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Pyriproxyfen Netting Suppresses Reproductive Output and Oviposition in Anopheles Stephensi

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Abstract

General Background: Insecticide resistance among malaria vectors poses a critical threat to global disease control strategies. **Specific Background:** Pyriproxyfen, a juvenile hormone analogue, interrupts insect growth and reproduction through sublethal contact. **Knowledge Gap:** The combined effects of pyriproxyfen-treated netting on adult fecundity, egg hatchability, and oviposition choice in *Anopheles stephensi* remain insufficiently characterized. **Aims:** This study evaluated reproductive performance and behavioral deterrence in adult females following exposure to pyriproxyfen-treated netting. **Results:** Exposure to 0.02 ppm pyriproxyfen reduced egg production by 80.7% and hatchability to 18.5%, while choice assays demonstrated 98.5% effective repellency. **Novelty:** The findings demonstrate a synergistic dual mechanism combining severe physiological sterilization with near-complete behavioral oviposition deterrence. **Implications:** Incorporating pyriproxyfen into long-lasting insecticidal nets provides an effective strategy to suppress resistant vector populations.

Keywords: Anopheles Stephensi, Insect Growth Regulator, Oviposition Deterrence, Pyriproxyfen, Vector Control

Key Findings Highlight

Short contact with treated netting reduced female egg production by up to 80.7 percent.

Sublethal exposure decreased egg hatchability down to 18.5 percent at 0.02 ppm.

Choice bioassays revealed an effective oviposition repellency rate reaching 98.5 percent.

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Introduction

Malaria remains one of the most intractable and lethal infectious diseases in the world, taking a heavy toll on human health, particularly in sub-Saharan Africa and Southeast Asia. In 2022, there were an estimated 249 million clinical cases and > 608,000 malaria-related deaths in the world, most of the mortality and morbidity in children < 5 years of age and pregnant women . The disease is caused by protozoan parasites of the genus *Plasmodium* and is only transmitted by the bite of an infected female *Anopheles stephensi* mosquito. Consequently, vector control has been a fundamental component of malaria prevention strategies for many years, and the use of the two major interventions - insecticide-treated nets (ITNs) and indoor residual spraying (IRS) - has contributed extensively to the decline in malaria incidence over the last 20 years . The rise of resistance to insecticides among *Anopheles* populations , especially against the pyrethroids used on insecticide treated nets (ITNs), is jeopardising the use of these interventions. The resistance crisis and changes in behaviours, such as early evening feeding and outdoor napping, have resulted in an urgent need for new chemical classes and innovative modes of action, which are able to avoid existing resistance mechanisms and target vulnerable life stages or behaviours never previously exploited.

Pyriproxyfen, a synthetic juvenile hormone analogue, has emerged as an appealing candidate for use in the next generation of vector control programs . Pyriproxyfen acts as a juvenile hormone mimic, a key regulator of insect metamorphosis and reproduction, disrupting normal endocrine regulation, while traditional neurotoxic insecticides kill adult mosquitoes instantaneously . Pyriproxyfen was originally developed and is widely used as a larvicide to control container-breeding mosquitoes, but more recently has attracted attention for its potent “adulticidal” and sublethal effects . Adult mosquitoes exposed to pyriproxyfen (directly, via treated nets, walls, or attractive toxic sugar baits, or indirectly, via auto-dissemination methods) can transfer pyriproxyfen to larval habitats, leading to widespread population suppression [8, 9].

In addition to its well-documented effects on larval development and adult longevity, there is mounting evidence of pyriproxyfen effects on two other, possibly synergistic, aspects of adult *Anopheles* biology: oviposition deterrence and female sterility. Oviposition deterrence is a behavioral response of gravid (egg-carrying) females to actively avoid deposition of their eggs on or around substrates contaminated with the compound and thus reduces the number of progenies entering the aquatic environment . This behavior is particularly useful because, even if adult survival is not immediately affected, it can prevent recolonization of treated sites. At the same time, pyriproxyfen may lead to decreased fertility in adult females that survive exposure. This is generally manifested as the inability to complete oogenesis, production of non-viable eggs or reduced oviposition when subsequently offered uncontaminated blood meals and oviposition sites . The combined effects of oviposition deterrence and sterility can result in a dramatic reduction in the effective reproductive rate of an *Anopheles stephensi* population and could potentially suppress malaria transmission without the need for immediate adult mortality.

