Laboratory evaluation of pyriproxifen treated bednets on mosquito fertility and fecundity. A preliminary study

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Summary

Aim: **The study evaluated the effect of pyriproxifen treated bednets on mosquito fertility and fecundity in the laboratory. Pyriproxyfen (insect growth regulator) has been considered as a possible tool for management of pyrethroid resistance in mosquitoes. There are documentations of the effectiveness of pyriproxifen in controlling larval development when applied directly to breeding site. Considerations have been given to the use of pyriproxyfen on bed nets for sterilizing effect on mosquitoes resistant to pyrethroids.**

Method: **Groups of mosquitoes (*Anopheles stephensi* - Beech) were exposed to bednet treated with 2% Pyriproxyfen and untreated netting 24 hrs after blood feeding. After oviposition, egg counts were done for each mosquito in the two groups. Larvae emerging from the laid eggs by each mosquito were counted. Three sets of experiments were successful.**

Results: **There was no significant difference in number of eggs laid between the two treatment groups (p=0.177). There was statistically significant difference in the number of laid eggs that hatched between the treatment types (p=0.0061). There was also statistically significant difference in the mean number of eggs that hatched between the experiments (p=0.0013). The mean number of eggs retained in the pyriproxyfen group was higher (70.3) than in the control group (41.6). This difference was not statistically significant with the small sample tested (p=0.08).**

Conclusion: **The results suggest that 2% pyriproxyfen on bed nets has no effect on *An. stephensi* fecundity. Reduced fertility of eggs laid by mosquitoes exposed to pyriproxyfen treated bed nets was observed.**

Key-words: *Pyriproxifen, Mosquito, Fecundity, Fertility.*

Résumé

Dessein: Evaluer l'effet du filet du lit médicalement traité avec pyriproxifene sur la fertilité et fécondité du moustique dans le laboratoire. Pyriproxyféne (régulateur de la croissance des insectes) a été considérée comme un outil probable pour la prise en charge de la résistance pyréthroide chez les moustiques. Il y a des documentations sur l'éfficacité de la pyriproxiféne dans le contrôle du développement de la larve quand elle est appliquée directement sur le lieu de production. On a mis une question à l'étude de l'utilisation de la pyriproxyféne sur les filets du lit pour l'effet de la stérilization sur la résistance des moustiques par rapport au pyréthroides.

Méthode: Groupe des moustiques (anophèles stephensi-Beech) ont été exposés au filet ddu lit médicalement traité avec 2% pyriproxyféne et un filet du lit sans traitement 24 heures après affourragement du sang. Après oviposition, on avait compté les oeufs de chaque moustique dans les deux groupes. On avait compté les larvae qui sortent des oeufs par chaque moustique. Trois séries d'epériences étaient connu du succés.

Résultats: Il n'y avait aucune différence importante dans les quantités des oeufs pondus entre les deux groupes traités (p 0,177). Il y avait une différence statistiquement differente dans la quantité des oeufs pondus qui étaient incubés entre les séries traitées (p0,0061). Il y avait également une différence statisquement importante dans les quantités moyenne des oeufs incubés entre les expériences (p0,0013). La quantité moyenne des oeufs gardés dans le groupe de pyriproxyfène étaient plus élevée (70,3) plus que dans le groupe témoin (41,6). Cette différence n'était pas statisquement importante par rapport au petit groupe d'échantillon étudié (p 0.08).

Conclusion: A travers le résultat, on peut dire que 2% de la pyriproxyfène sur filet du lit n'a aucun effet sur la fécondité d'anstephensi. Fertilité infériure des oeufs pondus par des moustiques exposés au filet du lit traité avec la pyriproxyféne était notée.

Introduction

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium* transmitted by anopheline mosquitoes. Of the four species that affect humans, *Plasmodium falciparum* is the most deadly and is associated with high morbidity and mortality in children under the age of 5 years and pregnant women due to its prevalence, virulence and drug resistance [1]. *Plasmodium. falciparum* causes approximately 1 million deaths and over 200 million clinical events among Sub -Saharan Africans every year[1]. It has been estimated that 90% of malaria deaths occur in sub-Saharan Africa and of this 80% are children below the age of 5 years[1].

Insecticide treated bed nets (ITN) reduce clinical malaria episodes by 48% and improve anaemia status by an average of 0.5 g/dl and regular use prevents approximately

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6 deaths for every 1000 protected children under the age of 5 years[2]. These gains are being threatened by the emergence of pyrethroid resistance in several anopheline species[3-6].

