Pyriproxyfen-treated bednets reduce reproductive fitness and longevity of pyrethroid-resistant Anopheles gambiae under laboratory and field conditions

Nelson Grisales
Liverpool School of Tropical Medicine
Rosemary S Lees
Liverpool School of Tropical Medicine
James Maas
Liverpool School of Tropical Medicine
John C Morgan
Liverpool School of Tropical Medicine
Dimitri W Wangrawa
CNRFP: Centre National de Recherche et de Formation sur le Paludisme
Wamdaogo M Guelbeogo
CNRFP: Centre National de Recherche et de Formation sur le Paludisme
Sagnon N'Fale
CNRFP: Centre National de Recherche et de Formation sur le Paludisme
Steven W Lindsay
Durham University
Philip J McCall
Liverpool School of Tropical Medicine
Hilary Ranson (Hilary.Ranson@lstmed.ac.uk)
Liverpool School of Tropical Medicine https://orcid.org/0000-0003-2332-8247

Research

Keywords: Pyriproxyfen (PPF), Anopheles gambiae, insecticide-treated nets (ITNs), juvenile hormone (JH), Olyset Duo®, pyrethroid-resistance

Posted Date: March 10th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-290529/v1

Abstract

Background

The efficacy of insecticide treated nets (ITNs) containing the insect growth regulator pyriproxyfen (PPF) and pyrethroid insecticides (PPF-ITNs) is being assessed in clinical trials to determine whether they provide greater protection from malaria than standard pyrethroid-treated ITNs in areas where mosquitoes are resistant to pyrethroids. Understanding the entomological mode of action of this new ITN class will aide interpretation of the results from these trials.

Methods

Anopheles gambiae s.l. mosquitoes from a susceptible laboratory strain were exposed to PPF-treated netting 24 h, 6 h, and immediately prior to, or 24 h post blood feeding, and the impact on fecundity, fertility and longevity recorded. Pyrethroid-resistant populations were exposed to nets containing permethrin and PPF (PPF-ITNs) in cone bioassays and daily mortality recorded. Mosquitoes were also collected from inside houses pre- and post-distribution of PPF-ITNs in a clinical trial conduced in Burkina Faso; female *An. gambiae* s.l. were then assessed for fecundity and fertility.

Results

PPF exposure reduced the median adult lifespan of insecticide-susceptible mosquitoes by 4 to 5 days in all exposure times ($p < 0.05$) other than 6 h pre-bloodmeal and resulted almost complete lifelong sterilisation. The longevity of pyrethroid-resistant mosquitoes was also reduced by at least 5 days after exposure to PPF- ITNs compared to untreated nets but was unaffected by exposure to standard pyrethroid only ITNs. A total of 386 blood-fed or gravid *An. gambiae s.l.* females were collected from five villages between 1 and 12 months before distribution of PPF-ITNs. Of these mosquitoes, 75 % laid eggs and the remaining 25 % appeared to have normal ovaries upon dissection. In contrast, only 8.6 % of the 631 blood-fed or gravid *An. gambiae s.l.* collected post PPF-ITN distribution successfully oviposited; 276 (43.7%) did not oviposit but had apparently normal ovaries upon dissection, and 301 (47.7%) did not oviposit and had abnormal eggs upon dissection. Egg numbers were also significantly lower (average of 138/female prior distribution vs 85 post distribution, $p < 0.05$).

Conclusion

Exposure to a mixture of PPF and pyrethroids on netting shortens the lifespan mosquitoes and reduces reproductive output. Sterilisation of vectors lasted at least one year under operational conditions. Our findings suggest a longer effective lifespan of PPF-pyrethroid nets than reported previously.

Background

As pyrethroid resistance becomes more widespread among African malaria vectors existing tools reliant on this insecticide class, such as insecticide treated nets (ITNs), are losing efficacy, and may be contributing to a recent rebound in malaria cases (WHO, 2019). There is therefore a need for insecticides with novel modes of action for use in malaria vector control tools including ITNs (WHO, 2016).

Methoprene and pyriproxyfen (PPF) are biological insecticides that mimic the action of the insect juvenile hormone (JH), which is essential for normal physiological development and maturation of juvenile insects. In holometabolous insects like mosquitoes it is essential for larval development and its downregulation is critical for metamorphosis and adult

emergence (Sláma, 1971; Wilson, 2004). In anautogenous mosquito females oocyte development enters a 'resting stage' under the influence of JH (Gwadz and Spielman, 1973) and its synthesis stops shortly after the bloodmeal and fat bodies and ovaries become receptive to the hormone 20-hydroxyecdysone which promotes egg development (Ma et al. 1988; Shapiro *et al*. 1986). Hence raising levels of JH, or JH analogues/mimics such as PPF, at this stage will disrupt oogenesis, potentially providing an effective means of mass sterilisation, reducing the size of future vector populations (Devine et al., 2009).

Anopheles mosquitoes exposed to PPF-treated net samples in a cone test (WHO, 2013) show reduced fertility, longevity and lifetime fecundity compared to unexposed controls (Ohashi et al., 2012; Kawada et al., 2014). Combining PPF with pyrethroids in ITNs is one approach currently under evaluation to reduce transmission of malaria by pyrethroid-resistant anopheline mosquitoes; mosquitoes able to withstand exposure to the pyrethroid insecticide are expected to be sterilised, and potentially have shorter lifespans due to exposure to the insect growth regulator on the nets. The ability of these dual action nets to sterilise field populations has been demonstrated in experimental hut trials using PPF- ITNs from two manufacturers: Olyset Duo® from Sumitomo Chemical Ltd (permethrin and PPF) and Royal Guard® from Disease Control Technologies (alphacypermethrin and PPF). For both net types, mosquitoes collected from huts with PPF-ITNs had reduced fertility and fecundity compared to mosquitoes from huts containing pyrethroid only ITNs (Ngufor et al., 2014, 2020; Koffi et al., 2015). The potential of PPF-ITNs to reduce malaria transmission has been shown in a cluster randomised clinical trial conducted between 2014 and 2015, in an area of intense malaria transmission in Burkina Faso where vectors are highly resistant to pyrethroids (Tiono et al., 2018). The entomological inoculation rate in clusters receiving Olyset Duo nets was approximately half that in control arms, containing pyrethroid only nets (rate ratio 0.49, 95% CI 0.32–0.66; $p < 0.0001$) and this resulted in a reduction in clinical malaria in children (incidence rate ratio 0·88 [95% CI 0.77–0.99; $p = 0·04$]. Epidemiological trials of Royal Guard nets are ongoing in Tanzania and Benin (London School of Hygiene and Tropical Medicine, 2019).

To better understand the mode of action of PPF-ITNs we here report results from laboratory bioassays measuring the impact of PPF on the reproductive output and longevity of pyrethroid susceptible populations, and data on the impact of PPF-ITNs on survival and longevity of pyrethroid resistant populations from the laboratory and field. The study also sought to understand more about the biological efficacy of PPF- ITNs under field settings by measuring the fertility of mosquitoes collected from houses in the Burkina Faso clinical trial pre and post PPF- ITN distribution.

Methods

Mosquito strains

The Tiassalé 13 strain is a hybrid of *An. gambiae s.s.* and *Anopheles coluzzii* which was collected in Côte d'Ivoire in 2013 since when it has been maintained in the insectaries of the Liverpool Insect Testing Establishment at the Liverpool School of Tropical Medicine under conditions described previously (Williams et al., 2019). This strain is highly resistant to DDT, permethrin, deltamethrin, bendiocarb, dieldrin, partially susceptible to fenitrothion and regularly subjected to selection pressure with deltamethrin to maintain pyrethroid resistance. Hereafter this strain is referred to as the pyrethroid-resistant strain.

The Kisumu strain used was *An. gambiae s.s*. colony susceptible to all insecticides, originally collected in Kisumu, and obtained from the MR4 collections (BEI Resources, 2021). Hereafter this strain is referred to as the pyrethroid-susceptible strain. The strain was maintained in the insectary of the Centre National de Recherche et de Formation sur le Paludisme, (CNRFP) in Ouagadougou, Burkina Faso, held at temperatures of 27–30°C and relative humidity of 75% – 95% with 12 h light:12 h dark photoperiod. Larvae were fed TetraMin Baby® fish food, and adults provided with 10% sucrose solution. Bloodmeals were provided by placing an immobilised rabbit on top of the cage, using different animals to feed treatment and control groups to avoid contamination.

Adults were reared from larvae collected from aquatic habitats in the villages of Naniagara, Tiefora and Bakaridjan in the Cascades District of south-western Burkina Faso and maintained in the CNRFP insectaries in Banfora, Burkina Faso to produce adult female F_0 progeny for bioassays (coordinates of larval collection sites are provided in File A1). Larval collections were performed in water bodies around each village, including semi-permanent and temporary water bodies, between June 2013 and October 2015. *Anopheles* larvae of all stages were collected using hand dippers and transported to the insectaries in Banfora where they were reared as described above. Blood-fed females collected inside houses in the village of Naniagara were allowed to oviposit and their progeny reared to adults under the same conditions in the Banfora insectaries. Results from insecticide susceptibility tests with permethrin are reported in full elsewhere (Tiono et al., 2018) but can be summarised as mortality rates of less than 20 % in discriminating dose assays for mosquitoes from all three villages.

