1 **Pyriproxyfen is metabolized by P450s associated with pyrethroid resistance in**
2 ***An. gambiae***
3
4 **Short Title:** Pyriproxyfen P450 metabolism
5
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14
15 **Abstract**
16 Pyrethroid resistance is widespread in the malaria vector *Anopheles gambiae* leading
17 to concerns about the future efficacy of bednets with pyrethroids as the sole active
18 ingredient. The incorporation of pyriproxyfen (PPF), a juvenile hormone analogue, into
19 pyrethroid treated bednets is being trialed in Africa. Pyrethroid resistance is commonly
20 associated with elevated levels of P450 expression including CYPs 6M2, 6P2, 6P3,
21 6P4, 6P5, 6Z2 and 9J5. Having expressed these P450s in *E. coli* we find all are
22 capable of metabolizing PPF. Inhibition of these P450s by permethrin, deltamethrin
23 and PPF was also examined. Deltamethrin and permethrin were moderate inhibitors
24 (IC_{50} 1 – 10 μM) of diethoxyfluorescein (DEF) activity for all P450s apart from CYP6Z2
25 (IC_{50} >10 μM), while PPF displayed weaker inhibition of all P450s (IC_{50} >10 μM) except
26 CYP's 6Z2 and 6P2 (IC_{50} 1 – 10 μM). We found evidence of low levels of cross
27 resistance between PPF and other insecticide classes by comparing the efficacy of

PPF in inhibiting metamorphosis and inducing female sterility in an insecticide susceptible strain of *An. gambiae* and a multiple resistant strain from Cote d'Ivoire.

Keywords

Pyriproxyfen, insecticide resistance, P450, Olyset Duo

Abbreviations

PPF, pyriproxyfen; ALA, 5-Aminolevulinic acid; DEF, diethoxyfluorescein; CPR, cytochrome P450 reductase.

1. Introduction

Malaria control is reliant on the use of insecticides. The dramatic reductions in malaria cases in Africa that have occurred over the last 15 years have been largely attributed to methods targeting the adult mosquito, primarily via the use of long lasting insecticidal nets (LLINs) treated with pyrethroids and, to a lesser extent indoor residual spraying (IRS) with pyrethroids and DDT and, more recently, carbamates and organophosphates (Bhatt *et al*., 2015; Ranson and Lissenden, 2016). Resistance to pyrethroids is now widespread in the major malaria vectors in Africa with resistance to other classes of public health insecticides also on the increase (Ranson and Lissenden, 2016). There is therefore an urgent need both for new insecticides to maintain the efficacy of these proven tools, and for new tools to reduce malaria transmission by the mosquito.

Pyriproxyfen (PPF) is a juvenile hormone analogue that inhibits metamorphosis. It has been used for several decades to protect against cotton pests (Carriere et al., 2012) and its extremely low toxicity to humans has also enabled applications in public health such as addition to water storage containers to control *Aedes* populations (Darriet and Corbel, 2006; Lee, 2001). As PPF is active in very low concentrations, the active

56 ingredient can be disseminated by the insect itself; this autodissemination route was
57 shown to be effective at controlling *Aedes* populations in trials in Peru (Devine et al.,
58 2009). For malaria vectors, difficulties in identifying and treating the diverse breeding
59 sites for malaria vectors have so far largely confined larviciding for malaria control to
60 easy to reach urban areas but the possibility of using autodissemination strategies to
61 distribute PPF to target *Anopheles* oviposition sites in rural areas is being explored.
62 Currently, however, control of *Anopheles* mosquitoes is more commonly targeted at
63 the adult stage, hence the impact of PPF on embryogenesis, shows the greatest
64 promise for malaria control. Exposure to PPF effectively sterilizes female mosquitoes
65 and has also been shown to reduce adult longevity (Ohashi *et* al., 2012; Ngufor *et al*.,
66 2014). Sumitomo Chemicals Ltd has developed a LLIN incorporating both permethrin
67 and PPF. This Olyset Duo®, net has been shown to be effective in laboratory and
68 experimental hut trials (Aiku *et al*., 2006; Ngufor *et al*., 2014; Ohashi *et al*., 2012;
69 Tsunoda *et al*., 2013) and is currently being evaluated in a randomised control trial in
70 Burkina Faso to compare the efficacy of this combination net with conventional Olyset
71 nets (Tiono *et al*., 2015).
72
73 However, concerns have been raised about the performance of Olyset Duo against
74 pyrethroid resistant populations (Koffi et al., 2015). An experimental hut study of Olyset
75 Duo carried out in an area where the *Anopheles gambiae* population has high levels
76 of both target site and metabolic resistance to pyrethroids found no significant
77 difference in the number of sterile mosquitoes in huts with Olyset Duo compared to
78 control huts (Koffi *et al*., 2015).
79
80 Resistance to PPF has been reported in other insects including the greenhouse
81 whitefly *Trialeurodes vaporiarium* (Karatolos *et al*., 2012) and the sweet potato whitefly,
82 *Bemisia tabaci* (Rami Horowitz *et al*., 2003). Although the mechanisms of resistance

