Testing a pyriproxyfen auto-dissemination station attractive to gravid Anopheles gambiae sensu stricto for the development of novel attract-and-kill strategies for malaria vector control

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Abstract

Background Larval source management is an effective supplementary tool for malaria vector control although it is not used widely in sub-Saharan Africa. This study explored whether an attract-and-kill strategy could contaminate gravid Anopheles gambiae sensu stricto with the insect growth regulator, pyriproxyfen, at a bait-station, for dissemination to larval habitats. Methods A bait-station comprising an artificial pond, containing water was treated with 20 ppm cedrol, an oviposition attractant, was covered with pyriproxfen-treated netting. Three identical semi-field cages were used to assess the potential of gravid Anopheles gambiae sensu stricto to transfer pyriproxyfen from the bait-station to three open ponds. Gravid females were released in the test and one of the control cages that had no pyriproxyfen on its bait-station. No mosquitoes were released in the third cage with a pyriproxyfen-treated station. Transfer of pyriproxyfen to open ponds was assessed by monitoring emergence of late instar insectary-reared An. gambiae sensu stricto larvae introduced into the open ponds. Liquid chromatography-mass spectrometry was used to quantify the amount of pyriproxyfen carried by a mosquito and the amount transferred to water. Results 86% (95% CI 81-89%) of larvae introduced into the open ponds in the two control cages developed into adults. Transfer of pyriproxyfen to the test cage depended on the distance of the pond from the bait-station. While only 25% (95% CI 22-29%) adult emergence was observed in larvae introduced into ponds 4.4 m from the bait-station, the emergence rates increased to 92% (95% CI 89-94%) in larvae introduced in ponds 10.3 m away. Each mosquito was contaminated with 112 µg (95% CI 93-123 µg) pyriproxyfen, whilst 230 ng/L (95% CI 180-290 ng/L) was transferred by a single female to 100 ml of water. Conclusions Pyriproxyfen was auto-disseminated by gravid females from attractive bait-stations, but mainly to aquatic habitats near the bait station. To make this approach feasible for malaria vector control, stronger attractants and better pyriproxyfen delivery systems are needed.

Background

Improved access to the core malaria control interventions namely vector control, preventive therapies, diagnostic testing and effective treatment have greatly contributed to the global reduction in malaria morbidity and mortality (1–3). However, recent World Malaria Reports from 2017 and 2018 indicate that this remarkable progress has stalled (4,5). This worrying trend emphasizes the need to explore additional tools for malaria prevention and control to supplement the current frontline measures and ensure that the gains achieved in malaria reduction in the last decade are sustained (6–8).

Malaria control programs are encouraged to adopt integrated vector management strategies to increase efficacy, cost-effectiveness and sustainability of disease control (8,9) . Larval source management (LSM) targeting mosquito vectors in their aquatic habitats, such as larviciding and environmental management, can effectively serve as complementary vector control measures (10–12) . This is specifically since LSM strategies control vectors that exhibit exophagic and exophilic traits that are less controlled by long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) (10). Studies in East Africa demonstrated the benefit of targeting mosquito larval habitats with microbial larvicides as a complementary measure to the

use of LLINs in reducing malaria transmission (12–14). The challenge of conventional larviciding, however, is the requirement to distribute mosquito larvicides by ground teams to all available breeding habitats in an intervention area (15–17). Whilst it is hypothesized by some that the effective implementation of larviciding for malaria control relies upon the capacity to target insecticide application at aquatic habitats that are most productive in terms of adult emergence (18,19), habitat productivity is still poorly understood and hence non-predictable (20–22).

Auto-dissemination, a novel technique that exploits the adult insect as a 'vehicle' to deliver the insecticide to breeding habitats has been shown to be effective in the control of social insects, such as ants, termites (23–25). Auto-dissemination has shown great potential in the control of mosquitoes of different genera (26–29). Laboratory and field studies have demonstrated the potential of container breeding *Aedes* mosquitoes to transfer PPF from contaminated surfaces to larval habitats to reduce adult vector emergence rates by 42-100% (26). These successes have led to increasing interest in the evaluation of the strategy for the control of the Afrotropical malaria vectors (28,30,31). Recent studies conducted in semi-field tests in Tanzania provide evidence of the potential of *An. arabiensis* to transfer the insecticide pyriproxyfen (PPF) from resting surfaces to larval habitats, consequently inhibiting larval development (31,32). However, in these two studies 1500-5000 host-seeking females were released into a semi-field cage of approximately 360 m^3, a population density that is unlikely to occur under natural conditions. Furthermore, it is likely that a large proportion of host-seeking females became sterilized on contact with PPF and, since they would not become gravid, would not transfer PPF to aquatic habitats (33). Recently we showed that the optimum time for contaminating female malaria vectors is when they are gravid and close to oviposition. Thus, we hypothesized that the most effective approach for auto-dissemination is to target the gravid female (33). The aim of our study was to design an attractive bait-station where gravid females are exposed to PPF and disseminate it to open ponds under semi-field conditions.

Methods

Study site

Experiments were carried out in large netting-screened semi-field cages (10.8 m long × 6.7 m wide × 2.4 m high) on the Thomas Odhiambo Campus of the International Centre of Insect Physiology and Ecology (*icipe*-TOC), located on the shore of Lake Victoria in Mbita, Homabay county, western Kenya (geographic coordinates 0 26' 06.19" S, 34 12' 53.13"E; altitude 1,137 m above sea level). Mbita is characterized by tropical climate with an annual average minimum temperature of 16 ºC and maximum temperature of 29ºC. The area experiences two rainy seasons; the long rains between March and June and the short rains October and December.

Test insecticide

An experimental formulation of dust, with particles 12µm diameter, containing 10% of pyriproxyfen (PPF) (Sumilarv®, Sumitomo Chemical Company) was used in all experiments.