The effect of relative time of exposure to pyriproxyfen-treated netting and blood feeding on insecticide-susceptible *Anopheles gambiae*

Exposing and blood feeding mosquitoes

To evaluate if the relative timing of contact with the PPF-treated net and the provision of a bloodmeal had any effect on PPF efficacy, mosquitoes were exposed to the net and blood-fed according to different regimes. A 1% w/w PPF-treated net was provided by Sumitomo Chemical Co. LTD. (Tokyo, Japan), and designed to have a release rate of PPF as close as possible to that of the Olyset Duo® net. Mosquitoes were exposed to the net using a custom Deli pot bioassay: 25 ml clear plastic pots (height 28 mm, top diameter 50 mm base diameter of 40 mm) were prepared by cutting a large hole in the lid of the pot and a smaller (approximately 1 cm diameter) hole in the bottom. The lid and pot were assembled with a piece of either untreated or PPF-treated net between them. Groups of 10 mosquitoes were introduced by manual aspirator to the assembled plastic pot and exposed to untreated or PPF netting for three minutes.

To investigate the effect of time of PPF exposure relative to that of a bloodmeal, susceptible adult female mosquitoes were exposed to an untreated or PPF -treated net at 24 h, 6 h, and immediately (within 15 minutes) prior to, or 24 h post blood feeding. The 24-h pre-exposure treatment was to simulate mosquitoes exposed while trying unsuccessfully to blood-feed on a human host protected by a bed net and then succeeding on the following night. The 6 h pre-exposure treatment simulated an initial contact with a net followed by a successful feed on the same night. The 24 h post blood-feeding treatment represented mosquitoes that successfully took a bloodmeal and then were exposed to a net the following night. Each treatment group was matched with a negative control group exposed to an untreated net. Three-day old female mosquitoes were used for all experiments, except in the 24 h prior cohort where exposure occurred on day 3 and the bloodmeal offered on day 4 day.

Measuring mosquito longevity and lifelong fecundity

Mosquitoes from a single cohort of mosquitoes were used for this experiment, exposed as described above in pools of 10 mosquitoes with at least 10 replicates per treatment. After exposure to the net, groups of mosquitoes from replicate exposures were pooled by treatment, into a 30 x 30 x 30 cm cage. Mosquitoes that were unable to stand or were dead were removed. A 10% sucrose solution was provided *ad libitum*. Individual mosquito mortality was recorded daily and dead mosquitoes were removed from the cages.

A bloodmeal was offered to each cage every week until all mosquitoes died. The number of fully engorged mosquitoes was observed immediately post blood-feeding, but all females were retained in the cage. Two days after each bloodmeal, a plastic dish with a filter paper, partially submerged in distilled water, was introduced in each cage to encourage oviposition. Mosquitoes were allowed to lay eggs for three days, before the paper was removed and the eggs counted using a dissection microscope. Temperature and relative humidity were recorded daily.

Measuring the fecundity, fertility and offspring viability of individual mosquitoes

In this experiment, six replicate groups of 10 mosquitoes were exposed per treatment described above, using a single cohort of mosquitoes. After exposure, mosquitoes were individually transferred to flat-bottomed 50 ml plastic cell culture tubes, each containing a piece of filter paper over a wet piece of cotton for oviposition and sealed with a piece of mesh held by a rubber band to provide air. A piece of cotton soaked in 10% sucrose solution was put on the mesh on top of the tubes. Individual oviposition was recorded daily for five days post bloodmeal. After five days, all remaining live mosquitoes which failed to oviposit were dissected and their ovaries scored for follicular development using observation under a microscope. The ovaries were visually scored as comprising either normal or abnormal (with no follicular growth or yolk deposition). Dead mosquitoes were discarded.

Individual egg batches were placed in separate disposable plastic pots (height 42 mm, top diameter 115 mm, base diameter 85 mm) with approximately 50 ml of distilled water. A pinch of ground Tetramin fish food was added to the pots daily after larval hatching. The total number of 2nd instar larvae produced and the number of adults emerging were recorded as estimated measurements of hatching ratio and adult production ratio, respectively, for every treatment batch.

Blood feeding mosquitoes through the pyriproxyfen-treated netting

To simulate those mosquitoes which successfully feed on a human host through a PPF-treated net, a 15 x 15 cm piece of the cage top was replaced with a piece of PPF-treated net through which a bloodmeal was offered to around 100 adult female susceptible mosquitoes (Kisumu strain). A negative control treatment consisted of a parallel cage with mosquitoes fed through the untreated mesh top of cages. Mosquitoes were left to feed freely for up to 20 min to allow complete bloodmeals to be taken, therefore the precise length of exposure to the net was unknown.

Two experiments were performed in parallel to those described above using a subset of the same cohorts of mosquitoes. In the first experiment longevity and lifelong fecundity was measured in two pools of mosquitoes, one allowed to feed through a PPF-treated net and one through an untreated net. In the second experiment, mosquitoes were isolated to measure individual fertility, fecundity, and offspring viability, as described above.

Measuring the impact on 24 h mortality and adult longevity of exposure to pyriproxyfen and/or pyrethroids in pyrethroid-resistant *Anopheles gambiae* s.l. colonies and field collected mosquitoes

Pyrethroid-resistant mosquitoes were tested in LSTM laboratories against pyrethroid-treated netting (Olyset® Nets, 2% w/w permethrin, equivalent to 800 mg/m^2 of finished net), PPF-treated netting (1 % w/w PPF, equivalent to 400 mg/m^2) and PPF ITNs (Olyset Duo® nets, 2 % permethrin, 1 % PPF, equivalent to 800 and 400 mg/m^2). Naniagara F_0 adults reared from larval collections were tested in Banfora insectaries using PPF-ITNs and control nets only. Mosquitoes obtained from larval collections at Tiefora and Bakaridjan, between July and September 2014 were exposed to control nets, ITNs and PPF-ITNs .

Three to five day-old female adults were exposed to net samples in a WHO cone bioassay following the standard protocol (WHO, 2013) with the following modifications; first panels were not selected systematically from each side of the net, as suggested by the guidelines and second, 10 mosquitoes rather than the suggested five, were tested for each cone. Mosquitoes exposed in this manner were used to measure the immediate mortality effects and longer term impact on survival (Tiassalé and Naniagara only).

To measure the immediate effect of PPF- ITN exposure on mortality, mosquitoes were exposed to the nets for 3 min, with knockdown and mortality being recorded at 1 h and 24 h post-exposure. Untreated nets were used as negative controls. Mosquitoes were then offered a bloodmeal 24 h post net exposure. Unfed mosquitoes were discarded and engorged

mosquitoes pooled in empty polyethylene buckets (85 oz) covered by a fine mesh. Mortality was recorded daily until all mosquitoes died. A piece of cotton containing 10% sucrose solution was available *ad libitum* and a bloodmeal offered weekly using a Hemotek Membrane Feeding System (Hemotek Ltd., Blackburn, UK). In each net-treatment group, mortality comparisons were made with negative control nets (untreated nets).

Up to 10 replicate exposures of 10 individuals were performed per treatment for each experiment, although, as not all mosquitoes bloodfed, starting numbers were lower for some controls. Without the considerable resources needed to produce the high number of mosquitoes for this experiment, the cone bioassay experiments were not conducted for all nets simultaneously, but as paired tests comparing a treated net with an untreated control.

Determination of the impact of implementation of Pyriproxyfen ITNs on the reproductive output of Anopheles gambiae s.l. *in* Banfora district, Burkina Faso

Distribution of Olyset Duo nets and mosquito collection protocols

Five villages in the Cascades region of Burkina Faso were selected where ITNs (Olyset Nets®) were originally distributed to all households in May - June 2014 and replaced by PPF- ITNs (Olyset Duo®) from June 2014 to September 2015 in a stepped-wedge experimental design (Tiono et al., 2015). Mosquito collections were performed before and after replacement of ITNs with PPF-ITNs in five villages: Naniagara (Olyset Duo replacement in September 2014, mosquito collection in June 2014 and September 2015), Bakaridjan (Olyset Duo replacement in July 2015, mosquito collection in September 2014 and September 2015), Pont Maurice, Djomale and Sikané (Olyset Duo replacement in August 2015, mosquito collection in June and October 2015) (see Table A1 for coordinates of villages).

Female blood-fed anopheline mosquitoes were collected inside houses in each of the study villages. The number of houses visited depended on the density of adult female *Anopheles* and ranged from 23 (Sikané) to 140 (Pont Maurice). For the 'prior' collections, i.e. collections done when only ITNs were present, mosquitoes were captured in every house that collectors were allowed to enter. For the post-replacement collections, mosquitoes were only collected in houses where PPF-ITNs were present. Collections started at 06.00 h and residents requested to keep windows and doors closed until mosquito collections were completed. Mosquitoes were collected indoors using Prokopack aspirators (Vazquez-Prokopec et al., 2009), transferred gently into mosquito cages and transported to the Banfora insectaries. Collections continued until approximately 100 females had been assessed for oogenesis, per village, per collection period.

Measurement of individual mosquito fecundity, fertility and offspring viability of wild mosquitoes

Individual blood-fed mosquitoes were transferred to flat bottomed 50 ml plastic cell culture tubes to measure oviposition rates and individual fecundity and fertility as described above. As mosquito size and/or species could be confounding factors when measuring reproductive outputs, a sub-sample of individuals, collected post Olyset Duo distribution that either: a) laid healthy eggs, b) did not lay eggs but had normal ovary appearance on dissection, and c) did not lay eggs and had abnormal ovary appearance were taken for wing length measurement and species identification (determined following the SINE protocol (Santolamazza et al., 2008).

Statistical analysis

All statistical analyses were carried out using the R statistics package, v 4.02 (R Core Team (2020), 2020). Kaplan-Meier survival analyses were done using the survival (Therneau et al., 2020) and survminor (Kassambara et al., 2020) packages and graphs drawn using the ggplot2 package (Wickham, 2016). Fisher Exact Probability Test was used to compare proportions of mosquitoes blood-fed or ovipositing in the experiments performed in the laboratory. Wing length analysis was done using the t.test function in R to produce mean and 95% confidence interval values.