Previous studies have reported reproductive impairment and oviposition-related responses after exposure to pyriproxyfen, but a reduction in egg production does not necessarily mean oviposition deterrence, because reproductive physiology and oviposition behaviour are different biological processes. Furthermore, few studies have been done on the effect of pyriproxyfen-treated netting on egg production and egg hatchability of adult *Anopheles stephensi* under laboratory-controlled conditions.

Therefore, the present study aimed to evaluate the effects of pyriproxyfen-treated netting on reproductive performance in adult female *Anopheles stephensi* by quantifying egg production and egg hatchability following exposure. Understanding the reproductive consequences of pyriproxyfen will aid in developing newer vector control methods for situations where insecticide resistance is becoming widespread.

2. Materials and Methods

2.1 Mosquito Rearing

Anopheles stephensi (Diptera: Culicidae) were obtained from the laboratory colony kept at the insectary of our Institution. Mosquito larvae were nourished daily using a mixture of fish meal and yeast in 3:1 proportion. Setting up the experiments involved placing larvae into containers made of plastic, used de-chlorinated water and measuring approximately 30 × 20 × 8 (cm) . Pupae were collected daily using plastic pipettes and placed into adult rearing cages (30 × 30 × 30 cm). Adult mosquitoes were kept under controlled environmental conditions: temperature 27 ± 2°C, relative humidity 70 ± 10%, and a 12:12 h photoperiod (L:D) . Adults were fed ad libitum on 10% sugar solution soaked in cotton wool. All experiments were approved by the institutional animal care and use committee.

2.2 Preparation of Pyriproxyfen-Treated Netting

Technical grade pyriproxyfen (Sumilarv®, Sumitomo Chemical Co., Ltd., Tokyo, Japan) was diluted with isopropyl alcohol to prepare two concentrations, 0.005 ppm and 0.02 ppm (wt: vol) (Agbevo et al. 2024). The dosage of pyriproxyfen was based on previous studies demonstrating both sublethal and sterilizing effects on anopheline mosquitoes [14-16].

Polystyrene mesh (17 × 17 cm) with 75 holes per square inch (i.e. equivalent to Olyset Net, Sumitomo Chemical Co.) was soaked in each of the pyriproxyfen solutions for 1 hour . The untreated control netting was only dipped in isopropyl alcohol. After immersion, all net pieces were dried overnight at room temperature (25 ± 2°C) under a fume hood. Dried netting pieces were kept in sealed Aluminium foil packets at 4°C until use as described by .

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2.3 Exposure of Adult Females to Treated Netting

Three treatment groups were used, each with 45 non-bloods fed adult female *Anopheles stephensi* (2–4 days old). Each treatment group contained 15 females with three biological replicates of 5 females per cage. Females were exposed to pyriproxyfen-treated netting following standard World Health Organization cone bioassay protocols . Each treated net piece was mounted in a plastic panel at a 45° angle as described by .

Batches of 15 females were placed gently in WHO cones (11 cm in diameter; manufactured under standard conditions) and placed on the treated netting for 3 min . The reason for the 3-minute exposure was due to prior studies finding this exposure duration sufficient to allow sufficient pyriproxyfen absorption, while minimizing stress associated with handling. Three exposure groups were established:

- **Control group:** Exposure to untreated netting (only isopropyl alcohol)
- **Low concentration group:** Exposed to 0.005 ppm pyriproxyfen-treated netting
- **High concentration group:** Exposure to netting treated with 0.02 ppm pyriproxyfen

The females were collected from the cones using a mouth aspirator after exposure and transferred to holding cages (21 × 21 × 28 cm) with access to 10% sugar solution [6, 17].