Mosquitoes

Anopheles gambiae sensu stricto (Mbita strain) larvae and adults used in this study were obtained from the mosquito insectaries at icipe-TOC. Immature stages were reared in a semi-field cage at ambient conditions with average daily temperature of 25-28ºC, relative humidity of 68-75% and natural lighting. Mosquito larvae were reared in round plastic tubs (diameter 60 cm) filled with 5 L water (5 cm deep) from Lake Victoria filtered through a charcoal-sand filter. Mosquito larvae were fed with a pinch of fish food (Tetramin©Baby) twice daily. Third instar mosquito larvae were randomly selected from different tubs to ensure that those introduced into experimental ponds were the same size. Adult mosquitoes were held in mosquito-netting covered cages (30 cm x 30 cm x 30 cm) in a holding room with ambient climate conditions and provided with 6% glucose solution ad libitum. Three day old females were allowed to feed on a human arm on two consecutive nights. Only gravid female mosquitoes were used for experiments in this study.

Development of a bait-station

Contamination of adult *An. gambiae s.s.* with PPF. Since water vapour is a general attractant for malaria vectors (34), we considered it was essential to have water in our bait station. Females were prevented from accessing the water to lay eggs using fly gauze (black fibre-glass netting gauze (1.7x1.5 mm) treated with PPF. Two methods of applying the PPF to the netting material were tested in cage experiments. First, the netting gauze (diameter 7 cm) was treated with 1 g of PPF dust applied with a soft brush to ensure uniform spreading of PPF over the netting surface. The amount of PPF on netting gauze was 1.3 g/m^2 after weighing. Second, PPF was mixed with cooking oil (Ufuta Pure Vegetable Cooking Oil, Bidco Africa) and applied to the netting. Here 1 g of PPF dust was mixed in 2 ml of oil and this formulation applied to a netting gauze with a brush. The netting treated with PPF served as the dissemination station. The control netting gauze was untreated and was used in control cages.

These experiments were conducted in mosquito-netting covered cages (30 cm x 30 cm x 30 cm) to determine which of the two methods of treating the netting gauze contaminated gravid *An. gambiae s.s.* with lethal doses of PPF for transfer to water. Each cage was provided with two glass cups (Pyrex®, 100 ml, diameter 7 cm). The first cup in each cage was filled with 100 ml non-chlorinated tap water while the second cup that served as the bait station was filled with 100 ml of six-day old soil infusion that has been shown previously to attract gravid females in cages (35) to lure gravid females. The top of the cup that served as the bait-station in the control cages was covered with untreated netting while in the test cages it

was covered with netting gauze treated with either PPF dust or PPF dust formulated in oil. The top of the other cup filled with water was left open in all cages to allow for egg-laying by gravid females.

In each cage five gravid *An. gambiae s.s.* were released at 18:00 h and left overnight. The following morning the number of eggs in each open cup was counted. To confirm the transfer of PPF in test cages, 10 insectary-reared late instar *An. gambiae s.s.* larvae were introduced into all open cups with water in all cages and monitored for adult emergence. Larvae were fed daily on a pinch of Tetramin®Baby fish food. Because PPF does not produce acute toxicity on mosquito larvae but prevents emergence of adults from exposed pupae (36), any pupae that developed were transferred into plastic cups (diameter 7 cm) and monitored for emergence. It took 6-7 days for all larvae introduced into the cups to develop into adults or die. These experiments were conducted in three rounds on separate dates. There were five replicate cages per treatment in each experimental round, thus in total there were 15 cages with untreated netting gauze, 15 cages with netting gauze treated with PPF dust and 15 cages with netting treated with PPF dust formulated in oil. Both cups were randomly allocated to one of the four corners in the first cage. The positions of the cups in subsequent cages were rotated to the next corner in a clockwise direction relative to the starting position.

Soil infusion was prepared by incubating 15 L of non-chlorinated tap water with 2 kg of soil collected from a known *An. gambiae s.l.* aquatic habitat. Infusions were prepared in round plastic tubs (diameter 0.42 m) and left for six days before use in experiments. During the six-day incubation period tubs were covered with mosquito netting and kept in sheds, to protect them from rain.

Luring gravid *An. gambiae s.s.* to a pond. These experiments were conducted in a semi-field cage (Figure 1). Four artificial ponds were created by digging down round enamel tubs (diameter 0.42 m, depth 8 cm) at the four corners of the semi-field cage. The tubs were dug 1 m away from the nearest wall. During each experimental round three of the ponds were filled with 7 L of non-chlorinated tap water while the fourth pond was filled with a test substrate to attract gravid females.

Two test substrates were tested based on previous published work that showed their potential in attracting gravid female *An. gambiae s.s.*: a six-day old soil infusion (35) and the sesquiterpene alcohol, cedrol (Cedrol ≥99.0% (sum of enantiomers, GC, Sigma-Aldrich, Steinheim, USA) (37). Both substrates were evaluated separately on different dates. Thus, during the experiments the test pond was either filled with either 7 L of six-day old soil infusion or 7 L of non-chlorinated tap water treated with cedrol. Two concentrations of cedrol were tested sequentially: 5 ppm and 20 ppm. Cedrol was prepared in ethanol by first preparing a stock solution of 10,000 ppm by dissolving 150 mg of cedrol to 15 ml of absolute ethanol (≥99.8% (GC), Sigma Aldrich). Dilutions were made by adding the appropriate volume of stock solution to water in the pond. For instance, 5 ppm cedrol was prepared by adding 3.5 ml of stock solution into 7 L of

water in the dug down tub. Similarly, 20 ppm cedrol was prepared by adding 14 ml of stock solution into 7 L of water in tub.

To simulate the natural environment, where female *An. gambiae* s.s. take a blood-meal indoors and rest till they are gravid, we released gravid females inside a small wooden hut (1.78 m long x 1.73 wide x1.80 m high) that was set up in the centre of the semi-field cage (Figure 1).

The hut had a door and two windows that were shut when the experiment was in progress. The hut had two open eaves (1.70 m x 0.18 m) located at opposite sides which served as exit points for the gravid females. In each experimental night 200 gravid *An. gambiae s.s.* were released at 18:00 h in the centre of the hut. To measure the number of mosquitoes visiting a pond, the top of each pond was covered by a black fibre-glass netting gauze cut to size (diameter 0.42 m) on which a fine film of insect glue was sprayed (Oeco insect spray, Oecos, UK) to trap the mosquitoes as they searched for oviposition substrate to lay eggs. At 6:00 h the following morning the number of mosquitoes trapped on the sticky screens placed on top of each pond was counted. Each of the test substrates were evaluated during 12 nights with fresh substrates and fresh batches of mosquitoes. The four ponds were randomly allocated in all four corners of the semi-field cage using a randomized complete block design.