Results

The effect of relative time of exposure to pyriproxyfen-treated netting and blood feeding on insecticide-susceptible *Anopheles gambiae*

Pyriproxyfen exposure reduces longevity in pyrethroid susceptible mosquitoes

Kaplan-Meier survival curves for each of the groups of insecticide susceptible mosquitoes exposed to PPF-treated nets or untreated nets are shown in Fig. 1. In all cases, except the 6 h pre-bloodmeal treatment, exposure to the PPF- treated net increased the rate of mortality and decreased the lifespan of the mosquitoes.

Pyriproxyfen exposure reduces lifelong fecundity in pyrethroid susceptible mosquitoes

Exposure to PPF-treated netting resulted in few eggs being laid (0–5 eggs/test), regardless of the timing of exposure in relation to the first bloodmeal (Table 1). Blood feeding rates were similar between PPF-exposed and those exposed to untreated netting ($p > 0.1$).

Table 1

Blood feeding rates and egg production in different treatment groups. The number of female mosquitoes that blood-fed, and the number of eggs they produced from successive opportunities to blood feed. Control mosquitoes were exposed to untreated nets, and PPF treated were exposed to PPF-treated nets at 24 h prior, 6 h prior, immediately before, or 24 h post the first blood feeding opportunity, respectively. The number of mosquitoes offered a bloodmeal (Total) was recorded at the first blood feed, and the number of females that took a bloodmeal were recorded at each blood feeding. An error in recording resulted in missing data for the immediately prior cohort (ND = not determined).

Time bloodmeal was offered relative to exposure	Netting Type	1st Bloodmeal		2nd Bloodmeal		3rd Bloodmeal		4th Bloodmeal		5th Bloodmeal	
		Blood-fed (n/N), (%)	**Eggs Laid (N)**	**Blood-fed (N)**	**Eggs Laid (N)**	**Blood-fed (N)**	**Eggs laid (N)**	**Blood-fed (N)**	**Eggs Laid (N)**	**Blood-fed (N)**	**Eggs Laid (N)**
24 h prior	Untreated	149 / 187 (80)	6,551	127	5,215	12	160	7	216	3	122
	PPF-treated	125 / 167 (74)	0	55	0	1	0	1	0	0	0
6 h prior	Untreated	102 / 162 (62)	1,645	37	1,447	16	226	4	199	0	0
	PPF-treated	95 / 171 (55)	0	64	5	14	0	1	4	0	0
Immediately prior	Untreated	ND / 126	5,241	104	3,782	38	1,175	23	384	16	0
	PPF-treated	ND / 158	0	107	0	47	63	23	32	0	0
24 h post	Untreated	98 / 98 (100)	3,534	47	1,888	0	0	0	0	0	0
	PPF-treated	100 / 100 (100)	0	53	0	0	0	0	0	0	0

Pyriproxyfen exposure reduces the fecundity, fertility and offspring viability of individual mosquitoes

In experiments where mosquitoes were able to oviposit separately, the oviposition rate in the groups exposed to untreated netting ranged from 76 % to 88 % but none of the mosquitoes exposed to PPF laid any eggs (Table 2). The morphology of the primary follicles was assessed in individuals which did not oviposit (Fig. 2). In the control group, 3 % (1/35) had abnormal ovaries, compared to 94 % (119/126) of mosquitoes exposed to PPF ($p < 0.01$). Larval hatch rates and adult emergence were recorded but, as none of the treatment group laid any eggs these results are not presented here.

Table 2

Number of eggs oviposited per individual female after a single blood feeding opportunity in mosquitoes exposed to a pyriproxyfen treated net at different time points. Mosquitoes were exposed to untreated netting or PPF-treated netting at 24 h or 6 h before, or 24 h after a bloodmeal was offered.

Time bloodmeal was offered relative to exposure	Netting type	Blood - fed (N)	No. (%) ovipositing	Mean no. eggs/mosquito	95 % Confidence Interval
24 h prior	Untreated	41	36 (88%)	78.5	64.6–92.4
	PPF-treated	19	0 (0%)	0	0
6 h prior	Untreated	42	34 (81%)	88.4	75.3–101.6
	PPF-treated	26	0 (0%)	0	0
Immediately prior	Untreated	46	35 (76%)	58.1	39.7–76.5
	PPF-treated	36	0 (0%)	0	0
24 h post	Untreated	59	48 (81%)	69.7	57.6–81.9
	PPF-treated	59	0 (0%)	0	0

Blood feeding mosquitoes through the pyriproxyfen-treated netting reduces longevity and lifelong fecundity in pyrethroid susceptible mosquitoes

In the previous experiments, mosquitoes were exposed to a net for 3 min. To assess the impact of exposure during blood feeding, mosquitoes were presented with a bloodmeal for 20 min through either an untreated or PPF-treated netting. Exposure to PPF-treated netting resulted in a reduction in median adult longevity of 5 days (Fig. 3). Sterilisation was not complete with this exposure regime but there was a 5.4 -fold reduction in average eggs/blood-fed female after the first bloodmeal, increasing to an 18 -fold reduction after the second bloodmeal and null egg batches thereafter (Table 3)). When blood-fed females were separated for oviposition, 81 % (total n = 54) laid eggs in the control group compared to 29 % (n = 48) in the PPF exposed group ($p < 0.01$).

Table 3

Number of blood-fed mosquitoes and number of eggs produced after different blood meals after feeding through PPF-treated and untreated netting.

Netting Type	1st Bloodmeal		2nd Bloodmeal		3rd Bloodmeal		4th Bloodmeal		5th Bloodmeal	
	No. blood-fed / Total (%)	No. of eggs	No. blood - fed	No. of eggs	No. blood - fed	No. of eggs	No. blood-fed	No. of eggs	No. blood-fed	No. of eggs
Untreated	107 / 112 (96%)	4,218	49	4,269	43	3,022	20	1,525	17	1,514
PPF-treated	91 / 91 (100%)	654	59	287	25	0	6	0	0	0

Survival and adult longevity are reduced by exposure to pyriproxyfen treated net in pyrethroid-resistant *Anopheles gambiae* s.l. colonies and field collected mosquitoes

Total mosquito survival 24 h post exposure was lower ($p < 0.05$, Fisher exact test) when field collected mosquitoes were exposed to PPF-ITNs (76 % survival, n = 454) in WHO cone assays compared to standard ITNs (94 % survival, n = 294)

(Supplementary Fig. 1) although, when separated by village, the difference was only significant for mosquitoes from one of the three collection sites. Mortality was low following exposure to both net types in all villages (< 11 % for ITNs and < 33 % for PPF-ITNs (Supplementary Fig. 1). The pyrethroid resistance status of the laboratory resistant mosquito strain was confirmed by the very low mortality observed 24 h after exposure to an ITN (3 %).

The lifespan of the laboratory resistant strain was unaffected by exposure to the ITN (Fig. 4, panel A). However, the lifespan of both the resistant strain and field collected Naniagara mosquitoes was significantly reduced by exposure to a PPF-ITN (Fig. 4, panels C and D, respectively). These reductions were not just statistically but biologically significant, with median lifespan being reduced by > 5 days in PPF-ITN exposures resulting in a median lifespan in the pyrethroid-resistant laboratory strain and Naniagara field population of 15 days (95% CI 14–16) and 10.5 (95 % CI 9–14) days respectively after exposure to PPF-ITNS (compared with those exposed to a control net of 21 (95 % CI 18–23) and 16 days (95 % CI 16–18), respectively).

Determination of the impact of implementation of Pyriproxyfen ITNs on the reproductive output of Anopheles gambiae s.l. in Banfora district, Burkina Faso

Blood-fed, half-gravid and gravid mosquitoes from five clusters within the Olyset Duo clinical trial site, were collected at two time points, one pre- and one post-Olyset Duo® distribution. Approximately 5 % of the total mosquitoes did not show any signs of oogenesis, and a further 5% did contain eggs but the morphology of the ovaries could not be clearly discerned (i.e. eggs were too small so they could be still in development, or simply no clear decision could be made upon dissections). These mosquitoes were excluded from the subsequent analyses and the remaining mosquitoes classified into three categories: 1) mosquitoes that laid eggs, 2) mosquitoes that retained normal eggs and 3) mosquitoes whose ovaries had no follicular growth or yolk deposition (defined as ‘abnormal’).

Of the 515 mosquitoes collected from five villages prior to Olyset Duo® distribution, the proportion of female *An. gambiae* s.l. that oviposited varied by site from 53% in Bakaridjan (the only site where collections were performed in September and not June) to 85% in Pont Maurice (Fig. 5). Of these, 386 (75%) oviposited eggs normally and 129 (25%) did not oviposit but upon dissection appeared to have normal eggs. None of the non-ovipositing mosquitoes were scored as having abnormal ovaries. In contrast, of the 631 mosquitoes collected after PPF-nets were distributed only 54 (9 %) successfully oviposited; 276 (44 %) did not oviposit but were scored as having normal ovaries upon dissection, and 301 (48 %) did not oviposit and had abnormal eggs (as in Fig. 5). Most mosquitoes collected (> 74 % in each collection round) were blood-fed (Supplementary Table A2), and only small percentages were non-blood-fed and gravid.

In four of the five villages surveyed, the mean number of eggs laid by each female mosquito was significantly lower in all sites in the latter collections, post distribution of the PPF-ITNs (Fig. 6). When combining data from all five villages, the overall reduction in number of eggs laid was significant (p = 0.023, paired t-test). The hatch rate of these eggs was also lower in all sites after PPF-ITN distribution (Supplementary Fig. 2); on average 42 % of eggs laid from females collected before PPF-ITN distribution hatched vs 15 % after distribution but large variations in hatch rate were observed and the difference pre and post distribution was only significant in two of the five villages.