2.4 Blood Feeding and Oviposition

After exposure, all females within each of the experimental treatment groups were maintained in three replicate cages, each of which contained five females, and also had 10 males (adults) for mating purposes and to permit multiple opportunities for copulation. Blood meal was collected from domestic pigeons (Columba livia). The male pigeons were used in the study as the removal of feathers from the breast allows easy access of the mosquitoes to the blood vessels, as reported earlier . The females were available overnight for blood-feeding by pigeons.

Eggs were collected using three 200 mL plastic cups per cage that each contained 150 mL of dechlorinated (untreated) water for five days after receiving blood meals . Eggs were counted each day.

All oviposition cups were placed in untreated water and therefore the females lacked choice between treated and untreated oviposition sites. This design is consistent with the study’s intent to investigate reproductive performance rather than oviposition site preference or deterrence behavior.

2.5 Oviposition Deterrence Assay

This experiment was done according to the method of Soonwera & Phasomkusolsil (2017) with slight modifications . In the rearing cages (25□20□20) cm, 20 pairs of mosquitoes were introduced aging 6-8 hours were put in cages, the adults were fed 10% sugar solution. The females were fed blood by putting a pigeon on the top of the cage overnight after removing its chest feather and tiding its wings and feet. A plastic container of 200 ml fill with 150 ml of the higher concentration (0.02 ppm) of diluted pyriproxyfen with isopropyl alcohol and lower concentration (0.005 ppm), with control (untreated just water) and with three replicate for each concentration, were put randomly inside the cage as number from 1 to 9 to nullify any effect of their locations on oviposition. The cages were monitored daily for 5 days to see the laid eggs. After that, the eggs laid by the females in the containers were calculated.

The oviposition experiments were expressed as mean number of eggs and the Oviposition Activity Index (OAI) was calculated using the following formula:

OAI = (NT - NC) / (NT + NC)

Where:

- OAI = NT = the total number of eggs/females in the treatment container
- NC = the total number of eggs/ in the control container

OAI: value from -1 to +1, with 0 indicating normal response. OAI positive values means that more eggs were deposited in the treatment than in the control, and that the tested treatment were attractive. OAI negative value means that if there are more eggs in the control beakers than in the treatment, thus the tested treatment is deterrent. The effective repellency ER% as percentage for each treatment was calculated as a deterrent using the following formula :

ER% = [(NC - NT) / (NC + NT) × 100

2.6 Egg Count and Hatchability Evaluation

Eggs collected daily from each oviposition cup using a small paintbrush and were counted under a stereomicroscope (Olympus SZ61, Tokyo, Japan) at magnification 40×. *Anopheles stephensi* lay their eggs individually (not grouped), and each egg was counted individually .

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After counting, eggs from each replicate were placed separately into rearing trays (25 × 15 × 5 cm) containing 500 mL of dechlorinated water to rear and kept under similar environmental conditioning (27 ± 2 °C, 70 ± 10 % RH, 12:12 l:d); and the number of first-instar larvae that hatched from each egg batch was counted every day for 7 days . Percent hatchability was worked out as:

Hatchability (%) = (Number of eggs hatched / Total number of eggs) × 100

Embryonic development was monitored by observing unhatched eggs under the microscope .

2.7 Statistical Analysis

Egg production and hatchability were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons between treatment groups . $P < 0.05$ was considered statistically significant. Normal distribution of the data was verified by Shapiro–Wilk tests ($P > 0.05$ for all groups) and homogeneity of variances was confirmed by Levene's test ($P = 0.23$). Analyses were performed with SPSS version 25.0 (IBM Corp., Armonk, NY, USA).

2.8 Ethical Recommendation

All procedures on pigeons were carried out in accordance with institutional guidelines for the care of animals and were approved by the relevant ethical review committee.