83 have not been fully described, elevated levels of genes involved in insect P450 and
84 GST activity appear to be involved.
85
86 Several P450 enzymes have been implicated in the development of metabolic
87 insecticide resistance in *An. gambiae* but a relatively small subset of this large enzyme
88 family are consistently found up-regulated in pyrethroid resistant populations (David *et*
89 *al*., 2013, Inhgam *et al.,* 2015). This candidate list is predominated by three subfamilies
90 of the CYP6 P450s: CYP6P, CYP6M and CYP6Z, but also includes CYP9J5
91 (Hemingway et al., 2013; Toé et al., 2015) and the two CYP4G enzymes, CYP4G16
92 and 17 (Jones et al, 2013; Toé et al., 2015). CYP6M2 and CYP6P3 are confirmed
93 pyrethroid metabolisers but are also active against insecticides from other insect
94 classes (Mitchell *et al*., 2012; Muller *et al*., 2008; Stevenson *et al*., 2011). In contrast
95 the CYP4G enzymes do not have detectable activity against insecticides but instead
96 are believed to confer resistance via reducing insecticide uptake (Balabanidou *et al*, in
97 press).
98
99 Here, we expressed the CYPs 6M2, 6P1, 6P2, 6P3, 6P4, 6P5, 6Z2 and 9J5 in
100 *Escherichia coli* and assessed their ability to metabolize PPF. We also assessed the
101 likelihood of cross resistance between PPF and other insecticide classes by comparing
102 the efficacy of PPF in inhibiting metamorphosis and inducing female sterility in an
103 insecticide susceptible strain of *An. gambiae* and a multiple resistant strain from Cote
104 d'Ivoire.
105
106 **2. Material and methods**
107 **2.1 Reagents**
108 Oligonucleotides were synthesized by Eurofins genomics and enzymes for DNA
109 manipulation were supplied by Thermo Scientific. Isopropyl-ß-D-thio-
110 galactopyranoside (IPTG), 5-aminolevulinic acid (ALA), and 3-[(3-cholamidopropyl)-

111 dimethylammonio]-1-propanesulfonate (CHAPS) were supplied by Melford (UK).
112 Insecticides were supplied by ChemService: 3-phenoxybenzyl (1*R,S*)-*cis,trans*-3-(2,2-
113 dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (permethrin, mixture of isomers),
114 (*S*)-α-cyano-3-phenoxybenzyl (1*R*,3*R*)-*cis*-2,2-dimethyl-3-(2,2-dibromovinyl)-
115 cyclopropanecarboxylate (deltamethrin) and pyriproxyfen. HPLC solvents were
116 supplied by Fisher Scientific. Other chemicals were obtained from Sigma-Aldrich
117 unless indicated otherwise.
118
119 **2.2 Gene cloning**
120 Total RNA was extracted with either the Arcturus PicoPure Kit (Applied Biosystems)
121 or TRI reagent (Sigma-Aldrich) from ten adult *An. gambiae* mosquitoes from the
122 Kisumu strain. Complementary DNA was prepared using Superscript III (Invitrogen)
123 with an oligo(dT)$_{20}$ primer and used as a template for amplifying full-length genes with
124 KOD DNA polymerase (Merk Chemicals). The gene-specific primers used in these
125 high-fidelity PCRs were designed according to the *An. gambiae* genome sequence
126 (Table S1). PCR products from P450s, summarized in Table S2, were ligated into
127 pGEM T-easy (Promega) and sequenced. For expression, the ompA leader sequence
128 (ompA), was engineered onto the amino-terminus to direct the P450 to the *E. coli* outer
129 membrane during expression as previously described (Mclaughlin *et al*., 2008;
130 Stevenson *et al*., 2011). The ompA-leader was fused to the P450 cDNA in frame with
131 the P450 initiation codon by fusion PCR. The ompA-leader was fused to the P450
132 cDNA in frame with the P450 initiation codon by fusion PCR. The ompA P450 fusion
133 was flanked at the 5' and 3' ends with NdeI and NotI respectively for ligation into
134 NdeI/NotI digested pCWori+.
135
136 For CYP6M2 and CYP6P3 a single plasmid expression system for the P450 and CPR
137 was constructed by ligating the expression cassette (cDNA and *tactac* promotor)
138 containing the *An. gambiae* NADPH P450 reductase (CPR) cDNA from the

139 pACYC:AgCPR plasmid described above into the pCW:P450 expression plasmid. In
140 the new construct the expression of each protein is under the control of its own *tactac*
141 promoter so expression of both proteins is induced by the addition of IPTG to the
142 culture.
143

144 **2.3 Preparation of membranes expressing P450 and AgCPR**

145 For dual plasmid co-expression of P450 and AgCPR (all P450s except CYP6M2 and
146 CYP6P3), competent *E. coli* DH5α cells were co-transformed with pCW:P450 plasmid
147 and pACYC:*AgCPR* (Stevenson *et al.*, 2011). Cultures, generally 0.2 l, were
148 supplemented with 1.0 mM ALA and incubated at 23°C for 18 – 24 hours after 1mM
149 IPTG induction. P450 expression, *E. coli* membrane isolation and determination of
150 P450 and AgCPR content was performed as previously described (Mclaughlin *et al.*,
151 2008; Stevenson *et al.*, 2012). Samples were stored in aliquots at -80 °C. *An. gambiae*
152 cytochrome b_5 (b_5) was prepared as described previously to supplement enzyme
153 reactions at a 10:1 molar ratio, b_5:P450 (Stevenson *et al.*, 2011).
154

155 For CYP6M2 and CYP6P3, *E. coli* JM109 were transformed with the single plasmid
156 system for the co-expression of AgCPR. Cultures were grown in 10 l stirred batch,
157 glass fermenters and were harvested and processed to produce membrane fractions
158 containing the CYP and CPR using the procedures described above, scaled to account
159 for the larger culture volumes. Cytochrome b_5 was added to the CYP6P3 bacterial
160 membrane preparations at a 10:1 molar ratio, b_5:P450. After the addition of the
161 cytochrome *b5* the membrane preparation was gently stirred at 4°C for 30 min to allow
162 time for the incorporation of the cytochrome *b5* into the membranes before aliquotting
163 and freezing at -80 °C.
164