Mean wing length was 3.18 mm (95 % CI 3.16–3.21 mm, n = 190) and there was no significant association between wing length and the probability of a mosquito developing normal eggs (p = 0.197). There was also no significant difference between species composition in the subset that had normal ovary development (2 *An. arabiensis*, 9 *An. coluzzii* and 54 *An. gambiae s.s.*) compared to those with abnormal ovaries (11 *An. coluzzii* and *63 An. gambiae s.s.*).

Discussion

Our findings show that PPF is a potent sterilising agent for *An. gambiae s.l.* in the laboratory and field and hat PPF exposure reduces the longevity of mosquitoes by .

Impact of PPF exposure on reproductive output

Exposure to PPF-treated nets in the laboratory completely prevented egg laying when mosquitoes were exposed 24 h prior to blood feeding or 24 h post bloodmeal, and this sterilising effect was retained after five successive rounds of weekly blood feeding. PPF exposure closer to the time of blood feeding (6 hours or immediately prior) also resulted in complete loss of oviposition for the 1st gonotrophic cycle, with only a very small number of eggs laid (maximum average of 1.4 eggs/blood -fed female) following subsequent bloodmeals. The decline in egg production was not due to a reduction in blood feeding and is indicative of a direct impact of PPF on egg development. In mosquitoes exposed to PPF, those that did not oviposit failed to develop morphologically normal eggs as seen previously (Koama et al., 2015; Yadav et al., 2019). The mechanism by which PPF achieves lifelong sterilisation may be due to permanent disruptions in JH-mediated gene regulation (Wilson, 2004), absence of nurse cells degeneration in follicle development (Judson and De Lumen, 1976), lack of follicle reabsorption (Judson and De Lumen, 1976), irreversible damage of the reproductive organs (Ohashi et al., 2012) or a combination of these.

When mosquitoes were exposed to PPF during a bloodmeal, sterilisation was partial, but the total number of eggs per original blood-fed mosquito over 5 successive bloodmeals reduced by > 13-fold from 136 in mosquitoes exposed to untreated netting to 10.3 in those exposed to PPF netting. In this experiment, the length of exposure was not controlled as mosquitoes were allowed to feed naturally until replete. Reduced impact of exposure to pyrethroids in groups who also received a blood meal has been observed in laboratory trials (Glunt, Thomas and Read, 2011; Hauser, Thiévent and Koella, 2019) and in experimental hut trials (Hughes et al., 2020), possibly due to the upregulation of detoxification enzymes during digestion of a blood meal providing some protective effect (Spillings et al., 2008; Oliver and Brooke, 2016). It is possible that a similar phenomenon is being observed here.

Lifelong, almost complete sterilisation, occurs after PPF exposure, and is consistent with previous studies of insecticide susceptible *An. gambiae* exposed to concentrations of PPF that were orders of magnitude lower than the 1 % PPF on the nets in our study (Ohashi et al., 2012). Mbare and co-workers (Mbare, Lindsay and Fillinger, 2014) also recorded a strong sterilising effect on *An. gambiae s.s.* females exposed within 24 h before or after a bloodmeal. However others found that sterilisation in *An. arabiensis* only occurred in mosquitoes exposed to PPF 24 h after blood feeding, but not 24 hours before (Harris et al., 2013). Jaffer (Jaffer et al., 2015) found that greatest fertility inhibition was induced in *An. gambiae* when females were blood-fed 1 hour after exposure, compared to 24 and 120 h after, concluding that the effects of pyriproxyfen are partially reversible through the action of metabolism or excretion. The differences observed on the impact of timing of exposure on sterilisation effect between the studies could have multiple explanations including differences in species or strain sensitivity, duration of exposure, the formulation and concentration of pyriproxyfen, the surface mosquitoes were exposed to and the specific nature of the bioassay. However, our laboratory results that showed substantial sterilisation regardless of the timing of exposure were encouraging as under operational settings, female mosquitoes may encounter active ingredients on bednets whilst host seeking (i.e. prior to blood feeding), during the act of feeding, or whilst trying to escape a holed net after successfully blood feeding.

As far as we are aware, our study is only the second study to measure the impact of PPF-ITNs on mosquito reproduction under field conditions. The earlier study was a small-scale study involving collections in 15 households which found an overall reduction in the proportion of blood-fed mosquitoes ovipositing one week after the introduction of PPF-treated nets from 77 % to 45 % with a nearly 50 % reduction in number of eggs/female (Kawada et al., 2014). The current study was considerably larger than the previous study and involved collections from 286 households using PPF-treated nets. Importantly, we were able to show the sterilisation persisted for one year after the PPF-treated nets were deployed in the field. In our studies the average number of eggs laid by female *An. gambiae s.l.* and the egg hatching rate declined after

deployment of PPF-treated nets compared with the period before deployment when standard pyrethroid-treated nets were used. This effect was not related to the size of the mosquitoes or species composition at the different collection time points. The absence of abnormal ovaries before deployment of PPF-treated nets is striking.

Impact of PPF exposure on adult longevity

In our laboratory assays, PPF exposure resulted in significantly reduced median lifespan of 2–5 days in all but one treatment, in agreement with earlier studies (Ohashi et al., 2012). A similar life shortening effect of between 5.5 and 7 days was observed after pyrethroid resistant populations were exposed to the mixture of permethrin and PPF. Similar findings were found with unfed mosquitoes exposed to PPF-treated netting (data not shown). Whilst exposure to pyrethroid only nets has previously been shown to reduce adult longevity (Viana et al., 2016), in the current study, exposure to pyrethroid-treated ITNs had no effect on longevity, strongly suggesting that the reduction in lifespan seen from exposure to PPF-ITNs is caused by PPF. In order for a mosquito to transmit malaria parasites they must survive the 10–14 day intrinsic incubation period for the parasite to develop between first and subsequent bloodmeals (Detinova, 1962). Hence if the reductions in lifespan observed in the laboratory were indicative of a similar effect in the field, nets containing PPF would be expected to lead to major reductions in malaria transmission by reducing the proportion of infected mosquitoes. This is supported by a decline in parous mosquitoes observed in villages with PPF-treated nets (Odds ratio = 0.69 (0.52–0.91)) in a cluster randomised controlled trial in Burkina Faso (Tiono et al., 2018). Mathematical modelling shows that a decrease in mosquito longevity would have a greater impact on transmission than sterilisation. It is important, however, to note that daily mosquito survival in the field is much lower than observed in the laboratory. In nature few females survive long enough to become infectious (Matthews, Bethel and Osei, 2020), even in the absence of PPF. Thus the impact of PPF-treated netting in the field is likely to be greater than indicated by our laboratory experiments.

Other impacts of PPF exposure

Previous studies have shown that the mortality of mosquitoes exposed to PPF-ITN netting was greater than when exposed to pyrethroid only treated nets (Ngufor et al., 2014, Koffi et al., 2015, Toé et al. 2019) and a similar result was observed in our study when results were pooled for the three villages. Although the concentration of permethrin in the standard ITNs and PPF-treated ITN were identical, the bleed rate in the PPF-treated ITNs was higher than the standard net, suggesting that there was likely to be higher concentrations of permethrin on the PPF-treated ITNs than the standard net. Alternatively, the increased mortality observed when permethrin is combined with PPF may be due to competitive metabolism delaying the detoxification of the pyrethroid insecticide; this hypothesis is supported by studies showing that certain mosquito P450s can efficiently metabolise both permethrin and PPF (Yunta et al., 2016).

In addition to incorporation into bednets, PPF is already used to suppress mosquito populations either by directly applying to breeding sites, or by adding PPF powder to odour baited traps. Both approaches can effectively reduce adult emergence and the latter has the added advantage of using the mosquito itself to autodisseminate PPF to a wide range of breeding sites, including cryptic sites difficult to reach with conventional larvicides (Maoz et al., 2017). We did not directly test for autodissemination of PPF by mosquitoes exposed to the PPF nets as the chemical steps needed to incorporate PPF (or any active ingredient) into the net are intended to release the chemical in a slow controlled manner and we anticipated that the formulated product would not be as readily disseminated as the powdered PPF used in traps. We also did not look at effect of PPF exposure on the development of Plasmodium in the mosquito; this should be the subject of further studies given the impact that agonists of the steroid hormone 20-E have been shown to have on prevalence of establishment of Plasmodium infections in the mosquito midgut (Childs et al., 2016).

Implications for use of PPF in insecticide treated nets

The results of this study show that PPF is highly effective in reducing reproductive outputs and adult longevity of both insecticide susceptible and pyrethroid resistant mosquitoes. A clinical trial in the study area showed that the addition of

PPFs to ITNs can reduce the clinical burden of malaria in an area of high intensity transmission by 12%. This reduction in malaria was associated with fewer vectors and a decrease in the survival of the vector population, leading to a halving in the entomological inoculation rate (EIR) in villages with PPF-treated nets compared to those with standard ITNs (Tiono et al., 2018). The observation of reduced adult female density in clusters of villages with PPF-ITNs suggests that the reduction in the number of immature *Anopheles* caused by the impact of PPF on mosquito fertility was not fully compensated for by any increase in larval productivity in the natural aquatic habitats caused by reduced larval densities. Whether a similar relationship between female fertility and adult population size would be observed in other transmission settings remains to be seen. Linking the entomological effects of PPF exposure with epidemiological outcomes is the subject of ongoing studies and may inform future development of nets containing insect growth regulators. Laboratory tests of an agonist of the steroid hormone 20-ecdysone (Childs et al., 2016) recorded four distinct effects (reduced insemination, reduced egg production, shortened lifespan and impaired *Plasmodium* development) not all of which have been investigated for PPF. Determining the importance of each potential entomological outcome on the epidemiological impact of nets containing PPF and pyrethroids under field settings, with different species compositions and differing levels of pyrethroid resistance, is important for establishing where and when this potential new net class may be most effective.