3. Results

3.1 Effect of Pyriproxyfen on Oviposition (Egg Laying)

Exposure of adult female *Anopheles stephensi* to pyriproxyfen-treated netting had a significant effect on egg production (Table 1). Females subjected to untreated netting produced 643 eggs that averaged 42.9 ± 3.8 eggs from each female and had a hatch rate of 96.6%, indicating normal embryonic development and successful hatching of larvae. Exposure to pyriproxyfen at 0.005 ppm lowered total egg production to 276 eggs, which is 18.4 ± 1.9 eggs/female. Exposure to 0.02 ppm reduced egg production to only 124 eggs, which is 8.3 ± 0.6 eggs/female.

Egg production was reduced by 57.1 % at 0.005 ppm and by 80.7% at 0.02 ppm compared with the control group. One-way ANOVA indicated significant differences among treatment groups ($F(2,6) = 38.4$, $P < 0.001$). Tukey's multiple comparison test showed significant reductions in egg production in both pyriproxyfen-treated groups compared with the control ($P < 0.01$ for 0.005 ppm; $P < 0.001$ for 0.02 ppm). These results show a concentration-dependent reduction of female reproductive output after short exposure to pyriproxyfen-treated netting.

Table 1. Effect of Pyriproxyfen Exposure on Egg Production of Adult Female *Anopheles stephensi*

Treatment	Total eggs	Mean eggs/female (± SE)	% Reduction
Control	643	42.9 ± 3.8a	—
Pyriproxyfen 0.005 ppm	276	18.4 ± 1.9b	57.1%
Pyriproxyfen 0.02 ppm	124	8.3 ± 0.6c	80.7%

Table 1.
Values with different letters are significantly different (Tukey test, $P < 0.05$).

3.2 Impact of Pyriproxyfen Exposure on Egg Hatchability

Egg viability was also significantly affected by pyriproxyfen exposure (Table 2). Eggs from control females had a hatchability of 96.6%, indicating normal embryonic development and successful emergence of larvae. Pyriproxyfen caused a significant reduction in hatchability. Egg hatchability from females exposed to 0.005 ppm was 52.5%. Hatchability of eggs from females exposed to 0.02 ppm was only 18.5%. Hatchability was decreased 45.7 % at 0.005 ppm and 80.8 % at 0.02 ppm compared to the control. The decrease in hatchability was statistically significant ($F(2,6) = 52.1$, $P < 0.001$) and showed a clear concentration-dependent response. Microscopic observation revealed that there were many unhatched eggs in the pyriproxyfen-treated groups, suggesting that the normal embryonic development was disrupted.

Table 2. Effect of Pyriproxyfen Exposure on the Egg Hatching of Adult Female *Anopheles stephensi*

Treatment	Total eggs	Hatched Larvae	Hatchability ± SE (%)
Control	643	621	96.6 ± 0.72%a
Pyriproxyfen 0.005 ppm	276	145	52.5 ± 3.01%b
Pyriproxyfen 0.02 ppm	124	23	18.5 ± 3.49%c

Table 2.
Values with different letters are significantly different (Tukey test, $P<0.05$).

3.3 Oviposition Deterrence Assay

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In order to assess whether pyriproxyfen provides a behavioral deterrent for ovipositing females, we performed a choice assay in which gravid female insects had the option of laying eggs in untreated, shallow water or in water treated with pyriproxyfen at either 0.005 ppm or 0.02 ppm (Table 3).

Table 3. Oviposition Deterrent Effects of Pyriproxyfen against *Anopheles stephensi* in Choice Assays.

Replicate	Control (untreated water)	0.005 ppm Pyriproxyfen	0.02 ppm Pyriproxyfen
1	150	25	2
2	175	10	0
3	210	27	2
Total eggs	535	62	4
Mean eggs ± SE	178.3 ± 17.6	20.7 ± 5.5	1.3 ± 0.7

Table 3.

At 0.005 ppm, the untreated containers had a total of 535 eggs laid while only 62 eggs were laid in the treated containers. There was an 88.4% reduction in oviposition at 0.005 ppm. At 0.02 ppm, a total of 4 eggs were laid in the treated containers compared to 535 in control containers, representing a 99.3% reduction in oviposition.