165 **2.4 Pyriproxyfen metabolism**

166 Incubations of a 200 µl reaction mix containing 20 µM insecticide, 0.1 µM of the
167 recombinant enzyme, 1.0 µM b_5, 0.2 M of TrisHCl at pH 7.4, 0.25 mM $MgCl_2$, 1 mM
168 glucose-6-phosphate, 0.1 mM NADP+ and 1 unit/mL glucose-6-phosphate
169 dehydrogenase (G6PDH) were carried out in the presence or absence of 10 µM
170 piperonyl butoxide (PBO) at 30°C with shaking (1200 rpm) for 90 min and stopped by
171 addition of 200 µl of methanol. Shaking was carried for an additional 10 min before
172 centrifuging the reactions at 20000 *g* for 20 min. 150 µl of supernatant was used for
173 HPLC analysis. Reactions were performed in triplicate and a paired T-test of sample
174 reactions (+NADPH) vs negative control (-NADPH) used for statistical measurements
175 of substrate depletion.
176
177 For reciprocal IC_{50} measurements of CYP6P3 metabolism of PPF and permethrin,
178 enzyme reactions were carried out with PPF or permethrin added as inhibitors (at
179 concentrations ranging from of 0 - 1.6 mM or 0 -3.2 mM respectively) to the reaction
180 mix to titer their effect on the insecticide turnover (fixed at 20 µM). Three replicates of
181 positive and negative control reactions were run for each P450/inhibitor combination.
182
183 **2.5 High-pressure liquid chromatography (HPLC) analysis**
184 Samples were analyzed by high-pressure liquid chromatography, HPLC (Agilent 1100
185 series). The quantity of insecticide remaining in the samples was determined by
186 reverse-phase HPLC with a monitoring absorbance at 232 nm using a C18 column,
187 Acclaim 120, Thermo Scientific. 100 µl of sample was loaded with a flow-rate 1 ml/min
188 at 23 °C into an isocratic mobile phase 90% methanol and 10% water. The retention
189 time for PPF is 7.4 min and for PBO 7.1 min.
190
191 The same conditions were applied for HPLC analysis for the PPF/permethrin inhibition
192 assays. Retention time for trans- and cis-permethrin is 11.8 min and 14.1 min
193 respectively.

194

195

196 **Mass spectrometry analysis for pyriproxyfen metabolism**

197 PPF metabolism by CYP6P3 was examined by mass spectrometry to confirm oxidation
198 and identify the metabolites produced. Aliquots (5 μl) of organic solvent-quenched
199 reaction supernatant were injected onto a high resolution Thermo Q-Exactive mass
200 spectrometer (MS) that was coupled to a 1290 series Agilent LC system. The
201 chromatographic separation was performed on a Waters Acquity BEH C18 (2.1 x 50
202 mm; 1.7 μm) analytical column at 30 ºC using a mixture of water (0.1% formic acid) (A)
203 and acetonitrile (0.1% formic acid) (B) as mobile phase. In the 12 minutes run time the
204 gradient program was as follows: 5% B in 0-1 minute; 5% B to 100% B in 1-8 minutes;
205 100% B in 8-10 minutes; 100% B to 5% B in 10-10.1 minutes; 5% B in 10.1-12 minutes.
206 During the analysis the MS was operated in positive ion full scan mode (mass range:
207 100-1000 m/z) at 35K resolution using a constant heated electrospray capillary
208 temperature (320 ºC), spray voltage (3500 V), sheath gas (55 arbitrary units) and
209 auxiliary gas flow rate (10 arbitrary units).

210

211 **2.6 Diethoxyfluorescein metabolism**

212 Diethoxyfluorescein (DEF) substrate was dissolved in DMSO, with final concentration
213 of 2 % per assay. All test compounds were dissolved in DMSO, with a final solvent
214 concentration <2 % per assay. For calculation of the kinetic parameters (K_M and V_{max}),
215 each P450 was used at a final concentration of 10 nM (1 pmol/reaction) and DEF
216 concentrations in the range: 0, 0.31, 0.63, 1.25, 2.5, 5, 10 and 20 μM. DEF reactions
217 were carried out at 25 °C in 50 mM KPi at pH 7.4 containing 1 mM glucose-6-
218 phosphate (G6P), 0.1 mM $NADP^+$, 0.25 mM $MgCl_2$, and cytochrome b_5 at a 10:1 molar
219 ratio, b_5:P450. $NADP^+$ and G6P were excluded from the minus NADPH controls.

220

Variable ligand concentrations were used for IC_{50} calculations with DEF used at ~ K_M for each P450 (i.e. 0.5, 1.4, 0.7, 1.0, 3.5 and 0.5 µM for CYP6M2, CYP6P2, CYP6P3, CYP6P4, CYP9J5, and CYP6Z2 respectively) and 0.1 µM P450. Three replicates of positive and negative control reactions were run for each P450/substrate combination in opaque white 96-well (flat-based) plates in triplicate. The fluorescent reactions were monitored in a fluorescence plate-reader (Ex = 485 nm, Em = 520 nm) continuously over 20 minutes time period after the addition of NADPH regenerating system. The rate of fluorescent molecules produced per P450 molecule per min (turnover) was determined by linear regression of the measurements between 3 min and 10 min after the reactions began. The Michaelis-Menten and IC_{50} fitting calculations were performed using Graphpad Prism 6. Data were fitted to the dose-response model and plots with R^2 <0.95 were rejected.