In the current study we provide evidence that PPF-ITNs remained effective in reducing *Anopheles gambiae* reproductive output for up to a year after distribution. However, a net durability study on Olyset Duo®, performed alongside the clinical trial found that impacts on mosquito fertility were lost after one month of operational use (although the increase in 24 h mortality in PPF-ITNs compared to standard ITNs was retained for the duration of the study) (Toé et al., 2019). The disparities between the two studies may reflect differences in the bioassays deployed; understanding the correlation between entomological outcomes may help guide the development of simple robust protocols to measure PPF bioefficacy on nets. It should also be noted that these results refer to specific net formulations. Other nets containing PPF will have differing release profiles and so will perform differently, meaning that additional studies would be needed in order to evaluate their efficacy.

There are a number of limitations to our study. Firstly, the evaluation of the efficacy of PPF-ITNs in reducing reproductive output was a pilot study; available resources did not permit collection of mosquitoes from additional replicate villages at each time interval which would have supported more robust conclusions. Secondly, we did not include a further follow up beyond 12 months to test whether the sterilisation effect lasted for the full expected lifespan of the net. Thirdly, although mosquitoes were only sampled from houses containing PPF-ITNs at the ‘post distribution’ time point, we have no way of knowing whether these mosquitoes actually came into contact with the nets prior to sampling. Finally, the lack of reliable methods to age adult mosquitoes precluded us from determining whether the reduction in longevity following PPF-ITN exposure that we observed in the laboratory was replicated under operational conditions.

Conclusions

The mixture of PPF and pyrethroids in a net can increase contact mortality, reduce reproductive outputs and shorten the lifespan of pyrethroid-resistant mosquitoes. In laboratory colonies of *An. gambiae s.l.* these effects are largely independent of the time of exposure relative to a bloodmeal. Importantly the PPF in PPF-ITNs remained active on the nets for at least a year under operational settings; this contrasts with results from laboratory cone bioassays which found the sterilising effect of PPF was lost after one month of net use and highlights the importance of developing and utilising assays that are reflective of the performance of products in the field. The data presented will guide the development of laboratory assays to assess efficacy and durability of different products and provide encouragement that the public health value of this potential new net class may be realised in future clinical trials.

Abbreviations

EIR - entomological inoculation rate

ITN – insecticide treated net

LSTM - Liverpool School of Tropical Medicine

ND – not determined

PPF – pyriproxyfen

WHO – World Health Organization

Declarations

Ethics approval and consent to participate of data and material

Blood feeding is required in female mosquitoes to initiate and maintain the process of oogenesis. All work with animals conformed to national regulations of Burkina Faso (LOI N°048-2017/AN PORTANT CODE DE SANTE ANIMALE ET DE SANTE PUBLIQUE VETERINAIRE) regarding the Protection of Animals

Consent for publication

Not applicable

Availability of data and material

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests. Nets used in this study were provided free of charge by Sumitomo Chemical Ltd but the manufacturers had no role in the study design and implementation or interpretation of data.

Funding

The research leading to these results has received funding from the European Union Seventh Framework Programme FP7 (2007-2013) under grant agreement no 265660 AvecNet. NG was supported by the ‘Francisco Jose de Caldas’ PhD fellowship from the Colombian Administrative Department of Science, Technology and Innovation (COLCIENCIAS). JM and HR are supported by Partnership for Increasing the Impact of Vector Control (PIIVeC), funded by the Medical Research Council of the UK (grant number MR/P027873/1) through the Global Challenges Research Fund.

Authors’ contributions

HR, PJM and SWL designed the study. NG conducted the laboratory work and the majority of the field work, supported by DWW and JCM and supervised by WGM and SNF. NG, RL and JM analysed the data and NG, RL, JM and HR drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We are very grateful to Dr. Hyacinthe Kobié Toe and the rest of the field team at CNRFP for assistance with field collections and to the insectary teams at CNRFP for mosquito rearing and bioassay support. We thank Manuela Bernardi for preparation of Figure 5.

References

BEI Resources (2021) *BEI Web Resources: MR4*. Available at: https://www.beiresources.org/MR4Home.aspx (Accessed: 23 February 2021).

Childs, L. M. *et al.* (2016) 'Disrupting Mosquito Reproduction and Parasite Development for Malaria Control', *PLoS Pathogens*, 12(12), pp. 1–20. doi: 10.1371/journal.ppat.1006060.

Detinova, T. S. (1962) 'Age-grouping methods in Diptera of medical importance with special reference to some vectors of malaria.', *Monograph series. World Health Organization*. doi: 10.2307/3275215.

Devine, G. J. *et al.* (2009) 'Using adult mosquitoes to transfer insecticides to Aedes aegypti larval habitats', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.0901369106.

Glunt, K. D., Thomas, M. B. and Read, A. F. (2011) 'The Effects of Age, Exposure History and Malaria Infection on the Susceptibility of Anopheles Mosquitoes to Low Concentrations of Pyrethroid', *PLoS ONE*, 6(9), p. 24968. doi: 10.1371/journal.pone.0024968.

Gwadz, R. W. and Spielman, A. (1973) 'Corpus allatum control of ovarian development in Aedes aegypti', *Journal of Insect Physiology*. doi: 10.1016/0022-1910(73)90174-1.

Harris, C. *et al.* (2013) 'Sterilising effects of pyriproxyfen on Anopheles arabiensis and its potential use in malaria control', *Parasites and Vectors*. doi: 10.1186/1756-3305-6-144.

Hauser, G., Thiévent, K. and Koella, J. C. (2019) 'The ability of Anopheles gambiae mosquitoes to bite through a permethrin-treated net and the consequences for their fitness', *Scientific Reports*, 9(1), pp. 1–8. doi: 10.1038/s41598-019-44679-1.

Hughes, A. *et al.* (2020) 'Anopheles gambiae populations from Burkina Faso show minimal delayed mortality after exposure to insecticide - treated nets', *Parasites & Vectors*. BioMed Central, 13(1), p. 17. doi: 10.1186/s13071-019-3872-2.

Jaffer, A. *et al.* (2015) 'Evaluating the sterilizing effect of pyriproxyfen treated mosquito nets against Anopheles gambiae at different blood-feeding intervals', *Acta Tropica*. doi: 10.1016/j.actatropica.2015.07.011.

Judson, C. L. and De Lumen, H. Z. (1976) 'Some effects of juvenile hormone and analogues on ovarian follicles of the mosquito Aedes Aegypti (Diptera: Culicidae)', *Journal of Medical Entomology*. doi: 10.1093/jmedent/13.2.197.

Kassambara, A. *et al.* (2020) 'survminer'. Available at: https://cran.r-project.org/package=survminer.

Kawada, H. *et al.* (2014) 'A small-scale field trial of pyriproxyfen-impregnated bed nets against pyrethroid-resistant anopheles Gambiaes. S. In Western Kenya', *PLoS ONE*. doi: 10.1371/journal.pone.0111195.

Koama, B. *et al.* (2015) 'The sterilizing effect of pyriproxyfen on the malaria vector Anopheles gambiae: Physiological impact on ovaries development', *Malaria Journal*. doi: 10.1186/s12936-015-0609-3.

Koffi, A. A. *et al.* (2015) 'Efficacy of Olyset® Duo, a permethrin and pyriproxyfen mixture net against wild pyrethroid-resistant Anopheles gambiae s.s. from Côte d'Ivoire: An experimental hut trial', *Parasite*. doi: 10.1051/parasite/2015028.

London School of Hygiene and Tropical Medicine (2019) *Efficacy of Two Dual Active Ingredient Long Lasting Insecticidal Nets for Control of Malaria Transmitted by Pyrethroid Resistant Vectors in Benin (NNP)*, *https://clinicaltrials.gov/*. Available at: https://clinicaltrials.gov/ct2/show/NCT03931473 (Accessed: 15 October 2020).

Ma, M. *et al.* (1988) 'Permissive action of juvenile hormone on vitellogenin production by the mosquito, Aedes aegypti', *Journal of Insect Physiology*. doi: 10.1016/0022-1910(88)90064-9.

Maoz, D. *et al.* (2017) 'Community effectiveness of pyriproxyfen as a dengue vector control method: A systematic review', *PLoS Neglected Tropical Diseases*. doi: 10.1371/journal.pntd.0005651.

Matthews, J., Bethel, A. and Osei, G. (2020) 'An overview of malarial Anopheles mosquito survival estimates in relation to methodology', *Parasites and Vectors*. doi: 10.1186/s13071-020-04092-4.

Mbare, O., Lindsay, S. W. and Fillinger, U. (2014) 'Pyriproxyfen for mosquito control: Female sterilization or horizontal transfer to oviposition substrates by Anopheles gambiae sensu stricto and Culex quinquefasciatus', *Parasites and Vectors*. doi: 10.1186/1756-3305-7-280.

Ngufor, C. *et al.* (2014) 'Olyset Duo® (a pyriproxyfen and permethrin mixture net): An experimental hut trial against pyrethroid resistant Anopheles gambiae and Culex quinquefasciatus in southern Benin', *PLoS ONE*. doi: 10.1371/journal.pone.0093603.

Ngufor, C. *et al.* (2020) 'Efficacy of Royal Guard, a new alpha-cypermethrin and pyriproxyfen treated mosquito net, against pyrethroid-resistant malaria vectors', *Scientific Reports*. doi: 10.1038/s41598-020-69109-5.