Evaluation of the auto-dissemination of PPF by gravid *An. gambiae s.s.* from a bait-station to larval habitats

The experimental set-up used in this test was modified from Lwetoijera *et al*. (31). These experiments were conducted in three identical semi-field cages which included a small wooden hut at the centre and four ponds in the corners (Figure 2). In the first semi-field cage, three ponds were filled with 7 L of non-chlorinated tap water and left open for mosquito oviposition, whilst the fourth pond served as the bait-station which consisted of 7 L of water treated with 20 ppm cedrol as described above. On top of the cedrol-treated pond a netting gauze of diameter 0.42 m was placed and treated with 3.5 g PPF (25.3 g PPF/m^2). The three open ponds were 4.4 m, 8.4 m and 10.3 m from the bait-station (Figure 2). Two hundred gravid *An. gambiae s.s.* were released at 18:00 h per experimental night in the centre of the hut. The set-up in the second semi-cage was the same as the first, except that no mosquitoes were released in the cage. The aim here was to investigate if PPF might be distributed by air movement to neighbouring ponds, rather than mosquitoes. In the third semi-field cage, mosquitoes were released but the netting gauze placed on top of the pond serving as the bait station was untreated. This last set-up served to investigate natural adult mosquito emergence rates from ponds when no insecticide was present in the semi-field cage. The set-ups in the second and third semi-field cages thus served as controls.

The following morning the number of eggs laid in each open pond was counted. To ensure sufficient replication of the experiment the impact of PPF was not assessed by monitoring the development of eggs that were laid by the exposed females which would have taken approximately two weeks to complete one experiment (38). Instead, the possible transfer of PPF by females to the ponds was assessed by monitoring the adult emergence of 50 insectary-reared late instar *An. gambiae s.s.* larvae that were introduced into the open ponds in all three set-ups in the morning after gravid females were released. Introduced larvae were fed daily with a pinch of Tetramin®Baby Fish food. Any pupae that developed in the three ponds were transferred into 200 ml plastic cups (diameter 7 cm) and monitored for emergence. It took 6-7 days for all introduced larvae to develop into adults or die. Thereafter the ponds and hut were cleaned and all remaining alive adult mosquitoes aspirated using a motorized backpack aspirator (John W. Hock Company, USA). A new set of experiments was set-up with fresh substrates, fresh batches of adult gravid mosquitoes and mosquito larvae. The experiments were conducted for 12 rounds with each round lasting seven days. The four ponds were randomly allocated in all four corners of the three semi-field cages in a randomized complete block design. To avoid contamination, the semi-field cages in which the test and the two control experiments were conducted were not changed.

Liquid-chromatography-mass spectrometry (LC-MS) quantification of the amount of PPF carried by an individual mosquito and transferred to a water sample

An enamel bowl (diameter 0.42 m) filled with 7 L of non-chlorinated tap water was introduced into a 60 x 60 x 60 cm cage (BugDorm-2120F; MegaView Science Taiwan). Above the water a netting treated with 3.5 g PPF dust (25.3 g PPF/m^2 after weighing amount retained on netting) as described above was fixed. Two gravid *An. gambiae s.s.* were introduced at a time into the cage and observed. Two different tests were conducted with females that contacted the PPF-treated netting. Firstly, 200 females that contacted the PPF-treated netting were individually transferred into 1.5 ml Eppendorf tubes and frozen at -70°C until they were used for quantification of PPF on their bodies.

Secondly, 30 females that contacted PPF-treated netting in the BugDorm cage were used to determine the amount of PPF that a single mosquito transfers to water. For this, bioassays were conducted by introducing individual females into 15 x 15 x 15 cm cages containing a glass cup (Pyrex®, 100 ml, diameter 7 cm) filled with 100 ml of non-chlorinated tap water. The females were left overnight in the cages to lay eggs. The following morning the number of eggs laid by each female was counted. To confirm the transfer of PPF into the water in the cup, 10 late instar *An. gambiae s.s.* larvae were introduced and monitored for adult emergence as described above. Comparisons were made to a control group of gravid females that were unexposed to PPF. Thirty replicates of test and control cages were done. When all larvae had died or emerged as adults, the water from the cups was transferred into 50 ml glass jars. The water samples were frozen at -70°C until used for chromatographic quantification of PPF.

For quantification of the amount of PPF that contaminates a mosquito when a female makes contact with a PPF-treated netting material, PPF was washed off the body of an individual mosquito in an Eppendorf tube using 1.5 ml methanol (Sigma Aldrich, 99.9% HPLC grade). The content of the Eppendorf tubes was agitated in a sonicator (Branson 2510 Ultrasonic cleaner, Eagle Road, Danbury) at 25 ºC for 5 minutes. It was then centrifuged at 13,000 revolutions per minute (rpm) for 5 minutes in a microcentrifuge (PRISM^{TM}). The supernatant was transferred into 2 ml glass vials and used for detection of PPF by liquid chromatography-mass spectrometry (LC-MS).

To detect PPF in water samples used in bioassays, the samples were first pooled into groups of 10 before extraction (10 x 50 ml). Thus, there were six pools of water samples in which females that contacted PPF laid eggs and another six pools of water samples in which females unexposed to PPF laid eggs. Each pool of water samples was extracted separately. For each pool of water sample, approximately 500 ml of the sample was extracted in 200 ml chloroform (Sigma Aldrich, 99.9% HPLC grade) to separate the aqueous and organic layers. The organic layer, where PPF was expected to dissolve, was concentrated by evaporating it to dryness in a rotary evaporator (Heidolph Instruments, Germany). The residue was dissolved in 1 ml methanol and stored at 4°C awaiting analysis. To assist in quantification of PPF, a known concentration (0.00002 µg) of 4-benzylbiphenyl (99%, Sigma Aldrich) was added into each extracted water sample as internal standard just before the LC-MS run. The LC-MS run was performed using electron spray ionization (LC/ESI-MS). First, the standards of pure 10% PPF and 4-benzylbiphenyl were initially run separately in the LC-MS system to confirm the retention times of PPF and the internal standard. PPF used as standard was prepared by dissolving 40 mg of PPF (10%) in 1.5 ml ethanol in a 2 ml glass vial. This was agitated in a sonicator at 25 ºC for 5 minutes. The mixture was centrifuged at 13,000 rpm for 5 minutes. The supernatant was transferred into 2 ml glass vials ready for detection of PPF. The peaks of PPF and 4-benzylbiphenyl at the retention times were identified based on the molecular masses of their individual ions (molecular masses of PPF-322 and 4-benzylbiphenyl-247).