2.8 *In vivo* studies

Two strains of mosquitoes were used to assess the impact of exposure to PPF on life history. The Kisumu strain of *An. gambiae* originates from Kenya and is susceptible to all insecticide classes used in public health whereas the Tiassalé strain from Cote d'Ivoire shows resistance to four classes (pyrethroids, carbamates, organophosphates and the organochlorine DDT) (Constant *et al*, 2012). Resistance in the Tiassalé strain is mediated by multiple mechanisms, including the overexpression of cytochrome P450s, notably CYP6P3, 6P4 and 6M2 (Constant et al, 2014). Both mosquito strains were reared in the insectaries at the Liverpool School of Tropical Medicine under a 12:12 photoperiod at 27°C and 70-80% humidity.

To measure the effect of PPF on metamorphosis, SumiLarv®0.5G (Sumitomo Chemicals Ltd) was ground into a fine powder and dissolved in water to prepare a stock solution of 1000 ppm SumiLarv (50 ppm active ingredient). The solution was left overnight dissolving on a magnetic stirrer, protected from light. Serial dilutions were

249 prepared and the following PPF concentrations were tested: 0.001 ppb, 0.005 ppb,
250 0.07 ppb, 0.1 ppb, 1 ppb, 5 ppb and 10 ppb. Four replicates of 25 3rd instar mosquitoes
251 were exposed to each of the SumiLarv concentrations in paper cups for up to 8 days.
252 Larvae were fed Tetramin® baby fish food every day and cups covered with netting to
253 prevent adults escaping. The number of live and dead larvae, pupae and adults was
254 recorded every 24 hours until all individuals were emerged as adults or dead. Adults
255 and dead pupae were removed daily. The Dose Effect function on XLSTAT (Microsoft)
256 was used to estimate the concentration resulting in 50 % emergence inhibition (EI50).
257
258 To compare the impact of PPF on adult mosquitoes of the two strains, we measured
259 the ability of this compound to impair ovary development. Borosilicate glass tubes (30
260 cm long, 11 mm wide) were impregnated with three different concentrations of PPF
261 (ai): 0.55 mg/m^2, 2.75 mg/m^2 and 5.5 mg/m^2. An additional tube impregnated only with
262 the solvent (acetone) was used as a negative control. Tubes were used on the day of
263 preparation. Two groups of fifteen 5-7 days old female mosquitoes from Tiassalé and
264 Kisumu strains were tested for each concentration (n= 30). After 60 minutes
265 acclimation in paper cups, they were transferred to the glass tubes and exposed for 3
266 minutes. Mosquitoes were then returned to the paper cups and left for 24 hours with a
267 10% sucrose solution. 24 hours after exposure the mosquitoes were bloodfed and any
268 mosquitoes which did not feed were removed. Mosquitoes were retained in insectary
269 conditions with access to sugar water for five days and then dissected and the
270 morphology of the ovaries assessed as normal (loose, well developed eggs) or
271 abnormal (non-detachable, bubble-like eggs). Dead mosquitoes or mosquitoes not
272 presenting egg development were discarded and removed from the analysis.
273
274 To confirm that formulated products containing PPF also impaired egg development,
275 mosquitoes were exposed to Olyset Duo nets, nets containing 1 % PPF only (supplied
276 by Sumitomo Chemicals Ltd), conventional Olyset nets or untreated nets. Batches of

277 10 3-5 days old Tiassalé mosquitoes were exposed to the nets for 3 minutes following
278 WHO standard protocols for cone bioassays. Twenty-four hours after the exposure,
279 mosquitoes were bloodfed and left in paper cups with 10% sucrose solution. Dead and
280 non-bloodfed mosquitoes were removed from the experiment. After 5 days surviving
281 mosquitoes were dissected and the morphology of the ovaries examined as described
282 above. To assess the effects of short exposures to PPF LLINs, an additional set of
283 cone bioassays was performed, as described above, except exposure time was
284 reduced to 30 seconds.

285

286 **3. Results and Discussion**

287 This study was motivated by concerns over cross resistance between pyrethroid
288 insecticides and the insect growth regulator, PPF. Cross resistance between
289 insecticide classes with different modes of action, mediated by cytochrome P450s, has
290 previously been demonstrated in *An. gambiae* (Constant *et al.*, 2014; Mitchell *et al.*,
291 2012) and here we investigated whether these same P450 enzymes can also
292 metabolize PPF. CYP6M2, CYP6P3 and CYP6Z2 have previously been expressed in
293 bacterial expression systems but we extended the panel of recombinant enzymes to
294 include a further three *An. gambiae* CYP6P P450s implicated in pyrethroid resistance
295 in Cote d'Ivoire (Constant *et al*, 2014) and CYP9J5, which has been found over
296 expressed in pyrethroid resistant populations from Bioko Island (Hemingway *et al.*,
297 2013) and Burkina Faso (Toé et al., 2015).