Effective resistance management requires identifying alternative classes to pyrethroids for use on nets. Insect growth regulators (IGR) such as pyriproxyfen, have been shown in several studies to be effective in controlling larvae of various species of mosquitoes[7-10]. Use of granular formulations of pyriproxyfen at 0.01 to 0.1 mg/l has been documented to inhibit adult emergence when applied for larval control[11-14]. Miller[15] using bed net treated with pyriproxyfen at 0.5g/m^2 showed a reduction in fecundity in pyrethroid susceptible *An. stephensi* (Beech) and resistant *An. stephensi* (Dub/Apr) which were fed on human subjects through nets.

It is considered necessary to exploit the female sterilizing property of insect growth regulators such that when a pyrethroid -IGR mixture is applied to nets, pyrethroid resistant mosquitoes making prolonged contact with the net will pick up particles of IGR and will be sterilized and therefore will be unable to pass their pyrethroid resistant genes to the next generation.

Aims of this study are to

1. Test the effect of pyriproxifen on female fertility (egg fertility assessed as percentage of laid eggs which hatch) and
2. Test the effect of pyriproxifen on female fecundity (number of eggs laid per female).

Materials and Methods

A bednet treated with 2% Pyriproxyfen used in this study was provided by the Sumitomo Corporation Ltd.,Osaka, Japan. A laboratory colony of *Anopheles stephensi* (Beech) was used in the study. Eggs were obtained from the Insectary at the London School of Hygiene and Tropical Medicine. They were reared at room temperature of 25°C± 2°C and a high relative humidity, maintained by placing water soaked sponges under polythene covers of the cages and there was a photophase: scotophase of 16:8hours. Eggs were placed in ovipostion bowls containing one-day-old tap water for 48 hours and then transferred to breeding bowls. Larvae were fed with sprinkles of Farley's baby food once daily. Pupae were collected with a pipette and transferred to cages for adult emergence. The adults were fed with 10% glucose solution by wick. Adult females were fed by membrane feeding using defibrinated horse blood[16]. Forty-eight hours after the blood meals oviposition bowls were provided. The colonies were self-perpetuating.

Test of effect on fecundity and fertility

Three sets of experiments were successfully completed. In the first 2 experiments, 24 hrs after blood feeding groups of 20 female mosquitoes were exposed for 30 minutes to untreated (control) and pyriproxyfen treated bed netting. In the 3rd experiment groups of 20 mosquitoes were exposed for 1 hour. The exposed batches of insects were then placed in separate cages. After 48 hrs the exposed mosquitoes were placed individually in glass tubes that were lined with cotton wool and filter paper and filled with enough water to allow egg laying. Eggs were left to hatch and later counted using a dissecting microscope. The total number of eggs and hatched larvae per female mosquito were recorded. In the 2nd and 3rd experiments mosquitoes that did not lay eggs were dissected to record the presence of eggs in ovaries.

Eggs laid and hatched were recorded for each experiment and treatment group. Results were analysed using Statistical packages (Excel, STATA and Epi info). P value of 0.05 was taken as significant.

Results

Effect of pyriproxifen on mosquito fecundity and egg fertility

In the first two weeks of the study,newly emerged mosquitoes aged 1-2 days were exposed unfed to pyriproxifen treated bed nets or untreated bed net. In both groups there was high mortality (70-80%) immediately after exposure and those that survived in both groups refused to feed. These were thought to be too young hence disinclined to feed. The experiment was repeated. Groups of 2-3 days old unfed mosquitoes were exposed and a high mortality (70%) post exposure was noted. The mosquitoes in both groups also refused to feed. In the third batch, 3-4 days old unfed mosquitoes were exposed and they too had a high mortality after exposure, this was presumably due to handling.

Table 1 Egg laying /oviposition rate

	Age of used mosquitoes	Control: No females tested	Control: No. surviving 3days	Control: No. laying eggs(%)	Pyriproxifen: No. of females tested	Pyriproxifen: No surviving 3 days	Pyriproxifen: No laying eggs (%)
Exp. 1	5-6 days	20	10 (50%)	7 (70)	20	15 (75%)	12 (80)
Exp. 2	8-9 days	20	15(75%)	5 (33.3)	20	9 (45%)	5 (55.5)
Exp. 3	7 days	20	14(70%)	9 (64.2)	20	17(85%)	8(47.0)
Total		**60**	**39(65%)**	**21(53.8)**	**60**	**41(68.3%)**	**25 (60.9)**

In the fourth attempt a group of 2-3 days old female mosquitoes were exposed for 30 minutes after blood feeding. There were 20 mosquitoes each in the treatment group. In the control group, 14 (70%) mosquitoes survived, while in the pyriproxifen group 15 (75%) survived. None of the mosquitoes in both groups laid eggs. Dissection revealed eggs in 3 mosquitoes in control group and in 4 of the pyriproxyfen treated. Therefore a high proportion in control (85%) and in pyriproxyfen (80%) groups failed to produce eggs despite blood feeding. From this point onwards the outcomes were more successful.