The LC/ESI-MS used consisted of a quaternary LC pump (Model 1200) coupled to Agilent MSD 6120-Single quadruple MS with electrospray source (Palo Alto, CA). The MS component of the system was used to verify the peak assigned to PPF or 4-benzylbiphenyl as the active ingredients based on the identification of molecular masses of the ions. The system was controlled using ChemStation software (Hewlett-Packard). Reverse-phase liquid chromatography was performed using an Agilent Technologies 1200 infinite series LC, equipped with a Zorbax Eclipse Plus C_{18} column, 4.6 x 100 mm x 3.5 µm (Phenomenex, Torrance, CA). The following gradient using A (5% formic acid in LC-grade ultra pure H_2O) and B (LC-grade methanol) (Sigma, St. Louis, MO) was used; 0-5 min, 95-100% B; 5-10 min, 100% B; 100-5 min. The mobile phase liquid was acetonitrile (Sigma Aldrich). The flow rate was held constant at 0.7 mL min^{-1}. The sample injection volume was 100 µl, and data were acquired in a full-scan positive-ion mode using a 100 to 500 *m/z* scan range. The dwell time for each ion was 50 ms. Other parameters of the mass spectrometer were

as follows: capillary voltage, 3.0 kV; cone voltage, 70 V; extract voltage, 5 V; RF voltage, 0.5 V; source temperature, 110ºC; nitrogen gas temperature for desolvation, 350ºC; and nitrogen gas flow for desolvation, 400 L/h.

Data analysis

Data were analysed in R statistical software package version 2.13. Generalized estimating equations (GEE) were used to analyse all data with experimental round/night included as repeated measure in the models. Data collected in cage and semi-field experiments that determine the transfer of PPF to water were analysed as proportions. Proportions were analysed by fitting a binomial distribution with a logit function and an exchangeable correlation matrix assumed. In analysing data performed in cage experiments to determine if mosquito can get contaminated with PPF dust or PPF dust formulated in oil from treated netting, the cage (control or test) was included as fixed factor with the control cage used as the reference. In semi-field experiments to evaluate the potential of gravid female to transfer PPF to open ponds, the open pond ID identified by its distance from the bait station was used as the fixed factors with the pond closest to the bait station used as the reference.

Count data evaluating the number of mosquitoes visiting ponds treated with soil infusion or cedrol were fitted to a Poisson distribution with a log link function. Here the ponds were included in the model as fixed factors with the pond serving as the bait station used the reference. All means (proportions or counts) per treatment and their corresponding 95% confidence intervals (CIs) were modelled as the exponential of the parameter estimated for the individual models with no intercept included.

Results

Gravid *Anopheles gambiae s.s.* gets contaminated with more PPF when the PPF is only dusted on netting than when PPF is formulated in oil

Both application methods of PPF on the netting of the bait-stations led to the transfer of PPF to the open cup by gravid females and significant reduction in the emergence of adults from introduced larvae (Figure 3 and Table 1). However, the adult mosquito emergence rates were five times lower with PPF dust than the oil formulation.

Oviposition attractants can lure gravid *An. gambiae s.s.* to a bait-station under semi-field conditions

The number of mosquitoes trapped on the sticky screens placed over ponds containing soil infusion or cedrol at 5 or 20 ppm was significantly higher than the number trapped over ponds with untreated water (Table 2). The attractiveness of soil infusion and water treated with 5 ppm cedrol was similar and not very strong; a female was only approximately 1.3 times more likely to land on the test pond that control pond (Table 2). When the water in test pond was treated with 20 ppm of cedrol, however, it was approximately twice as likely for a female to be trapped in test ponds compared to control ponds with untreated water (Table 2).

Transfer of PPF by gravid *An. gambiae s.s.* is dependent on the distance of the habitat from the dissemination station

In all semi-field cages, where gravid females were released inside the hut, eggs were observed the following morning in all three open ponds at any experimental night. However, egg numbers were not further quantified, or larvae followed up. The potential transfer of PPF was evaluated based on the adult emergence rate from introduced insectary-reared third instar larvae.

In the absence of PPF on the bait-station as well as in the absence of gravid females in the semi-field cage, 86% (95% CI 81-89%) of introduced larvae emerged in both experiments. For some unexplained reason there were differences in the emergence rates of larvae introduced into the ponds in the two control semi-field cages. The emergence of larvae in the control experiment where no mosquitoes were released but PPF was present on the bait station was consistently higher than in control experiment where mosquitoes were released but no PPF was present in the cage (Table 3). This might have been due to some microclimate differences in the two semi-field cages or might have been due to some unexplained interaction between the early instars originating from the oviposition and the introduced larvae. Importantly, in both control experiments, emergence rates were similar in all three open ponds in the cages (Table 3) and it can be excluded that wind transferred PPF from the bait-station to the open ponds.

The presence of a PPF-treated bait-station reduced the emergence of adults from the three open ponds (Table 3) in the test semi-field cage confirming that PPF was transferred by the released gravid females. On average, only 25% (95% CI 22-29%) of introduced larvae emerged from the pond closest to the bait station. However, the greatest inhibition occurred in ponds close to the bait station than those further away. When comparing the emergence rates from the ponds in the test cage with the average emergence rate from ponds in the control cages, significant emergence inhibition was only observed for the two ponds closest to the bait-station. It was approximately 23 times less likely for an adult to emerge from the ponds closest to a bait-station (located 4.4 m away) and 6 times less likely from the ponds that were approximately twice as far away from the bait-station (located 8.4 m away) as the closest pond than it was for an adult to emerge from any pond in the control experimental cages (Table 3). No emergence inhibition was recorded

from the open pond that was furthest away from the bait-station and located in the opposite corner on the other side of the hut suggesting that no or insufficient PPF was transferred to this pond.