298

299 **3.1 Functional expression of P450s in *E. coli***

300 P450s require electrons from NADPH-cytochrome P450 oxido-reductase (CPR) for
301 catalysis, thus new candidate *An. gambiae* P450s were co-expressed with AgCPR in
302 *E. coli* using ompA and pelB leader sequences to direct the enzymes to the inner
303 bacterial membrane as previously with CYP6M2 and CYP6P3 (Muller *et al.*, 2008;
304 Stevenson *et al.*, 2011). CYP's 6P1, 6P2, 6P4, 6P5, 9J5 and 6M1 were co-transformed

305 with AgCPR-pACYC for *E. coli* expression, producing characteristic CO-reduced
306 spectra indicative of active P450 (Fig. S1). The yields of P450 were in the range 10 –
307 100 nmol/L, with CYP6P5 producing the lowest quantities of P450 (~ 10 nmol/L; Table
308 S3). CYPs 6P1 and 6M1 failed to express functional P450.
309
310 CYP6M2 and CYP6P3 have previously been expressed following co-transformation
311 with AgCPR on separate plasmids. Here, these enzymes were co-expressed in
312 tandem with AgCPR on a single from the P450 expression plasmid, pCWori+ to
313 facilitate scaled 10 l fermentor expression. Tandem expression from the single plasmid
314 produced higher CPR:P450 ratios. The fluorogenic substrate DEF was used to
315 estimate the kinetic parameters of the tandemly expressed recombinant proteins
316 against dual plasmid protein expression (Table 1) and used in dose-response
317 experiments to determinate the inhibitory effect of permethrin, deltamethrin and
318 pyriproxyfen in its metabolism. As expected, since CPR is rate limiting, the V_{max} values
319 were 3 - 6 fold higher using the single plasmid in tandemly expressed membranes
320 compared with dual plasmid expression, consistent with the elevated levels of CPR.
321 The K_M value is the concentration of DEF required to reach max reaction velocity and
322 independent of the enzyme concentration. The single versus double plasmid K_M
323 values for CYP6M2 (0.4 vs 0.5 μM) and CYP6P3 (0.7 vs 0.9 μM) were similar, again
324 consistent with higher CPR levels increasing reaction rates through enhanced electron
325 transfer rather than effects on substrate binding.
326
327 **3.2 PPF metabolism**
328 The ability of the *An. gambiae* P450s to metabolize PPF was tested by measuring
329 substrate turnover (substrate disappearance over time) in the presence and absence
330 of NADPH. We also included PBO in parallel reactions as further validation of P450
331 induced substrate depletion (Fig. S2). PBO is an inhibitor of P450 monoxygenase
332 activity and a common insecticide synergist (Vijayan *et al.*, 2007). All seven P450s

333 metabolized PPF to some degree with the percentage PPF depletion ranging from
334 24.78 % for CYP9J5 to 100% for CYP6P3, and PPF depletion was inhibited by PBO
335 for each P450 tested (Table 2). Since *E. coli* membranes expressing CYP6M2 and
336 CYP6P3 had higher levels of CPR, the rates of activity were not comparable with the
337 rest of the P450s. However, it is notable that, with the exception of CYP6P5, all
338 members of the CYP6P family and CYP6Z2 produced high levels of PPF depletion (58
339 - 100%). CYP6Z2 is of interest since it is found overexpressed in pyrethroid resistant
340 populations of *An. gambiae*, but metabolises the pyrethroid metabolites 3-
341 phenoxbenzoic alcohol and aldehyde rather than the parent compound (Chandor-
342 Proust et al., 2013). Here CYP6Z2 appears to play a direct role in the primary
343 metabolism of PPF, thus may have an influential role in PPF clearance and potentially
344 insecticide resistance.

345

346 Mass spectrometry analysis of the PPF metabolites generated by CYP6P3, the
347 strongest metabolizer was carried out to confirm oxidation and identify possible
348 metabolites. Expected metabolites included 4`-OH-PPF, 5``-OH- PPF and 5``,4`-OH-
349 PPF, that have been previously identified from the *in vitro* metabolism of PPF by
350 microsomes from housefly larvae (Zhang et al., 1998). CYP6P3 generated three
351 metabolite peaks (Fig. S3). The extracted ion chromatograms of $[M+H]^+$ generated two
352 peaks (6.9 min and 7.1 min), with molecular mass (m/z=338.1387) corresponding to
353 the addition of a hydroxyl group (m/z=16), consistent with 4`-OH-pyriproxyfen and 5``-
354 OH-pyriproxyfen production. Furthermore, the data showed signs of a metabolite with
355 molecular mass 32 m/z larger than PPF, equivalent to a double hydroxylation,
356 potentially 5``,4`-OH-PPF resulting from secondary metabolism of 5''-OH-PPF and/or
357 4`-OH-PPF metabolites. This was, however, not confirmed chromatographically as
358 analytical reference standards were not available for the analysis. Further collision
359 mass spectrometry or NMR is required to confirm the identity of the metabolites.

360

361 Since PPF is being used in combination with pyrethroids in bednets we were interested
362 in potential synergistic effects. We therefore measured the IC_{50} values of PPF,
363 deltamethrin and permethrin to compare relative strengths of DEF metabolism
364 inhibition against the P450s (Table 3). The fluorescent substrate, DEF, was used for
365 monitoring P450 activity using a 96 well microtiter plate format. In drug screens,
366 compounds are generally categorized according to their activity as P450 inhibitors as
367 potent inhibitors (IC_{50} <1 μM), moderate (IC_{50} 1 – 10 μM) and weak inhibitors (IC_{50} >
368 10 μM) (Krippendorff *et al.*, 2007). Using these criteria, PPF displayed moderate
369 inhibition of DEF metabolism for CYP's 6Z2 and 6P2, with the remainder being weakly
370 inhibited. Deltamethrin and permethrin were moderate inhibitors of DEF activity for all
371 P450s apart from CYP6Z2, which was weakly inhibited by both pyrethroids. As Olyset
372 Duo nets contain both PPF and permethrin, we also measured PPF inhibition of
373 permethrin metabolism by CYP6P3 and vice versa (Fig. 1). CYP6P3 was chosen as it
374 is one of the P450s most frequently found at elevated levels of expression in pyrethroid
375 resistant populations of *An. gambiae*. Permethrin produced slightly stronger inhibition
376 of PPF metabolism (IC_{50} = 61.2 μM) than PPF inhibition of permethrin metabolism
377 (IC_{50} = 92.7 μM). Overall, the *in vitro* results suggest that the pyrethroids deltamethrin
378 and permethrin are slightly stronger inhibitors (2 – 3 fold) than PPF against the
379 pyrethroid metabolizing P450s tested.
380
381 These data indicate that PPF can be metabolized by a wide range of P450s associated
382 with pyrethroid resistance. In mosquitoes that have elevated levels of expression of
383 one or more of these enzymes, it is feasible that enhanced metabolism of PPF could
384 reduce the efficacy of this juvenile hormone analogue. To test this hypothesis we
385 performed PPF bioassays on insecticide susceptible and resistant strains.
386
387 **3.3 Bioefficacy of PPF against insecticide resistant mosquitoes**