Experiment 1: A group of 5-6 days old blood fed females (20 per group) were exposed for 30 minutes and of these 10 (50%) of the control group survived while 15(75%) in the pyriproxyfen group survived. In the control group 7 (70%) of surviving mosquitoes laid eggs (Table 1). The mean number of eggs laid was 93 and the mean number of hatched eggs was 76 (Table 2). In the pyriproxyfen group 12 (80%) of surviving mosquitoes laid eggs (Table 1). The mean number of eggs laid was 74.5 while the mean number that hatched was 46.0 (Table 2). Mortality of mosquitoes after exposure was noted to be higher in the control group than in pyriproxyfen group. The proportion of laid eggs that hatched was significantly lower in the pyriproxifen group (56.2 %) than in the control group (79.6%) ($t=2.5$, $df=17$, $p=0.02$) (KW 4.5, $df=1$, $p=0.03$). There was no statistically significant difference in the mean number of eggs laid in both groups ($t=1.2$, $df=13$, $p=0.24$). There was also no statistically significant difference in the mean number of laid eggs that hatched in both groups ($p=0.07$).

Experiment 2: A group of 8-9 days old blood fed females were exposed for 30 minutes, 15 (75%) survived in the control group while 9(45%) survived in the pyriproxyfen group. Of these, 5 laid eggs in both groups (33.33% in control and 55.55% in pyriproxyfen group) (Table 1). In the control group the mean number of eggs laid was 62.8 and the mean number of hatched eggs was 40.8. In the pyriproxyfen group the mean number of eggs laid was 67.4 and the mean number that hatched was 14.2.The proportion that hatched in the pyriproxyfen group (14.6%), was significantly lower than the proportion that hatched in the control group (66.0%), ($t=5.7$, $df=6$, $p=0.001$) (KW 6.81 $df=1$, $p=0.009$)(Table 2). There was no statistically significant difference in the mean number of eggs laid (0.19, $df=8$, $p=0.85$; KW 0.27 $df=1$ $p=0.60$). Kruskal Wallace non-parametric Anova test showed a significant difference in the mean number that hatched between the two groups a lower hatch rate was noted in the pyriproxifen group ($KW=3.93$ $df=1$ $p=0.04$). However this result was not confirmed by t- test ($t=1.8$, $df=8$, $p=0.1$). Fewer eggs hatched in experiment 2 compared to experiment 1 in both control and pyriproxifen groups.

Experiment 3: In the this group, 7 days old blood fed females were exposed for 1 hour and 14 (70%) survived in the control group while 17(85%) survived in the pyriproxyfen group. 9 (64.28%) of the surviving mosquitoes laid eggs in control group and 8 (47.05%) laid eggs in the pyriproxifen group (Table1). The mean number of eggs laid in the control was 92.8, the mean number of hatched eggs was 69.4. In the pyriproxyfen group the mean number of eggs laid was 88.6 while the mean number that hatched was 45.12. (Table 2). The proportion that hatched in the pyriproxyfen group (48.2%) was significantly lower than the proportion that hatched in the control group (78.6.0%) ($t=3.12$, $df=15$, $p=0.007$; KW 6.3, $df=1$, $p=0.012$) (Table 2). There was no statistically significant difference in the mean number of eggs laid in both groups (0,25, $df=15$, $p=0.8$; KW 0.002, df1, $p=0.96$). There was a statistically significant difference in the mean number of laid eggs that hatched in both groups ($t=2.1$, $df=11$, $p=0.05$; KW 4.0, $df=1$,$p=0.04$). 2-way analysis of variance using STATA was applied to data from all three experiments together. It showed no significant difference in number of eggs laid ($F=1.80$ $p=0.177$) between the two treatment groups. There was statistically significant difference in the number of laid eggs that hatched between the treatment types ($F=5.76$, $p=0.0061$). There was also statistically significant difference in the mean number of eggs that hatched between the experiments ($F=11.97$ $p=0.0013$). There were also significant differences between experiments ($F=27.8$, $p=0.0001$).