Ohashi, K. *et al.* (2012) 'Efficacy of Pyriproxyfen-Treated Nets in Sterilizing and Shortening the Longevity of <I>Anopheles gambiae</I> (Diptera: Culicidae)', *Journal of Medical Entomology*. doi: 10.1603/me12006.

Oliver, S. V. and Brooke, B. D. (2016) 'The role of oxidative stress in the longevity and insecticide resistance phenotype of the major malaria vectors Anopheles arabiensis and Anopheles funestus', *PLoS ONE*, 11(3), p. e0151049. doi: 10.1371/journal.pone.0151049.

R Core Team (2020) (2020) 'R: A language and environment for statistical computing.', *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria*.

Santolamazza, F. *et al.* (2008) 'Insertion polymorphisms of SINE200 retrotransposons within speciation islands of Anopheles gambiae molecular forms', *Malaria Journal*. doi: 10.1186/1475-2875-7-163.

Sláma, K. (1971) 'Insect juvenile hormone analogues.', *Annual review of biochemistry*. doi: 10.1146/annurev.bi.40.070171.005243.

Spillings, B. L. *et al.* (2008) 'The effect of a single blood meal on the phenotypic expression of insecticide resistance in the major malaria vector Anopheles funestus', *Malaria Journal*. doi: 10.1186/1475-2875-7-226.

Therneau, T. M. *et al.* (2020) 'survival'. Available at: https://cran.r-project.org/package=survival.

Tiono, A. B. *et al.* (2015) 'The AvecNet Trial to assess whether addition of pyriproxyfen, an insect juvenile hormone mimic, to long-lasting insecticidal mosquito nets provides additional protection against clinical malaria over current best practice in an area with pyrethroid-resist', *Trials*. doi: 10.1186/s13063-015-0606-4.

Tiono, A. B. *et al.* (2018) 'Efficacy of Olyset Duo, a bednet containing pyriproxyfen and permethrin, versus a permethrin-only net against clinical malaria in an area with highly pyrethroid-resistant vectors in rural Burkina Faso: a cluster-randomised controlled trial', *The Lancet*. doi: 10.1016/S0140-6736(18)31711-2.

Toé, K. H. *et al.* (2019) 'Assessing the impact of the addition of pyriproxyfen on the durability of permethrin-treated bed nets in Burkina Faso: A compound-randomized controlled trial', *Malaria Journal*. doi: 10.1186/s12936-019-3018-1.

Vazquez-Prokopec, G. M. *et al.* (2009) 'A New, Cost-Effective, Battery-Powered Aspirator for Adult Mosquito Collections', *Journal of Medical Entomology*. doi: 10.1603/033.046.0602.

Viana, M. *et al.* (2016) 'Delayed mortality effects cut the malaria transmission potential of insecticide-resistant mosquitoes', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1603431113.

WHO (2013) *Guidelines for laboratory and field-testing of long-lasting insecticidal nets*. Geneva: World Health Organization.

WHO (2019) *World Malaria Report 2019*. Geneva: World Health Organization.

Wickham, H. (2016) *ggplot2 Elegant Graphics for Data Analysis (Use R!)*, *Springer*. doi: 10.1007/978-0-387-98141-3.

Williams, J. *et al.* (2019) 'Characterisation of Anopheles strains used for laboratory screening of new vector control products', *Parasites and Vectors*, 12(1). doi: 10.1186/s13071-019-3774-3.

Wilson, T. G. (2004) 'The molecular site of action of juvenile hormone and juvenile hormone insecticides during metamorphosis: How these compounds kill insects', *Journal of Insect Physiology*. doi: 10.1016/j.jinsphys.2003.12.004.

World Health Organization (WHO) (2016) *Global report on insecticide resistance in malaria vectors: 2010–2016*. Geneva. Available at: https://apps.who.int/iris/bitstream/handle/10665/272533/9789241514057-eng.pdf?ua=1.

Yadav, K. *et al.* (2019) 'Pyriproxyfen treated surface exposure exhibits reproductive disruption in dengue vector Aedes aegypti', *PLoS Neglected Tropical Diseases*. doi: 10.1371/journal.pntd.0007842.

Yunta, C. *et al.* (2016) 'Pyriproxyfen is metabolized by P450s associated with pyrethroid resistance in An. gambiae', *Insect Biochemistry and Molecular Biology*. doi: 10.1016/j.ibmb.2016.09.001.

Figures

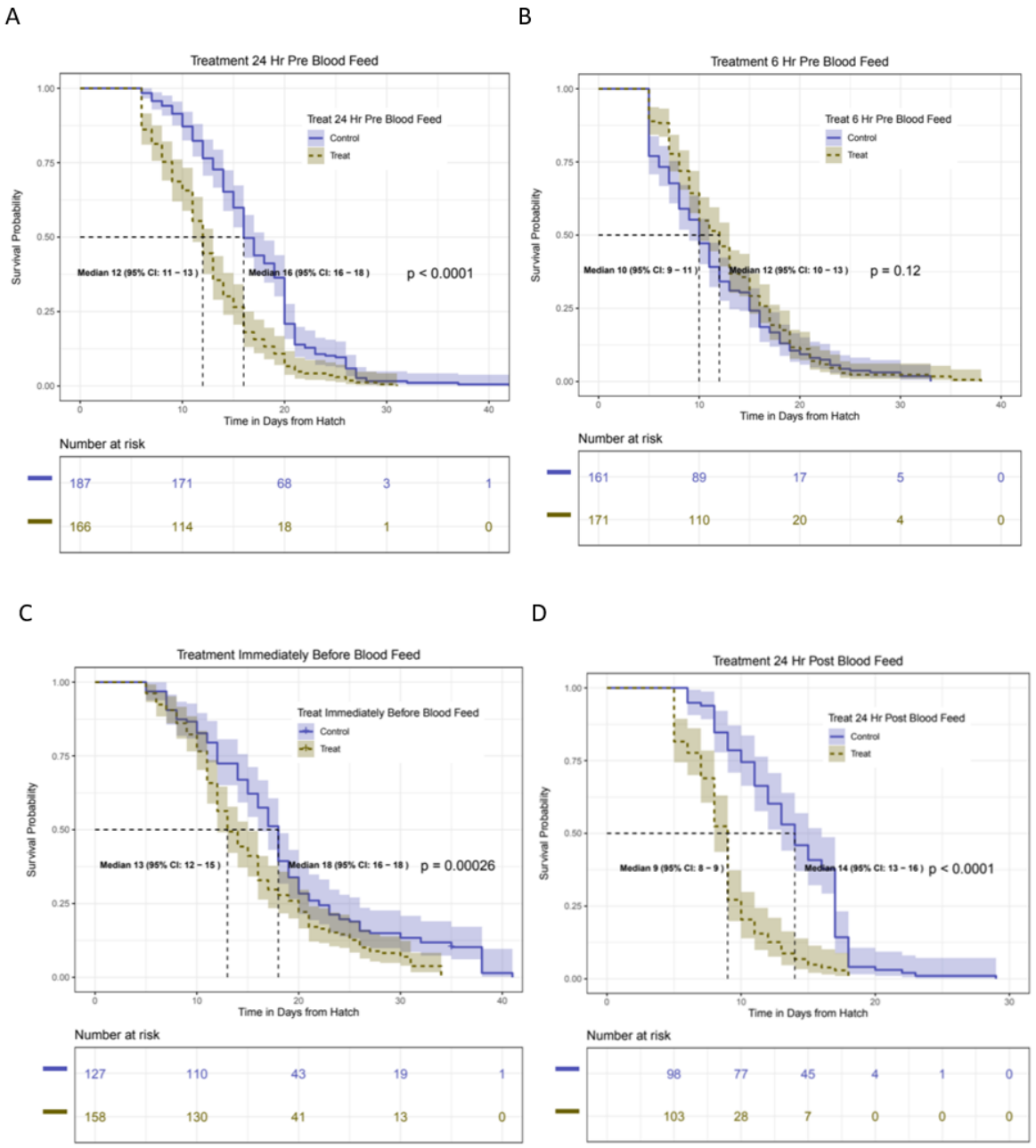

Figure 1

Survival curves for mosquitoes exposed to PPF at different times relative to being offered a bloodmeal. Daily survival of insecticide susceptible mosquitoes after exposure to 1% PPF-treated netting (brown) or untreated netting (blue) 24 h (A), 6 h (B) or immediately before (C) , or 24 h after a bloodmeal was offered (D). Mosquitoes were exposed to netting 4 days post-emergence.