388 The impact of exposure to PPF on metamorphosis and embryogenesis was compared
389 in the insecticide susceptible Kisumu strain and the multi resistant Tiassalé strain from
390 Cote d'Ivoire. The dose-response tests showed that SumiLarv® 0.5G affected adult
391 mosquito emergence in both strains but the minimum dose that inhibited 100 %
392 emergence was 1 ppb in the insecticide susceptible Kisumu strain and 10 ppb in the
393 Tiassalé strain. The concentration that resulted in 50 % inhibition of emergence (EI_{50})
394 for the susceptible Kisumu strain was 0.088 ppb (95 % confidence intervals 0.064 –
395 0.123 ppb) (Fig. 2), similar to values reported for *An. gambiae s.l.* in other studies
396 (0.025 ppb Kawada (1993), 0.13 ppb (Mbare et al 2013)). In contrast, the EI_{50} for the
397 Tiassalé strain was 0.356 ppb ai (0.274 – 0.463) approximately 4-fold higher than the
398 Kisumu strain. It is important to note that we cannot directly link the higher EI50 for
399 PPF to the presence of elevated P450s in the Tiassale strain given that only one
400 pyrethroid resistant population was evaluated and it is not known whether the same
401 P450s found elevated in adults of this strain are also up-regulated at the larval
402 stage. Furthermore, the field dose of SumiLarv 0.5G ranges from 10 - 50 ppb so it is
403 likely that the product would still inhibit development of the Tiassalé strain under field
404 conditions. However, given the trajectory of increasing pyrethroid resistance in both
405 *Anopheles* (Ranson and Lissenden, 2016) and *Aedes* mosquitoes (Vontas et al, 2012)
406 and the growing interest in use of PPF to target immature populations of these vectors
407 (Kiware et al, 2015, Abad-Franch et al., 2015;) it is important that further evaluation of
408 PPF efficacy against field populations is carried out.
409
410 As the sterilizing effect of PPF on mosquito populations has also received considerable
411 attention for malaria control, with clinical trials of the PPF/permethrin Olyset Duo LLIN
412 ongoing (Tiono et al, 2015, Sagnon et al., 2015) we also compared inhibition of
413 embryogenesis in our susceptible and resistant populations. In a narrow bore glass
414 tube assay, designed to ensure complete contact with the PPF for the duration of the
415 assay, mosquitoes from the insecticide susceptible Kisumu strain were completely

416 sterilized after a 3 minute exposure to 5.5 mg/m^2 whereas only 75% of Tiassalé
417 mosquitoes were sterilized by this dose. At half this dose, PPF had no impact on ovary
418 development in Tiassalé but resulted in 76 % of Kisumu mosquitoes being sterilized
419 (Fig. 3). Thus higher concentrations of PPF are needed to sterilize the pyrethroid
420 resistant strain, consistent with the effect on metamorphosis described above.
421

422 **3.4 Interactions between PPF and Permethrin.**

423 The *in vitro* analysis demonstrated that the same P450s can bind and metabolize PPF
424 and pyrethroids. This interaction could have a synergizing effect with one chemical
425 essentially reducing the rate of depletion of the other such that both chemicals are
426 more potent when used in combination. As the pyrethroid IC_{50}s were generally lower
427 than for PPF (with the exception of CYP6Z2), pyrethroids might be expected to have
428 a stronger enhancing effect on PPF activity than vice versa. However, the differences
429 in IC_{50} values were small (~2 fold) and, as the *in vivo* concentrations in mosquitoes
430 after exposure to products containing PPF and/or permethrin are unknown, such
431 predictions in isolation are highly speculative. Furthermore, as shown above, the
432 performance of PPF varies between strains and thus the impact of combining the two
433 chemistries in vector control products may depend on the level of expression of P450s
434 in the strain.
435

436 To investigate this further we evaluated the performance of LLINs containing a single
437 active ingredient versus the combination Olyset Duo LLIN in cone bioassays. The
438 Tiassalé strain was exposed to four net types, mortality recorded 24 hours after
439 exposure and surviving mosquitoes were offered a bloodmeal. Ovary development
440 was assessed after a further 5 days. As expected very low mortality was observed in
441 the mosquitoes exposed to untreated or PPF only nets (Table 4). Mortality was higher
442 after exposure to Olyset Duo nets than conventional Olyset nets (2-tailed z test,
443 p=0.02). All bloodfed mosquitoes exposed to the Olyset or untreated nets developed