The Oviposition Activity Index (OAI), a measure of oviposition activity, was calculated as:

OAI = (NT - NC) / (NT + NC).

Calculating OAI at 0.005 ppm results: OAI = (62 - 535) / (62 + 535) = -473 / 597 = -0.79.

Calculating OAI at 0.02 ppm results: OAI = (4 - 535) / (4 + 535) = -531 / 539 = -0.99.

Consequently, both concentrations produced strong deterrency as evidenced by the consistently negative OAI values, with the greatest deterrent effect occurring at the higher concentration. The effective repellency percentage (ER%) was calculated as follows:

ER% = [(NC - NT) / (NC + NT) × 100.

For 0.005 ppm, ER% = [(535 - 62) / (535 + 62)] × 100 = (473 / 597) × 100 = 79.2%.

For 0.02 ppm, ER% = [(535 - 4) / (535 + 4) × 100 = (531 / 539) × 100 = 98.5%.

These results confirm that pyriproxyfen is a potent ovipositional deterrent, as females are strongly deterred from depositing eggs into the treated water. The concentration-dependent deterrent effect was nearly complete at the higher concentration.

3.4 Combined Effect on Reproductive Success

The combined reduction in egg production and hatchability resulted in a major reduction in overall reproductive success. Females exposed to 0.005 ppm pyriproxyfen produced only 145 viable larvae, and those exposed to 0.02 ppm produced only 23 viable larvae, whereas control females produced 621 viable larvae.

In general, pyriproxyfen exposure decreased the production of viable offspring by approximately 76.7% at 0.005 ppm and 96.3% at 0.02 ppm compared to the untreated control. The results show that pyriproxyfen exposure can strongly reduce mosquito population recruitment by reducing both fecundity and fertility at the same time.

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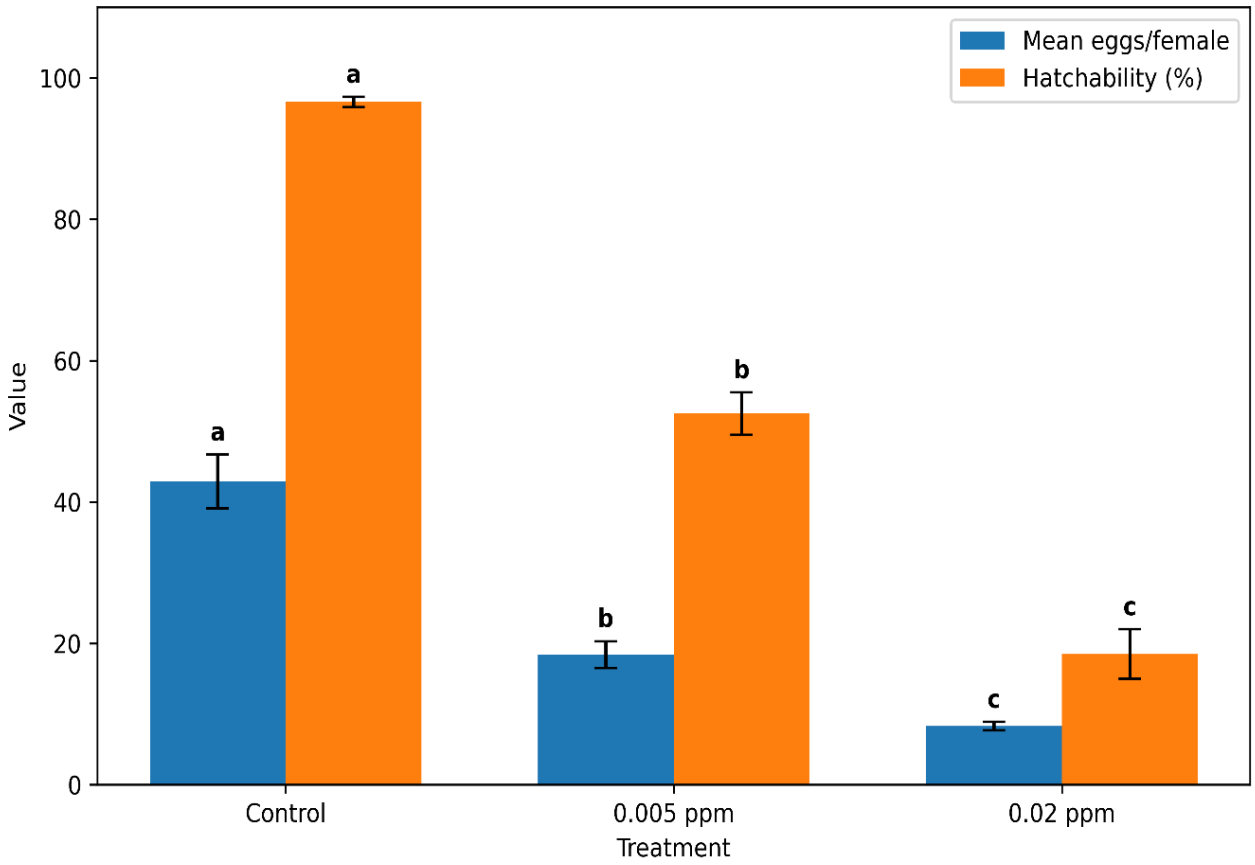