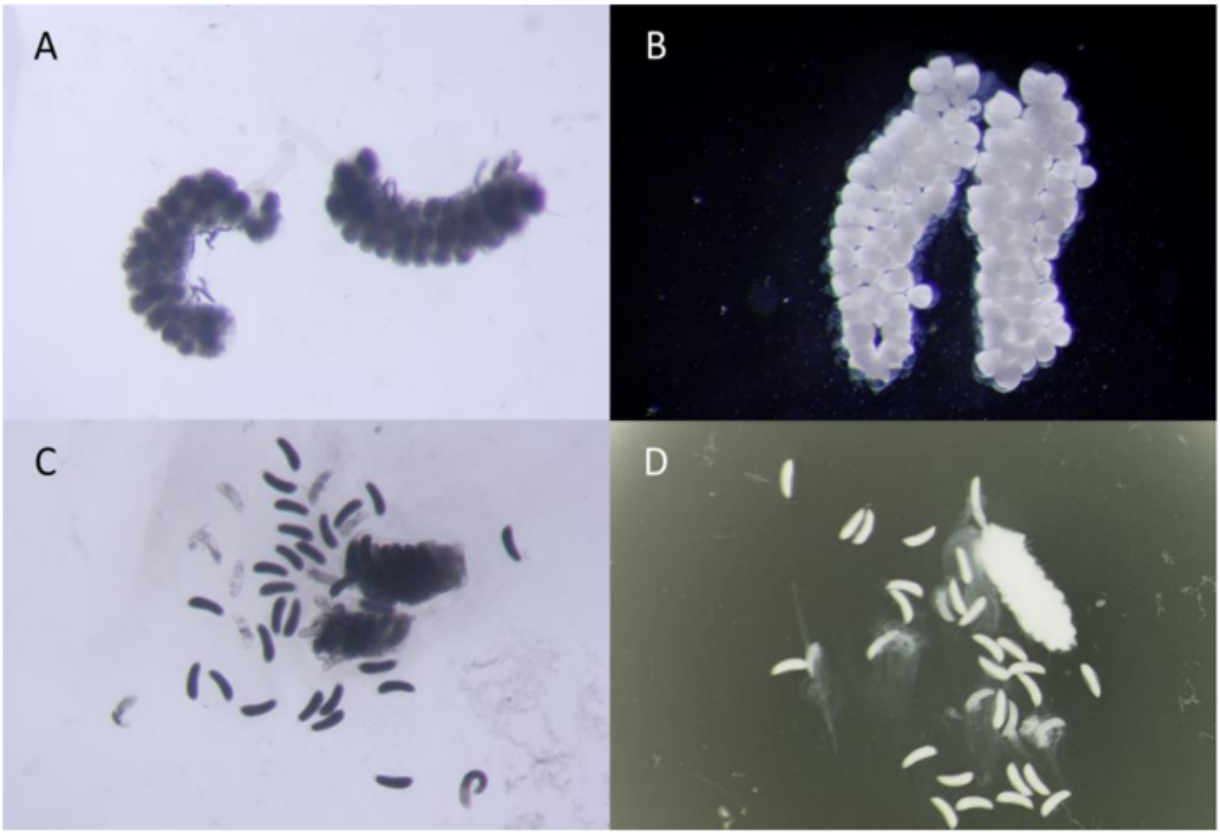

Figure 2

Morphology of eggs retained in ovaries of PPF exposed females compared to those in unexposed controls. Images are representative and were taken 4-5 days post exposure. A and B: Ovaries of mosquitoes exposed to PPF, showing round, non-detachable eggs. C and D: Ovaries of unexposed females, showing normal, oval-shaped mature eggs. Scale: all images at 200X magnification (approximate).

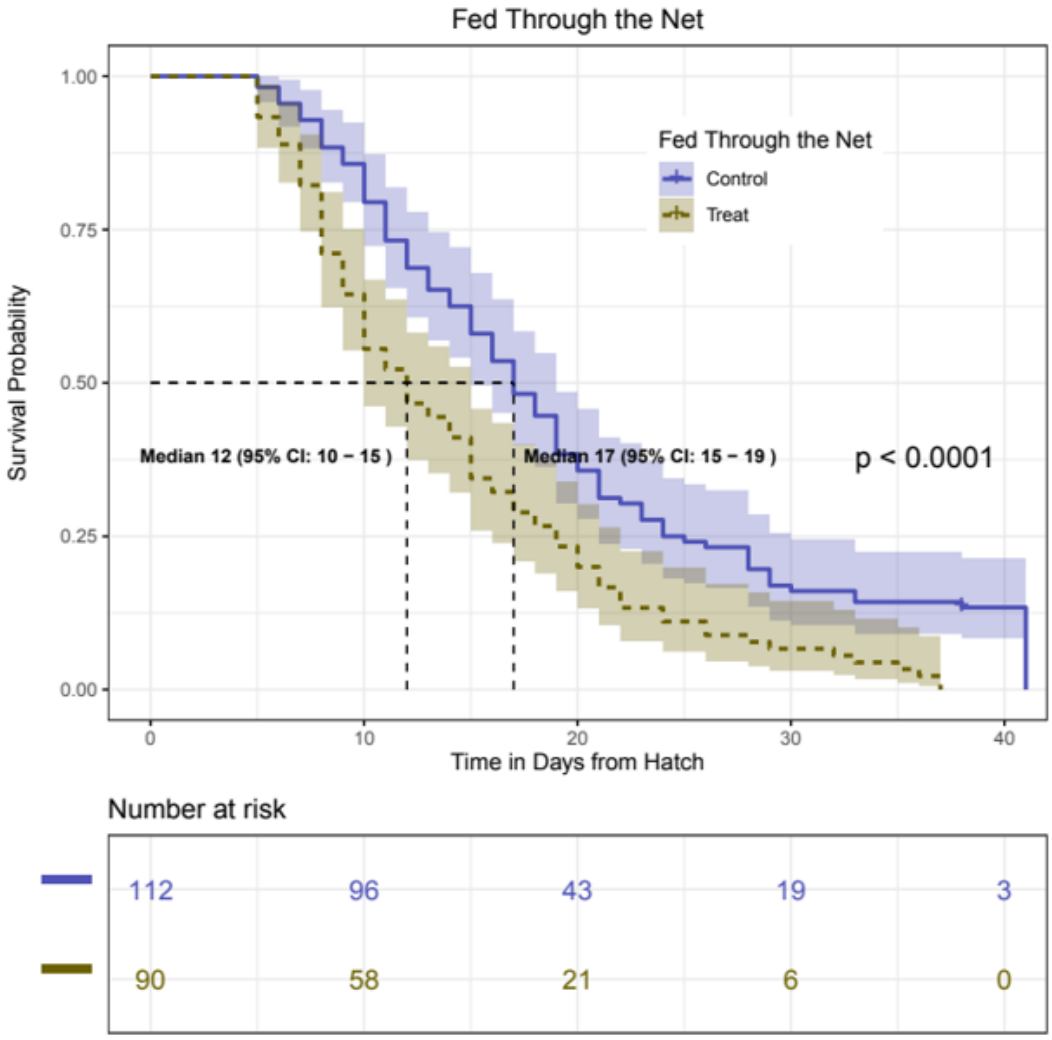

Figure 3

Daily survival of mosquitoes after feeding on blood through a 1% PPF (brown) or an untreated net (blue). Mosquitoes were exposed to netting 4 days post-emergence.

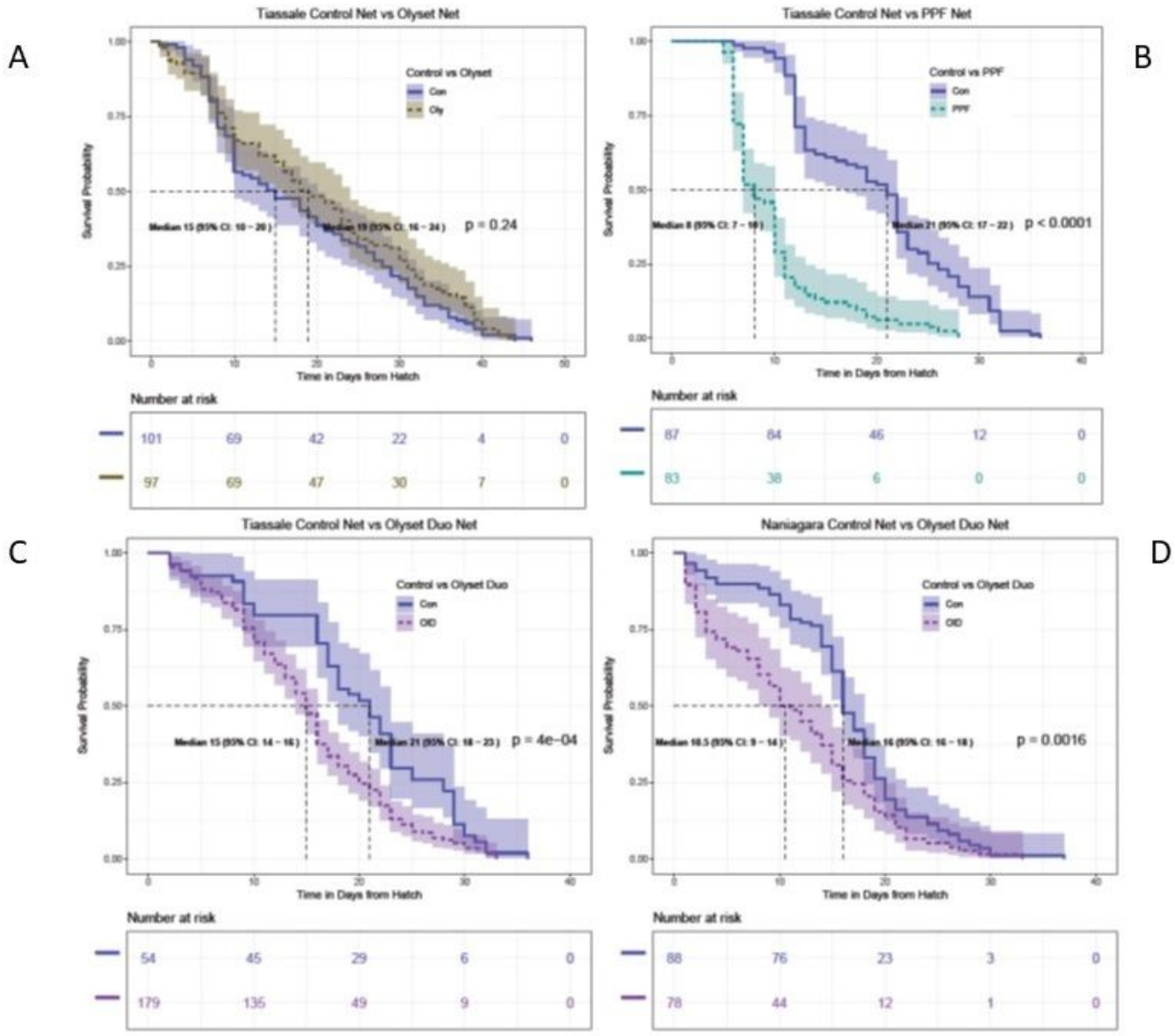

Figure 4

Survival curve of Anopheles gambiae mosquitoes from pyrethroid-resistant laboratory colony and field collected mosquitoes exposed to different treatments. A) Pyrethroid-resistant mosquitoes exposed to permethrin-treated nets (n=97) and untreated nets (n=101), B) Pyrethroid-resistant mosquitoes exposed to pyriproxyfen (PPF)-treated (n=83) and untreated nets (n=87), C) pyrethroid-resistant mosquitoes exposed to PPF-ITNs (n=179) and untreated nets (n= 54), and D) field caught mosquitoes exposed to PPF-ITNs (n=78) and untreated nets (n=88). Mosquitoes were exposed to a treated or untreated net 4 days post-emergence and offered a bloodmeal every 7 days.