444 normal ovaries. In contrast all of the mosquitoes exposed to the 1% PPF net were
445 sterilized. The number of surviving mosquitoes that successfully bloodfed from the
446 Olyset Duo arm was small but surprisingly only 60 % of these mosquitoes were
447 sterilized. The differential sterilizing effect of nets containing 1 % w/w PPF alone and
448 Olyset Duo (with 1% PPF and 2 % permethrin) was confirmed in follow up cone
449 bioassay study in which mosquitoes were only exposed for 30 seconds. Here > 87 %
450 of Tiassalé mosquitoes exposed to PPF nets (n=34 dissections) were sterilized versus
451 0% for untreated nets (n=39) and only 16 % for Olyset Duo nets (n= 38) (Table S4).
452
453 Taken together these results suggest that PPF *increases* the efficacy of permethrin
454 but permethrin *reduces* the efficacy of PPF. This is supported by data showing higher
455 mortality rates in huts with Olyset Duo than with Olyset in areas with resistant
456 mosquitoes (Ngfour et al 2014 in Benin, Koffi et al, 2015 in Cote d'Ivoire) (although no
457 increase in the proportion of mosquitoes sterilised in huts containing PPF only nets
458 versus Olyset Duo nets was observed, as our laboratory data would have predicted).
459 It is important to note that these experiments are conducted on formulated products,
460 and although the concentration of permethrin and PPF does not differ between net
461 types, they may differ in their bleed rates affecting the bioavailability of the two active
462 ingredients. Further laboratory and field evaluations, against vectors with differing
463 levels of metabolic resistance, are needed to better predict the performance of
464 combination products, such as Olyset Duo, in the field.
465
466 **Conclusions**
467 Given that pyrethroid resistant populations of malaria vectors are now ubiquitous in
468 Africa, it is important to evaluate the possible impact of this resistance on the
469 performance of any new vector control tools. In this study we demonstrated that a
470 subset of mosquito P450 enzymes responsible for elevated pyrethroid metabolism in
471 insecticide resistant mosquitoes can also metabolize PPF. As metabolic resistance is

472 an increasingly problematic resistance mechanism in African malaria vectors, there is
473 a very real concern that PPF resistance may already be present in field population of
474 *Anopheles* mosquitoes. Although the levels of PPF resistance we observed in the
475 current study are low, continual monitoring for resistance to this chemistry should be
476 undertaken in any area employing PPF as a larvicide or considering PPF use for adult
477 mosquito control.
478
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485
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489
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619

620 **Figure captions**

621

622 **Figure 1. Determination of IC_{50} values of permethrin and PPF in P450**
623 **metabolism.** Dose-response analysis of the inhibitory effect of (A) pyriproxyfen on
624 permethrin metabolism and (B) permethrin on pyriproxyfen metabolism.

625

626 **Figure 2. SumiLarv emergence inhibition curves for two strains of *An. gambiae*.**
627 Emergence inhibition dose response curves for the insecticide susceptible Kisumu
628 strain (continuous line) and insecticide resistant Tiassalé strain (dotted line). The grey
629 dotted lines represent 95 % upper and lower limits.

630

631 **Figure 3. Effect of pyriproxyfen on egg development for two strains of *An.***
632 ***gambiae*.** Proportions of normal/abnormal ovaries of mosquitoes from Tiassale and
633 Kisumu strains exposed to three different concentrations of pyriproxyfen. The number
634 over each bar corresponds to the sample size for each treatment.

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637 **Table 1. Kinetic parameters for DEF**

P450	Single plasmid expression		Dual plasmid expression	
	K_M (µM)	V_{max} (RFU/sec)	K_M (µM)	V_{max} (RFU/sec)
CYP6M2	0.4 ± 0.02	902.2 ± 106.99	0.5 ± 0.01	292.5 ± 38.05
CYP6P3	0.7 ± 0.10	54.4 ± 6.52	0.9 ± 0.09	14.8 ± 4.89
CYP6P2	nt	nt	1.4 ± 0.02	300.6 ± 12.00
CYP6P4	nt	nt	1.0 ± 0.04	24.2 ± 1.26
CYP9J5	nt	nt	3.4 ± 0.21	265.1 ± 28.75
CYP6Z2	nt	nt	0.5 ± 0.3	43.7 ± 8.9

Kinetic parameters for DEF

nt = not tested ; (mean ± SD)

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640 **Table 2. Pyriproxyfen metabolism by mosquito P450s.**

P450	% PPF depletion		Inhibition ratio (%)[a]
	- PBO	+ PBO	
CYP6M2	30.93 ± 4.65	1.44 ± 0.67	95.3
CYP6P2	58.03 ± 1.35	6.45 ± 3.22	91.0
CYP6P3	100.0 ± 0.01	8.68 ± 1.74	91.3
CYP6P4	81.63 ± 0.63	4.44 ± 1.99	94.6
CYP6P5	39.96 ± 1.04	2.40 ± 2.01	95.4
CYP9J5	24.78 ± 2.13	0.08 ± 0.15	99.6
CYP6Z2	66.26 ± 3.02	8.41 ± 1.58	87.3

[a] reduction of PPF depletion in percentage caused by the inhibitory effect of PBO; (mean ± SD)

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649 **Table 3. IC_{50} values for mosquito P450s.**

P450	IC_{50} (µM)		
	Pyriproxyfen	**Deltamethrin**	**Permethrin**
CYP6M2	14.14	4.24	8.07
CYP6Z2	2.23	13.99	13.72
CYP6P2	9.95	4.97	8.61
CYP6P3	15.82	3.17	6.77
CYP9J5	18.96	6.05	6.47

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659 **Table 4. Impact of exposure to LLINs containing permethrin and/or pyripoxyfen**
660 **on mosquito mortality and egg development.** *An. gambiae* Tiassalé strain were
661 exposed to the LLINs for 3 min. Mortality was measured 24 hours later and surviving
662 mosquitoes offered a blood meal. Ovary dissections were performed 5 days later. n
663 bloodfed accounts for the number of surviving mosquitoes that fed on blood and
664 survived for five days until ovary dissections.