Figure 1.

Figure 1. Pyriproxyfen Exposure Effect on Egg Production and Hatchability

Mean egg production and egg hatchability (%) of *Anopheles stephensi* females after exposure to untreated netting (control) or netting treated with pyriproxyfen (0.005 ppm and 0.02 ppm). Error bars are standard error of the mean. Letters above bars show significant differences among treatments ($P < 0.05$).

4. Discussion

The present study revealed that the egg production and egg hatchability of adult female *Anopheles stephensi* exposed to pyriproxyfen-treated netting were significantly reduced. Reproductive suppression increased in magnitude with increasing pyriproxyfen concentration showing a clear dose response. These findings corroborate the existing literature suggesting that pyriproxyfen may influence the reproduction capacity of mosquitoes and, therefore, overall reproductive success of the mosquito population [5-7, 19].

Disruptions along these critical biological pathways can result in lower egg production, and fewer viable eggs from blood meals [24-26]. Lower egg production and viability rates have been found for *Anopheles gambiae* as well as other mosquito species that have been exposed to surfaces/netting previously treated with pyriproxyfen [6, 27, 28].

Furthermore, the hatching rate of the eggs was also affected by pyriproxyfen's effects on their production & development, continuing to support previous research that addressed pyriproxyfen's effects on disrupting embryonic development as well as successful larval emergence [8, 11, 16]. There have been instances where embryonic development has occurred with very little to no viability due to pyriproxyfen transitioning from the adult female to oocytes . Despite this large decline seen with the hatch rate at 0.02 ppm, it would appear that pyriproxyfen has the potential to agnostically affect the reproductive success of female mosquitoes, but some eggs were still oviposited.

The reduced number of viable offspring produced by insects is an important consideration for vector control programs as it reduces the population size of subsequent mosquito generations. Pyriproxyfen differs from conventional adulticides in that it affects the population indirectly by affecting the reproductive and recruitment processes of mosquitoes after they have been exposed [6, 9]. This has potential implications on the long-term suppression of mosquito populations in regions where traditional interventions have been compromised by

pyrethroid-resistance .

Notably, the present study reports on an effective ovipositional inhibiting ability of pyriproxyfen. Results from the choice test suggest that female mosquitoes choose not to lay their eggs in water treated with pyriproxyfen at an OAI of –0.79 at 0.005 ppm and –0.99 at 0.02 ppm, where the corresponding effective repellent percentages (ER%) are 79.2% and 98.5%, respectively. This level of reptile avoidance from laying eggs in treated water supports previous findings indicating that pyriproxyfen will deter females from laying eggs in containers . Thus, the high level of deterrent activity at 0.02 ppm indicates that any water body contaminated with pyriproxyfen should be very unattractive to gravid females; consequently, the presence of this compound should decrease the number of eggs deposited into the water prior to the establishment of any physiological effects (such as mortality or infertility) on the mosquito population.