Gravid *An. gambiae s.s.* transfers less PPF to oviposition substrate than amount of compound that contaminates the body of female when she makes contact with PPF-treated netting

Ninety percent (n= 30) of females that landed on PPF-treated netting laid eggs when provided with water in a glass cup in a cage. A similar number (n= 30) of unexposed (control) females laid eggs. There was no difference in the mean number of eggs laid by females that were exposed to PPF and those that were not (p=0.78). The average number of eggs laid by all females was 61 (95% CI 50-76). Significant differences were, however, observed in adult emergence rates from larvae that were introduced into the cups (Table 4). It was 17 times less likely for a larva to emerge when it was introduced into water in which PPF exposed female had laid eggs than when introduced into a cup in which unexposed female had laid eggs (Table 4).

Based on the control emergence of 93% (95% CI 89-97%), the corrected percent emergence inhibition observed was 52% (95% CI 46-56%) (39); in other words an individual female transferred to 100 ml of water the concentration of PPF that inhibited emergence of approximately 50% of larvae (EI_{50}).

Preliminary chemical analysis showed that it was impossible to detect the low concentration of PPF that was washed off the body of a single mosquito. Thus, samples from 20 individuals were pooled for analysis with the LC-MS system. In total PPF was washed off the body of 140 individuals that had made contact with PPF and a similar number that did not make contact with PPF (controls). Consequently, there were seven pools of females that made contact and another seven pools that did not. No PPF was detected in any of the washes from control mosquitoes. PPF was below the detection limit in two of the pools from mosquitoes that made contact with PPF. The estimated amount of PPF washed off an individual female from the five pools in which PPF was detected was 141 µg, 120 µg, 93 µg, 117 µg and 89 µg. Thus, the average amount of PPF washed off an individual mosquito was 112 µg (95% CI 103-123µg). This is, however, an overestimate considering that PPF levels were below detection limits in two sample pools and were not included in calculating this average. Assuming that an individual female transfers this amount of PPF to 100 ml water, as used in our cage bioassays, we would expect a concentration of 1.12 mg PPF/L (1.12 ppm).

PPF was detected in three of the six water samples in which females exposed to PPF laid eggs but never in the control water samples. The estimated concentration of PPF detected in the individual water samples in the three positive pools were 330 ng/L, 160 ng/L and 190 ng/L. Thus, the average estimated concentration of PPF in a single oviposition cup used in our bioassay was 230 ng/L (95% CI 180-290 ng/L). This is equivalent to 0.00023 mg/L (0.00023 ppm). This is similarly an overestimate since in three of the test water samples PPF was below the detection limit and not included in estimating the average. This is the concentration that provided around 50% emergence inhibition of larvae introduced in water in our bioassays. Comparing the PPF concentration detected in water samples with the amount that contaminates a single female when she makes contact with a PPF dusted surface, revealed that an individual female transferred 4,869 times less PPF to oviposition substrate than the amount that contaminates the female from the treated surface.

Discussion

To our knowledge this is the first study to have developed a prototype bait-station for gravid *Anopheles gambiae s.s*. for the auto-dissemination of PPF to aquatic habitats. We show that gravid females can be lured to a target, where they are contaminated with PPF and then transfer PPF to an aquatic habitat while laying eggs. Although 200 gravid females were released in a relatively small space of approximately 170 m^3, adult emergence from larval habitats, was only inhibited by 72% (corrected based on control emergence) from ponds 5 m from the bait-station. However, adult emergence inhibition was not observed when larval habitats were 10 m away from the bait-station, when the hut acted as a barrier between the pond and bait station. These results strongly suggest that even if females can be lured successfully to a bait-station, they are only likely to transfer the PPF to the closest available and detectable oviposition sites.

Our proof-of-principle presented in this study highlights a number of challenges for developing the auto-dissemination approach for African malaria vectors that utilize a large range of habitats of variable size for oviposition (21). *Anopheles gambiae s.s.* can only transfer PPF to an aquatic habitat when a gravid mosquito is exposed to PPF, otherwise she would be sterilized and not visit an aquatic habitat (33). Therefore, it is the gravid female that must be targeted for contamination with PPF for eventual transfer to larval habitats. The aim here was therefore to develop a bait-station especially attractive for the gravid *An. gambiae s.s*. To date, only water-vapour (34), a soil infusion made from a specific habitat found at *icipe*-TOC campus (35) and a chemical compound, cedrol (37), attract gravid *An. gambiae s.s*. under experimental conditions. Our study confirms the previous findings that a six-day old soil infusion made from soil from a specific location at *icipe*-TOC (35) and cedrol-treated water (37) attract gravid *An. gambiae s.s.* under semi-field condition. Our study further highlights that these two oviposition attractants can be used in an attract-and-kill approach. However, contrary to findings by Lindh *et al*. (37) that water treated with 5 ppm cedrol doubled the catch of gravid *An. gambiae s.s*., the present study only achieved the same result with 20 ppm cedrol compared to the untreated control. This difference might be due to

absence of any air current or reduced airflow generated by the bait-station in our set-up. Lindh *et al.* (37) used modified BG-Sentinel traps that produce an air circulation with the help of a fan that is likely to release larger amounts of cedrol and water vapour by the trap, providing a stronger signal for oviposition site-seeking females. Under our experimental conditions, it is likely that the females that left the hut through the eaves on the side facing away from the bait-station went in similar proportions to the two ponds located on this side of the eave that were the closest. These females are unlikely to have visited the pond that served as bait-station as it was far away and not detectable from their exit point. This highlights the need to develop significantly more attractive substrates to add to bait-stations to lure gravid females at greater distances. This might be achieved through innovative release technologies (REF), and the formulation of more attractive chemical blends. However, this study also suggests that odour signals from an aquatic habitat are only detected and used at relatively short range, and so even a stronger signal might not be used over much long distances, especially in areas and during seasons without strong air movement at dusk and early evening when gravid females seek oviposition sites.

Gravid females transferred more PPF from treated surfaces when PPF was applied as a dust than when formulated in oil. There are two possible explanations for this. First, the oil might reduce the transfer of PPF to mosquitoes, with more of it adhering to netting. Second, it might also be that the oil contributed to a larger proportion of PPF remaining on the mosquito's body, thus limiting the chance of PPF getting in contact with water. Presumably, the hydrophobic oil attaches more strongly to the hydrophobic cuticle than to water. PPF in dust or powder form has been used previously in some of the successful studies that evaluated the potential of auto-dissemination for mosquito control (31,40), however, for large scale application and cost-effective use of the relatively expensive active ingredient there is need to investigate strategies that use PPF more efficiently. Previous studies show that electrostatic material are efficient surfaces for contamination of mosquitoes with insecticidal substances (28,41). Moreover, Swale *et al.*, (28) suggest that other insect growth regulators, such as novaluron, can be used in auto-dissemination for mosquito control due to their greater persistence in the environment as compared to PPF.

