulating the colour of the oviposition site/larval microcosm to increase the residence time of gravid females in the treated oviposition site/larval microcosm. The preference of gravid females to oviposit in dark coloured containers has been previously established (Wilton, 1968; Yap *et al.*, 1995). The lowest mortality (14.2%) was achieved when black paper wrapped both larval microcosms (BC), but their clear bottom was not covered (Table 5). When the treated microcosm was completely wrapped in black paper and the untreated microcosm was completely wrapped in white paper (BW), the resultant mortality in the untreated microcosm (21.7%) was not significantly different ($P > 0.05$) from the BC configuration. In the third experiment (BB), both microcosms were completely wrapped in black paper. With this design, mean mosquito mortality (37.8%) was significantly higher ($P > 0.05$) than was achieved with the other two microcosm configurations (Table 5). The level of mortality may be associated with mosquito residence time in a container, and the number of eggs laid in a container would be a logical surrogate measure of residence time. Accordingly, egg densities in the treated and untreated containers were compared to determine if percentage mortality could be related to the numbers of eggs deposited (Table 5). Although, for some experiments, differences between the numbers of eggs laid in the treated and untreated containers were significant ($P < 0.05$), no consistent trend in percentage mortality and egg densities in the untreated container was found. In fact, for the BW experiment there was a 30 times greater number of eggs deposited in the treated container, yet a 21.7% inhibition in mosquito emergence was achieved in the untreated container. Observational studies of the oviposition behaviours of these mosquito species would provide information that would be useful in explaining these results.

Effects of pyriproxyfen on fecundity and egg hatch from 1st and 2nd gonotrophic cycles

Comparisons were made only for progeny of females that completed both gonotrophic cycles. There was no significant difference between the 1st and 2nd gonotrophic cycles for mean number of eggs laid by untreated females

Table 4. Estimated amounts of pyriproxyfen transferred to larval microcosms by ovipositing *Aedes albopictus* and *Ochlerotatus triseriatus* that were forced to contact pyriproxyfen-treated surfaces.

	Estimated mean amount (±SE) of pyriproxyfen transferred (μg)		
mg/cm^2	One female/cage	Three females/cage	Five females/cage
Aedes albopictus			
0.2	0.0057 ± 0.0033	0.034 ± 0.0048	0.042 ± 0.0049
0.3	0.022 ± 0.0061	0.044 ± 0.0082	0.050 ± 0.0063
0.4	0.021 ± 0.0052	0.048 ± 0.0032	0.052 ± 0.0032
Ochlerotatus triseriatus			
0.2	0.0013 ± 0.00057	0.0012 ± 0.00021	0.0088 ± 0.0022

Table 5. Results of binary bioassays in which gravid *Aedes albopictus* had a choice of laying eggs in a larval microcosm containing an oviposition strip treated with pyriproxyfen (0.3 or 0.4 mg/cm^2) and a larval microcosm containing an untreated oviposition strip.

Test (treated vs. untreated)	Mean (±SE) percentage mortality[a]	Mean (±SE) no. of eggs laid in container				
		Treated	Untreated	n	t[b]	$P>\|t\|$
Black container–clear bottom vs. Black container–clear bottom	14.2 ± 4.4a	209.8 ± 19.9	184.0 ± 7.2	8	1.2	0.24
Black container–clear bottom vs. White container–clear bottom	21.7 ± 3.8a	291.9 ± 8.7	8.3 ± 4.4	12	−29.2	0.0001
Black container–black bottom vs. Black container–black bottom	37.8 ± 6.0b	144.2 ± 9.4	185.3 ± 12.0	16	2.7	0.011

[a]Mean percentage mosquito mortality in the control beaker. Mean values followed by the same letter are not significantly different ($P>0.05$). Probability of difference values were calculated in least significant difference tests for least square mean (LSM) mortality values under the hypothesis H_0: $LSM_{(i)} = LSM_{(j)}$.
[b]Tests the hypothesis that the mean difference between the numbers of eggs laid in the treated and control microcosms is 0.

Table 6. Results of paired t-test for effects of pyriproxyfen on hatch of 1st and 2nd gonotrophic cycle eggs of *Aedes albopictus* females that were not treated and females that were forced to contact surfaces treated with pyriproxyfen.