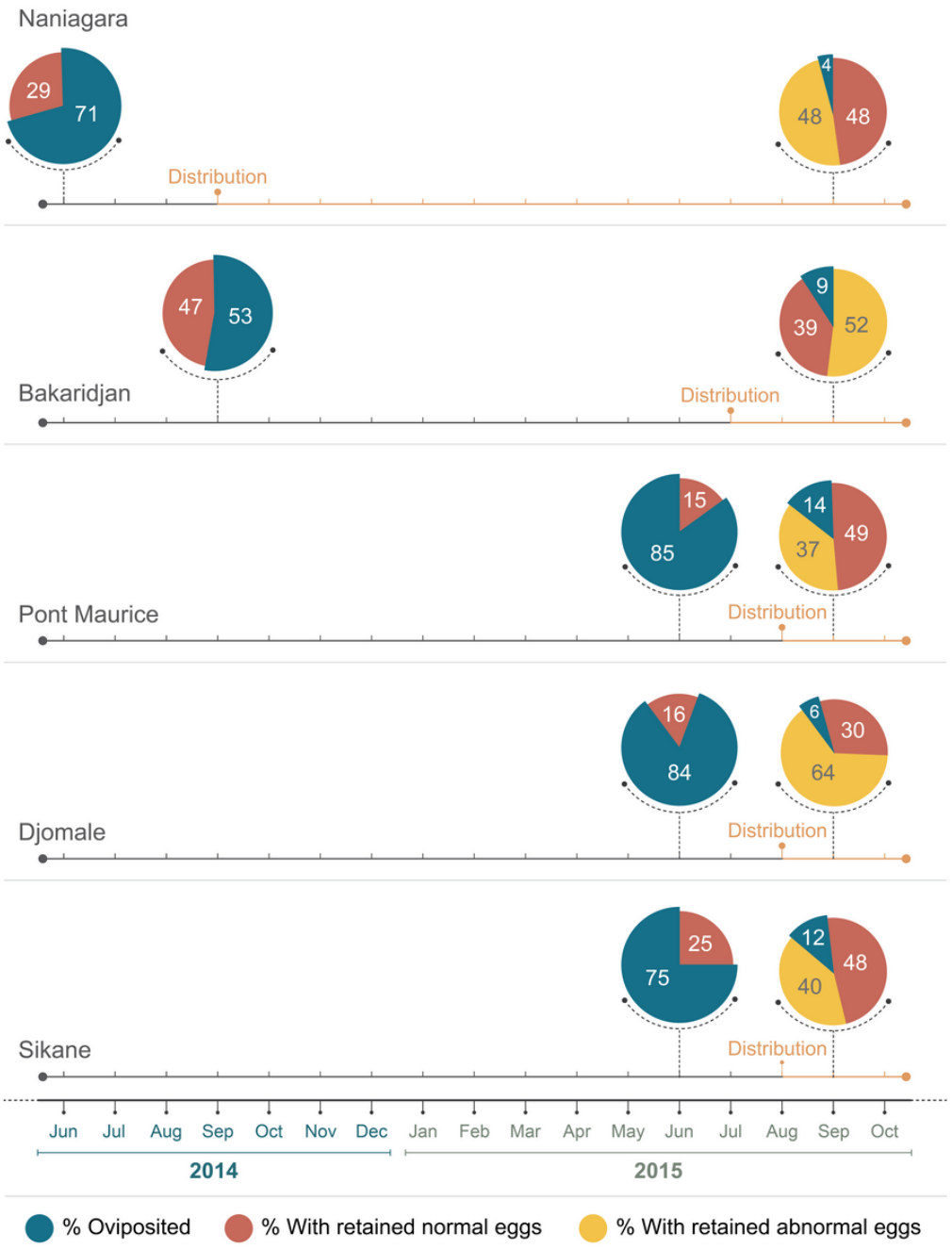

Figure 5

Oviposition rates and ovarian status of mosquitoes collected before and after the distribution of PPF-ITNs replaced pyrethroid only ITNs In Naniagara (n=72 before distribution; n=183 after) the second round of collections was completed one year after PPF-ITNs were distributed, in Bakaridjan (n=87 pre-distribution; n=127 post-distribution) the second round of collections was 3 months after the change in nets and in Pont Maurice (n=104 pre-distribution, n=107 post-distribution), Djomale (n=118 pre-distribution, n=90 post-distribution) and Sikane (n=134 pre-distribution, n=124 post-distribution) 1 month.

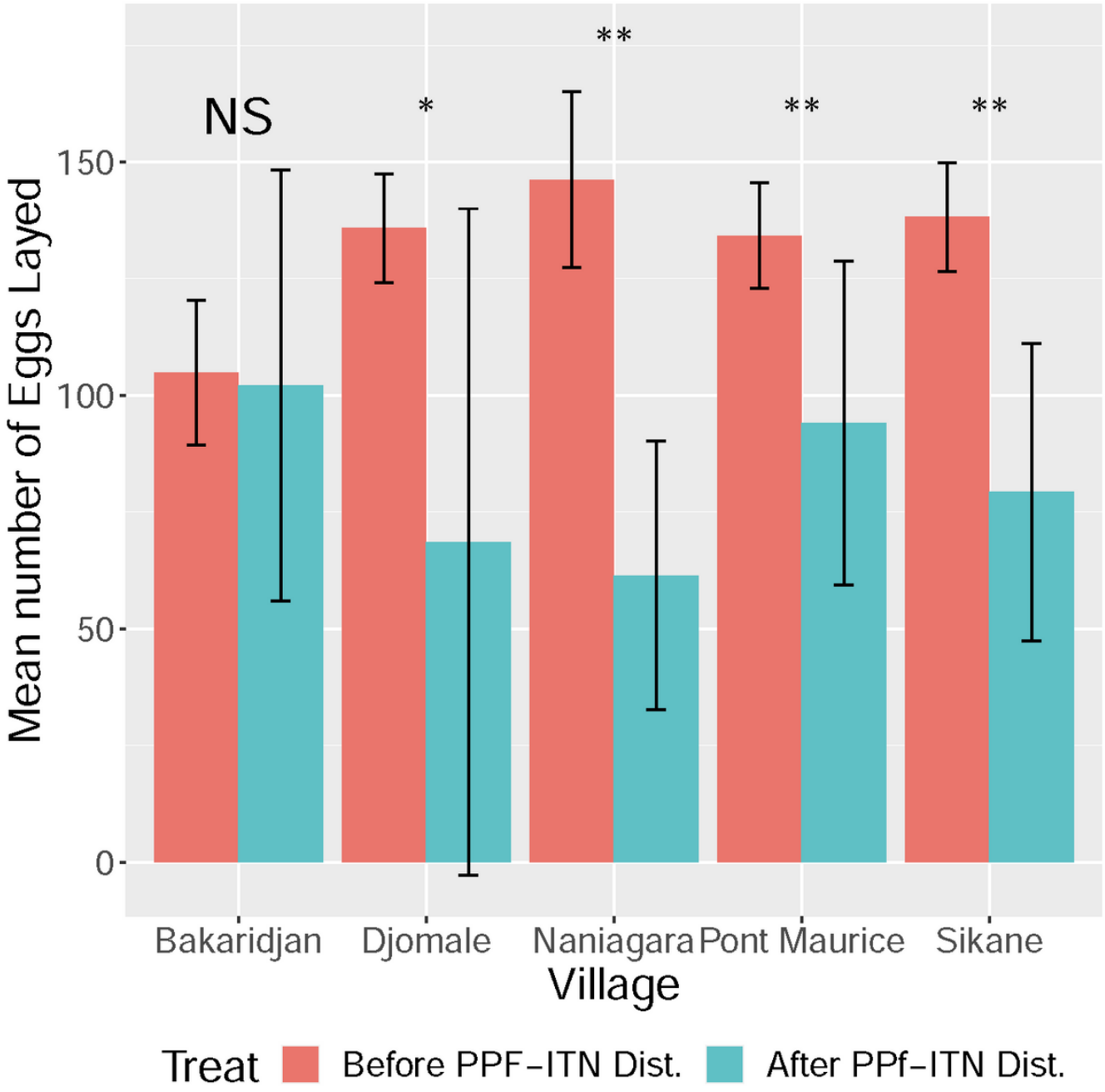

Figure 6

Mean number of eggs laid by Anopheles collected before and after the distribution of PPF-ITNs replaced pyrethroid only ITNs. Mosquitoes laying no eggs were removed from this analysis. The error bars show the Standard Error of Mean (SEM), and significant differences between collections within each village are $p< 0.05$*, and $p<0.001$**.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFilesGrisales.docx
- GrisalaesSuppFig1.pdf
- GrisalaesSuppFig2.pdf