In our approach, clearly, a lot of the active ingredient remained on the netting gauze and was wasted since not all the material was taken up. Furthermore, as shown in our chromatographic analyses, the amount of PPF that a gravid female transferred to an aquatic habitat was around 4,800 times less than that which contaminated a female when she made contact with a PPF-treated surface. This is not surprising as the amount of PPF on the insect cuticle is likely to decrease with time due to loss during flight and penetration through the insect cuticle (42,43). Furthermore, the lipophilic property of PPF (44) is likely to enhance its adherence to the hydrophobic insect cuticle, limiting the amount available for transfer to water.

It has been shown previously that the amount of PPF that adheres on a mosquito's body can be quantified by chemical analysis (45). The chromatography analysis confirms our findings from the bioassay, that a single female could transfer sufficient PPF to inhibit the emergence of approximately 50% (EI_{50}) of the larvae in 100 ml of water. The average concentration of PPF detected in water used in the bioassays was 0.00023 mg/L (95% CI 0.000180-0.000290 mg/L), which correlates well with our previous results from laboratory assays when the EI_{50} was established at 0.000120 ng/L (95% CI 0.000090-0.000160 /L) (46). The findings are also consistent with previous cage bioassays where females were contaminated in a plastic jar coated with PPF and a single female caused approximately 50% of the introduced larvae not to emerge (33). Taken together it appears that this is the maximum amount of our test formulation that a single female *An. gambiae s.s.* can transfer to an aquatic habitat using our system. Improved technologies of contaminating the female with PPF could include the electrostatic charging of the PPF particles to ensure a higher amount of PPF placed on an individual female (41). However, whether this would improve the amount transferred to water or only increase the overall amount carried by the individual mosquito would need to be tested.

Mathematical models show that the success of auto-dissemination for malaria vector control is dependent on the abundance of adult vectors, the number and stability of larval habitats and persistence of the insecticide used (47,48). Our study highlighted that the transfer of PPF to a larval habitat is dependent on the distance of the pond from the bait-station; the closer a pond is to the bait-station the more PPF gets transferred and therefore the higher the emergence inhibition rates. Similar habitats further away are less likely to be visited and therefore less PPF gets transferred. This suggests that numerous dissemination stations would be required in the field for gravid *An. gambiae s.l.* to transfer sufficient lethal doses of PPF to their larval habitats. This is a substantial challenge considering the large number and extensive nature of the larval habitats of *An. gambiae s.l.* in some areas (21,49).

To our knowledge the only other semi-field studies to evaluate auto-dissemination for control of *An. gambiae* species complex reported that *An. arabiensis* could transfer PPF from contaminated resting pots to cause more than 82% adult mosquito emergence from artificial larval habitats (30,31). The recent study by the authors indicate that under similar semi-field settings, auto-dissemination by *An. arabiensis* can cause complete reduction in the densities of this mosquito species (30). Several factors might explain the greater impact in these studies. In the first, the authors placed eight dissemination stations (resting pots) treated with PPF and provided only two small aquatic habitats (capacity 2.5 L). This makes a dissemination station to oviposition site ratio of 4:1; this is in comparison to a ratio of 1:3 in our study. In the same study a total of 5000 females were released in the semi-field cage increasing the likelihood of a mosquito visiting a dissemination station and the number of oviposition events in a single aquatic habitat. Furthermore, the capacity of the oviposition sites was three times smaller and much closer to the dissemination stations (1-8 m away) than in our study. Hence it can be hypothesised that the higher number of dissemination stations increased the chance of a mosquito resting on a PPF-treated surface

which subsequently increased the number of mosquitoes that were contaminated with PPF for transfer to the limited number of larval habitats.

A limitation of this study was the failure to assess the transfer of PPF from the dissemination station to the bait station with the eggs laid by female mosquitoes released in the semi-field cages. While PPF is a pupicide that has little impact on mosquito larvae, the impact in reducing adult emergence is higher in larvae that have prolonged exposure to the insecticide (36). It is likely that greater adult emergence inhibition rates would be observed if the effect on emergence inhibition was assessed by monitoring if eggs laid by females developed into adult mosquitoes.

Conclusions

Our study carried out under controlled conditions highlights potential limitations of the auto-dissemination strategy for the control of Afrotropical malaria vectors. Our findings indicate the need for further studies to explore the required ratio of dissemination stations to aquatic habitats for gravid *An. gambiae s.s.* to transfer sufficient quantities of PPF to larval habitats. The skip-oviposition behaviour that was recently observed in *An. gambiae s.s.* in cages (50) and in *An. arabiensis* in the field (based on microsatellite identification of families; Odero & Fillinger, personal communication) is likely to benefit the auto-dissemination strategy for malaria vector control since the gravids disseminate larvicides to more than one larval habitat. Mosquitoes of other genera such as *Culex* might be used to amplify the transfer of PPF to larval habitats of *An. gambiae s.s*. (33), given that culicine and *Anopheles* larvae frequently occupy the same aquatic habitats in the field (20,21). This might significantly improve the impact of such an intervention. Nevertheless, significantly more work is required in designing highly attractive bait-stations for gravid malaria vectors. This requires the identification of more attractants and composing highly attractive chemical blends, determining better mechanisms for optimum release of the attractants, identifying better and more cost-effective mechanisms for retaining and dispensing of PPF as well as improving the physical components to provide protective barriers from rain. Last but not the least, field evaluations are necessary to confirm the performance of such novel tools under natural conditions during both the dry and rainy seasons. Further studies are required before autodissemination could be used as a supplementary measure for malaria vector control in the field.