Group	Gonotrophic cycle	Mean (±SE) no. eggs per ♀	t[b]	$P>\|t\|$	Mean (±SE) percentage egg hatch per ♀	t[b]	$P>\|t\|$
Control ($n=4$)[a]	1st	87.8 ± 1.4			88.9 ± 4.4		
	2nd	90.5 ± 11.7	−0.23	0.83	78.5 ± 15.1	0.89	0.44
Treated ($n=6$)[a]	1st	89.8 ± 3.1			92.5 ± 3.9		
	2nd	74.5 ± 6.1	1.81	0.13	59.9 ± 9.4	3.12	0.026

[a]n = number of females that completed both gonotrophic cycles.
[b]Tests the hypothesis that the mean difference between the number of eggs laid or percentage hatch of eggs for the 1st and 2nd gonotrophic cycles is 0.

($P=0.83$). Fecundity of treated mosquitoes declined by an average of 15 eggs/female, but the difference in mean number of eggs laid between gonotrophic cycles was not significant ($P=0.13$) (Table 6). For control eggs, no difference ($P=0.89$) in the mean hatch rate of 1st and 2nd gonotrophic cycle eggs was found despite a decline in the mean hatch rate of 10% (Table 6). In contrast, the decline of 30% in mean hatch rate between eggs laid in the 1st and 2nd gonotrophic cycles by treated females was significant ($P=0.026$). This latter finding would be important under field conditions, because treated females that survive to complete a 2nd gonotrophic cycle would be contributing fewer viable eggs to sustain mosquito population growth. Pyriproxyfen treatments have reduced egg hatch in other insects (Hirano *et al.*, 1998). Because horizontal transfer of pyriproxyfen is dependent on skip oviposition behaviour, the insignificant effect of pyriproxyfen on fecundity of *Ae. albopictus* suggests that females would potentially visit an equivalent number of containers in each gonotrophic cycle.

Residual effectiveness of pyriproxyfen

Forcibly exposed *Ae. albopictus* females transferred amounts of pyriproxyfen that resulted in mortality levels (Table 7) comparable to those achieved in previous experiments (Fig. 1). A second cohort of larvae was introduced into the same oviposition containers 10 days after initial exposure in first experiment. For the second cohort of larvae, the residual efficacy of pyriproxyfen was substantially reduced at each population density of females that was evaluated (Table 7). Activity of pyriproxyfen against the 2nd cohort of larvae declined by 17-, 9- and 6-fold at one, three and five females/cage, respectively. This decrease in mortality was possibly due to absorption and metabolism of pyriproxyfen by the first cohort of larvae. The marked loss in activity in our experiments contrasts with results reported by Takagi *et al.*, 1995). They recorded high levels of activity for 4 weeks against *Ae. aegypti* larvae that were seeded into field containers treated with 10 p.p.b. of pyriproxyfen. In our experiments, even though mortality declined substantially for the 2nd cohort of larvae, exposure to sublethal amounts of pyriproxyfen would likely have had biological effects. Loh & Yap (1989) found that *Ae. aegypti* females surviving exposures to pyriproxyfen as larvae, laid eggs that exhibited significantly lower hatch rates.

Zeichner & Perich (1999) and Perich *et al.* (2003) developed a lethal ovitrap to control *Ae. aegypti*, using a pyrethroid-treated ovistrip causing mortality of gravid *Ae. aegypti* that become exposed during oviposition. Unlike

Table 7. Residual toxicity of pyriproxyfen to two cohorts of larvae. The first cohort of 3rd instars was treated by ovipositing *Aedes albopictus* that had been forced in contact with pyriproxyfen-treated ovistrips (0.3 mg/cm^2). After the first cohort had completed development, a second cohort of 1st instars was added to bioassay containers.

No. females per cage	Mean (±SE) percentage mortality	
	1st cohort of larvae	2nd cohort of larvae
1	30.6 ± 3.9	1.8 ± 1.7
3	56.4 ± 5.7	6.3 ± 2.5
5	46.9 ± 4.2	7.6 ± 2.7

their lethal ovitrap, which is based on acute effects, our IGR-treated ovitrap is not acutely toxic to adults. In the field, treatment of natural and man-made mosquito production sites would be dependent on horizontal transfer of biologically active amounts of pyriproxyfen from IGR-treated oviposition traps by gravid mosquitoes exhibiting skip oviposition behaviour (Trexler *et al.*, 1998). The management strategy is appealing and promises to be sustainable because it is based on mosquito biology and behaviour. Before field experiments are conducted, however, laboratory studies of the oviposition behaviours of *Ae. albopictus* and *Oc. triseriatus* are being conducted. The non-linear effects on mortality rates achieved across the population densities of females evaluated indicate that oviposition behaviour can be highly variable within and between mosquito species. These results provide justification for a detailed study of mosquito behaviours involved in oviposition and the interaction between females inside the oviposition container. Information derived from such observations would be useful in explaining results of the present studies, but also in designing a more efficient delivery system for pyriproxyfen.

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