Table 2 Egg laid and hatch count

	Control						Pyriproxifen	
	Mean eggs laid	Mean No. Hatched	Percentage hatched (%)	No. of mosquitoes	Mean No. laid	Mean No. hatched	Percentage Hatched(%)	N
Exp.1	93	76	79.6	7	74.7	46.0	56.2	12
Exp.2	62.8	40.8	66.0	5	67.4	14.2	14.6	5
Exp.3	92.8	69.4	78.6	9	88.8	45.1	48.2	8
Total	**85.8**	**77.7**	**74.0**	**21**	**64.8**	**39.4**	**45.6**	**25**

Table 3 Eggs retained by mosquitoes in Experiments 2 and 3

	Control		Pyriproxifen		
	Mean eggs retained	No of mosquitoes	Mean retained	No of mosquitoes	
Exp.2	25.8	8	43.0	4	
Exp.3	67.0	5	82.8	9	
Total	41.6	13	70.3	13	p=0.08

Overall trends

Effect on mosquito fecundity

In the experiments and between the two treatment groups there was no difference in mean number of eggs laid. This suggests that pyriproxyfen on bed nets has no effect on *An. stephensi* fecundity.

Effect on egg fertility

There were significant differences between the treatment groups in the mean number of laid eggs that hatched and in the proportion of eggs that hatched. This indicates a reduced fertility of eggs laid by mosquitoes exposed to pyriproxyfen treated bed nets.

Egg retention

Egg count by dissection was done in experiments 2 and 3, this was not done in experiment 1. The mean number of eggs retained in the pyriproxyfen group was higher (70.3) than in the control group (41.6). This difference was not statistically significant with the small sample tested (p=0.08) (Table 3).

Mosquito survival

Overall, 39 (65%) of the 60 mosquitoes held for 3 days after exposure in the control group survived, of those that survived 21 (53.8%) laid eggs. In the pyriproxyfen group 41 (70%) of the 60 exposed mosquitoes survived for 3 days after exposure. Of these 25 (60.9%) laid eggs. (Table 1). A low rate of oviposition was seen in both groups and appeared not to be associated with treatment types.

Discusssion.

The study was undertaken to observe the effect of pyriproxyfen on mosquito fertility and fecundity after tarsal contact with pyriproxyfen residues on bednet. Miller[15] showed a reduction in *An. stephensi* fecundity after exposure to pyriproxyfen treated bednet at 0.5g/m^2 and 1.0 g/m^2. In her work, a group of 15 mosquitoes exposed to untreated bed nets laid a mean number of 84.3 eggs per mosquito and the group exposed to pyriproxyfen treated bednet at 0.5g/m^2 laid a mean of 56.1 eggs per mosquito [17]. While this shows a reduction in mean eggs laid in the pyriproxyfen group, statistical significance of this difference is not known. A reduction in fecundity with topical application of IGRs has been reported [18]. However, Dell Chism and Apperson observed that " forcibly exposing gravid female mosquitoes to pyriproxyfen treated paper did not affect their fecundity" [19].In this study, no statistically significant effect of pyriproxifen on fecundity was found. It is to be noted that the dosages of pyriproxyfen used on bed nets in Miller's work were very high.

This work suggests that pyriproxifen on treated net has no effect on fecundity of mosquitoes at low doses. However there was a reduction in egg fertility in mosquitoes exposed after 24 hours to pyriproxifen.

In the last three weeks of the study, high mortality in newly emerged mosquitoes was noted, most of them were dead by the 4th day post emergence. The deaths of these mosquitoes most possibly were due to contamination of the breeding bowls by pyriproxifen through pipettes used in larvae counting. Vasuki [20] reported a drastic reduction in longevity of *An. stephensi* adult mosquitoes that were exposed to sublethal concentrations of insect growth regulator hexaflumuron at larval and pupal stage, he reported *Aedes aegypti* having survival duration of 2.74 days. It is interesting to note that the observed survival period for the set of mosquitoes contaminated was 3-4 days. This effect could be exploited in reducing the longevity of surviving adults and this could possibly impact on transmission of malaria.

Previous study showed that a dose of pyriproxifen as low as 0.000376ppm killed over 90% of larvae and was 2.2 and 21.5 times more toxic than the IGRs diflubenzuron and methoprene respectively[21]. Considering the high level of toxicity of pyriproxifen it will be necessary if studies of this nature are to be repeated that the control and pyriproxyfen treated groups be handled in separate rooms. In conclusion an effect on fertility was observed after 30min exposure to netting. The level of sterility demonstrated in this study is considered not enough to impact upon mosquito populations and therefore would be insufficient to reduce malaria. However the accidental finding of reduced longevity following exposure to sublethal doses of pyriproxyfen could possibly lead to reduction in survival of adult mosquitoes emerging from eggs laid by adult females who had been exposed to pyriproxifen treated nets, this needs to be explored further. It will be informative to study the effect of pyriproxifen treated nets using unfed mosquitoes, which will pick up residues at the time of feeding on an animal host, the effect of this contact on the fertility and fecundity will then be assessed in a manner that is realistic. Further work needs to be done to demonstrate tarsal transfer of pyriproxifen from treated bed nets to ovipostion sites for larval control.

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