Abbreviations

HPLC: High performance liquid chromatography icipe-TOC: International Centre of Insect Physiology and Ecology – Thomas Odhiambo Campus LC-MS: Liquid chromatography- Mass spectrometry LC/ESI-MS: Liquid chromatography/Electron spray ionization-Mass spectrometry

Declarations

Ethics approval and consent to participate

Ethical approval for this study was obtained from the Kenya Medical Research Institute's Ethical Review Committee (Protocol no. 422).

Consent for publication

Not applicable.

Availability of data and material

All data will be made available on reasonable request to the senior author.

Competing interests

Sumitomo Chemicals, Japan, provided the experimental formulation of the insecticide for this study free of charge. Nevertheless, neither the manufacturer nor any of the funders of this work had any role in the design, analysis or interpretation of the results, nor in the drafting of the manuscript.

Authors' contributions

UF conceived the idea for this research. OM, SWL and UF developed the experimental design and protocols. OM implemented the experiments, analysed the data and drafted the manuscript. All authors contributed to the final draft, read and approved the manuscript.

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Tables

Table 1: Adult emergence rates from larvae introduced into oviposition cups in cage experiments testing PPF formulations

Method of PPF presentation	Mean proportion emergence (95%CI)	Odds ratio (95% CI)	p-value
No PPF	0.89 (0.83-0.93)	1	
oil-formulated PPF	0.55 (0.35-0.62)	0.14 (0.07-0.28)	<0.001
PPF powder	0.11 (0.07-0.17)	0.02 (0.006-0.036)	<0.001

Table 2: Oviposition choice tests evaluating the attractiveness of three oviposition substrates in comparison to ponds filled with water only under semi-field conditions

Pond	Mean number of females attracted to pond (95% CI)	Rate ratio (95% CI)	p-value
Testing the attraction to soil infusion			
water+soil	39 (33 - 45)	1	
water 1	29 (25 - 33)	0.74 (0.59-0.93)	0.011
water 2	26 (22 - 31)	0.67 (0.54-0.82)	<0.001
water 3	27 (23 - 31)	0.70 (0.56-0.88)	0.002
Testing the attraction to water treated with 5 ppm cedrol			
water+5ppm cedrol	33 (30 - 35)	1	
water 1	25 (21 - 29)	0.76 (0.63-0.92)	0.005
water 2	26 (23 - 29)	0.80 (0.70-0.91)	0.001
water 3	26 (23 - 30)	0.81 (0.70-0.94)	0.005
Testing the attraction to water treated with 20 ppm cedrol			
water+20ppm cedrol	52 (46 - 60)	1	
water 1	28 (24 - 33)	0.54 (0.43-0.67)	<0.001
water 2	32 (28 - 38)	0.62 (0.48-0.80)	<0.001
water 3	27 (21 - 35)	0.52 (0.38-0.72)	<0.001

Table 3: Adult emergence rates of larvae introduced into open ponds in the three experiments to evaluate transfer of PPF in semi-field cages

Ponds	Mean proportion adult emergence (95% CI)	Odds ratio (95% CI)	p-value
Control 1- Mosquitoes released in semi-field cage & untreated netting gauze placed on top of bait station			
closest to bait station (4.4 m)	0.85 (0.82-0.87)	1	
medium to bait station (8.4 m)	0.83 (0.80-0.86)	0.87 (0.62-1.23)	0.443
furthest to bait station (10.3 m)	0.84 (0.81-0.87)	0.99 (0.71-1.37)	0.944
Control 2- No mosquitoes released in semi-field cage & netting gauze treated with PPF dust placed on top of bait station			
closest to bait station (4.4 m)	0.89 (0.86-0.91)	1	
medium to bait station (8.4 m)	0.89 (0.87-0.92)	1.03 (0.72-1.49)	0.854
furthest to bait station (10.3 m)	0.88 (0.85-0.91)	0.94 (0.65-1.34)	0.721
Test-Mosquitoes released in semi-field cage & netting gauze treated with PPF dust on top of bait station			
closest to bait station (4.4 m)	0.25 (0.22-0.29)	1	
medium to bait station (8.4 m)	0.58 (0.54-0.62)	4.07 (3.19-5.21)	<0.001
furthest to bait station (10.3 m)	0.92 (0.89-0.94)	33.89 (24.16-48.47)	<0.001
Emergence inhibition due to auto-dissemination – comparison of test with control 2			
control	0.89 (0.84-0.94)	1	
closest test pond (4.4 m)	0.25 (0.20-0.33)	0.042 (0.023-0.077)	<0.001
medium test (8.4 m)	0.58 (0.51-0.66)	0.173 (0.098-0.303)	<0.001
furthest test (10.3 m)	0.92 (0.85-0.98)	1.437 (0.846-2.444)	0.180

*Figure in parenthesis indicate the distance between the open pond and pond that served as the bait station

Table 4: Adult emergence rate of late instar larvae introduced into water in which females laid eggs

	Mean proportion emergence (95% CI)	Odds ratio (95% CI)	p-value
unexposed females	0.93 (0.89-0.97)	1	
PPF-exposed females	0.45 (0.39-0.51)	0.06 (0.03-0.10)	0.007

Figures

Figure 1

Semi-field cage showing artificial hut constructed at the centre of the semi-field cage