The dual role of pyriproxyfen—via its ability to act as both a physiological sterilizing agent and as a behavioral oviposition deterrent—creates a synergistic opportunity to suppress the mosquito population. Through a combination of the two actions, a female's reproductive potential is limited if she lays eggs in untreated water areas, while pyriproxyfen prevents females from depositing their eggs in treated areas a priori, resulting in more than one way to reduce the larval recruitment that ultimately will impact on the adult population.

The use of pyriproxyfen-treated nets can hinder the ability of mosquitoes to reproduce and survive, thus making it less likely that any mosquitoes that do survive will be able to transmit malaria [6, 29]. As a result, the use of pyriproxyfen in second-generation long-lasting insecticide-treated nets will create an additional effective mode of action for controlling mosquitoes that transmit malaria [5, 9]. Furthermore, Akram et al. (2025) demonstrated that pyriproxyfen (0.5 WDG) was significantly more efficacious than temphos in suppressing *Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus* in Pakistan and exhibited residual activity for 3.5 - 4 months when used at the recommended rates. These findings support the premise of utilizing pyriproxyfen for the production of LLINs .

Importantly, the current study measured reproductive outcomes and not oviposition-site selection behavior. Females were only provided with untreated oviposition cups so no conclusions can be made about oviposition deterrence or behavioural avoidance. The observed decreases in egg numbers are more properly interpreted as physiological effects on reproductive capacity rather than as evidence for altered oviposition preference.

Although the experiments were conducted under laboratory conditions, the results provide evidence that short contact with pyriproxyfen treated netting can result in a significant impairment of the reproductive performance of *Anopheles stephensi*. Further studies should assess how long these effects persist in semi-field and field settings and how much they contribute to the long-term reduction of malaria transmission.

5. Implications for Malaria Control

The sterilizing action of pyriproxyfen has far-reaching ramifications for the spread of malaria [6, 29]. After ingestion by the mosquito the *Plasmodium* parasite requires an incubation time of around 10-14 days (extrinsic incubation period) to mature before it can be spread . Even if a female survives long enough to become infectious, if she has been sterilized by exposure to pyriproxyfen, her blood meals will not create following generations of vectors . Pyriproxyfen has been shown to have both individual protection and population control when applied to treated nets [32-34]. Because of its dual action of providing individual protection and reducing mosquito populations, the use of pyriproxyfen should be seriously considered as a new generation tool for mosquito control.

6. Limitations of the Study

Several limitations need to be taken into account when interpreting the results of this study. The first limitation is that all the experiments were performed under laboratory control conditions; therefore the results may not account for environmental variability in field conditions , and the second limitation is that the study was performed with *Anopheles stephensi* that were susceptible and were maintained under laboratory controls; therefore, the results may not represent the response of an insecticide resistant population of *Anopheles stephensi*.

Third, the study was restricted to reproductive outcomes and oviposition preference, or deterrent behaviour were not directly tested as the females were only offered untreated oviposition sites. Finally, the persistence of pyriproxyfen activity on treated netting and long-term effects over multiple gonotrophic cycles were not explored. Further research is required to fill these gaps.

7. Conclusions

In the present study, a short exposure of adult female *Anopheles stephensi* to pyriproxyfen-treated netting caused a significant reduction in egg production and egg hatchability. Reproductive suppression was concentration dependent and the greatest reduction in fecundity, fertility and production of viable offspring was with the highest pyriproxyfen treatment.

These results suggest that pyriproxyfen may have important effects on mosquito reproductive success and recruitment of populations. The integration of pyriproxyfen into integrated vector management programmes and next-generation insecticidal nets may therefore provide an additional tool to suppress malaria vector populations

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particularly in areas of increasing insecticide resistance. Further laboratory, semi-field and field studies are required to confirm the long-term epidemiological relevance of these reproductive effects.

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