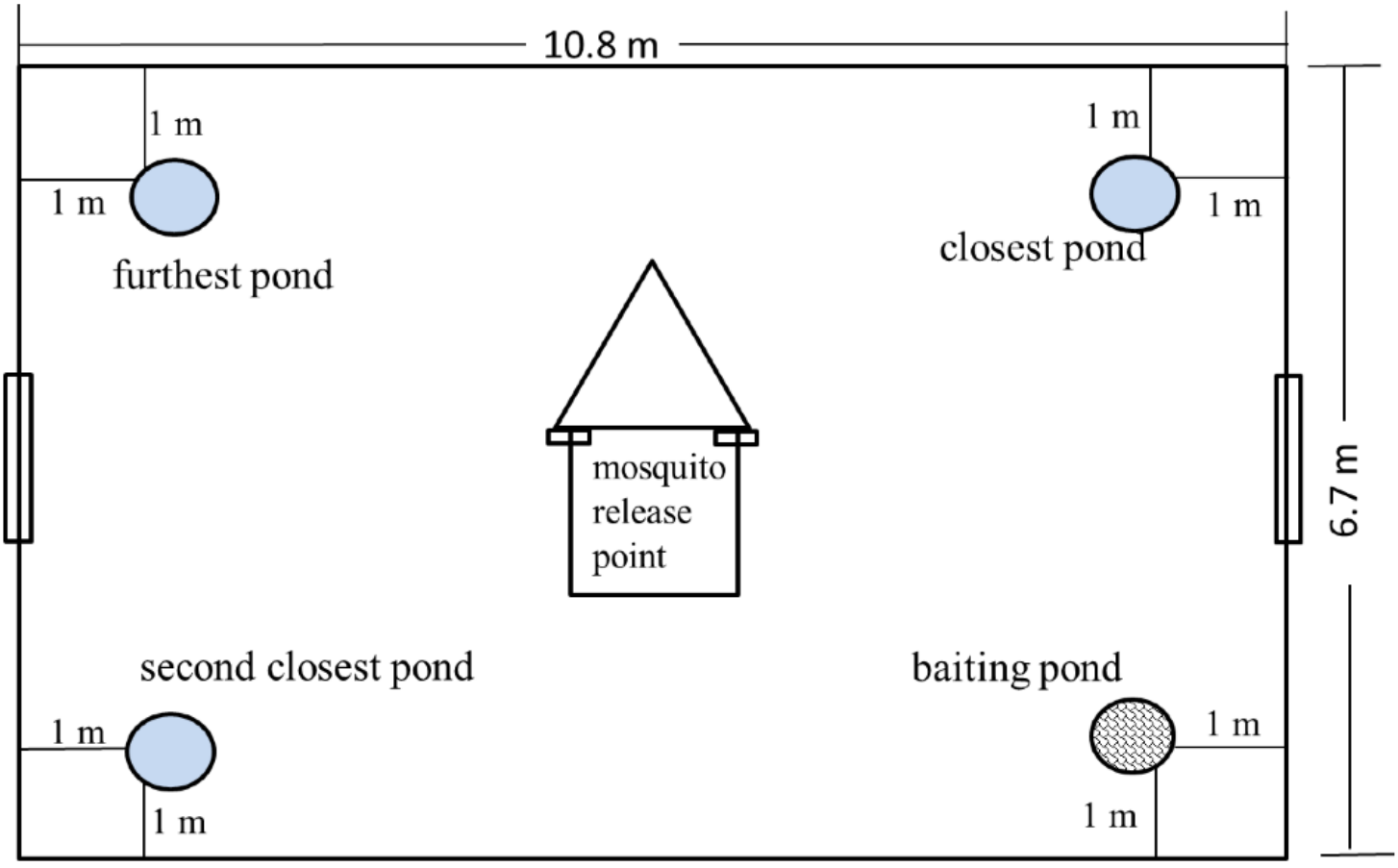

Figure 2

Schematic representation of semi-field cage showing location of ponds and the artificial hut that serve as release point of gravid mosquitoes

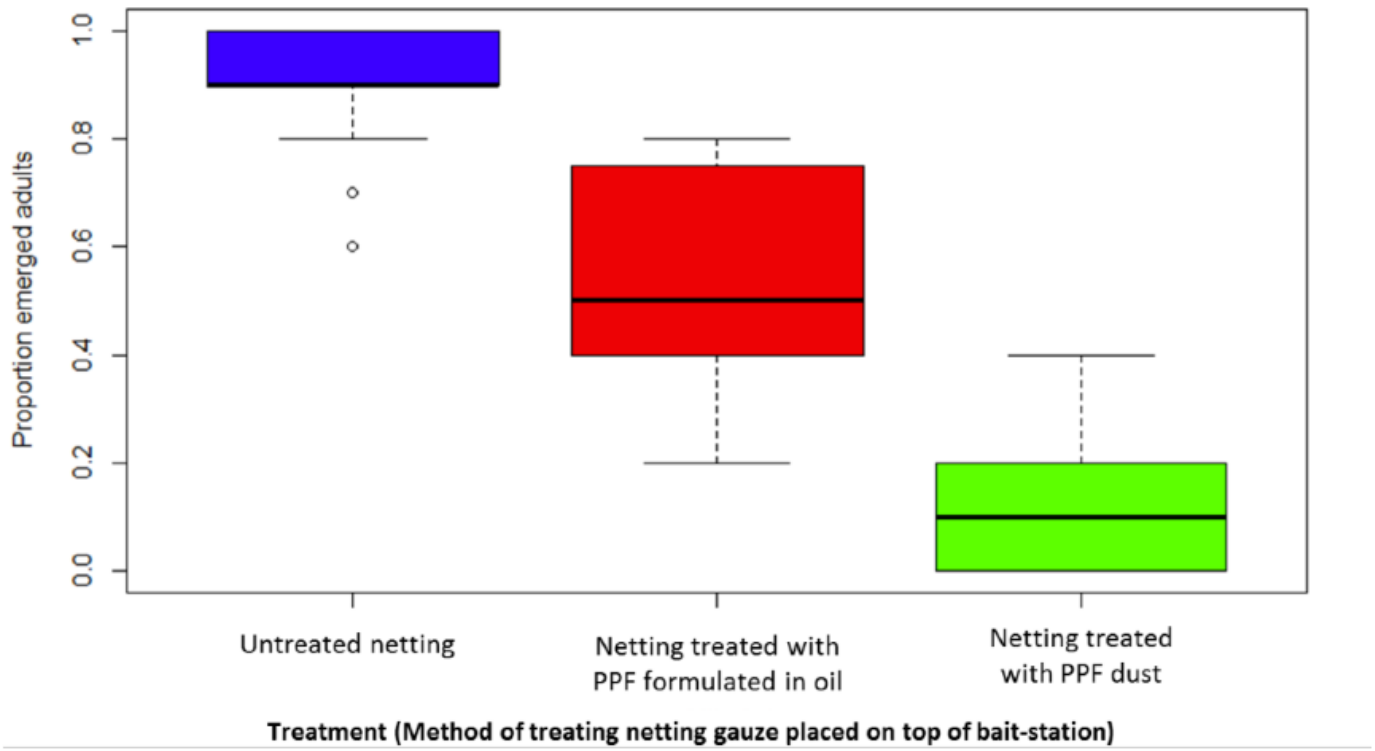

Figure 3

Box and whisker plots showing the median proportion and interquartile range of adult emerged in cage experiments to determine the best method to treat netting with PPF