665

LLIN	n	Mortality % (95%CI)	n bloodfed	Abnormal	% Sterilised
Untreated	45	2.22 (0.12-13.2)	23	0	0
Olyset	46	30.4 (18.2-45.9)	19	0	0
Olyset Duo	46	54.3 (39.2-68.8)	10	6	60
PPF	46	2.17 (0.11-13.0)	35	35	100

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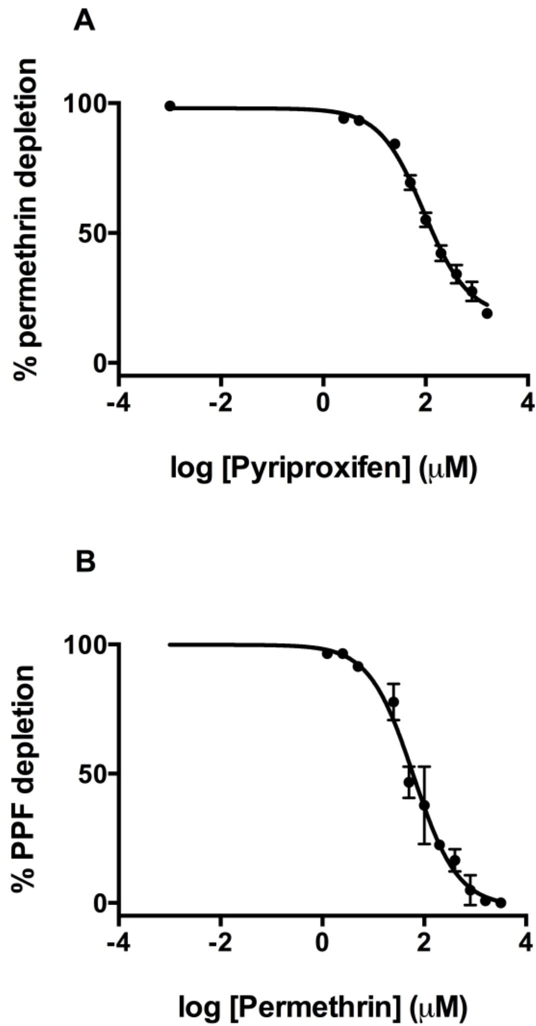

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673 **Figure 1. Determination of IC_{50} values of permethrin and PPF in P450**
674 **metabolism.** Dose-response analysis of the inhibitory effect of (A) pyriproxyfen on
675 permethrin metabolism and (B) permethrin on pyriproxyfen metabolism.

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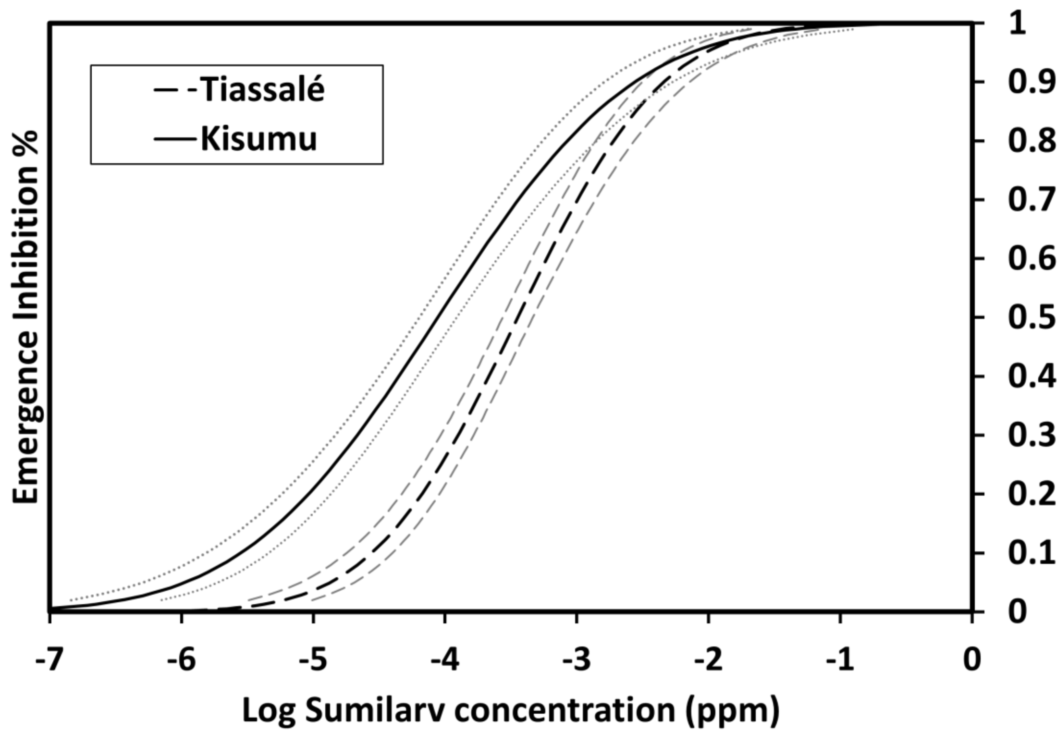

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680 **Figure 2. SumiLarv emergence inhibition curves for two strains of *An. gambiae*.**

681 Emergence inhibition dose response curves for the insecticide susceptible Kisumu

682 strain (continuous line) and insecticide resistant Tiassalé strain (dotted line). The grey

683 dotted lines represent 95 % upper and lower limits.

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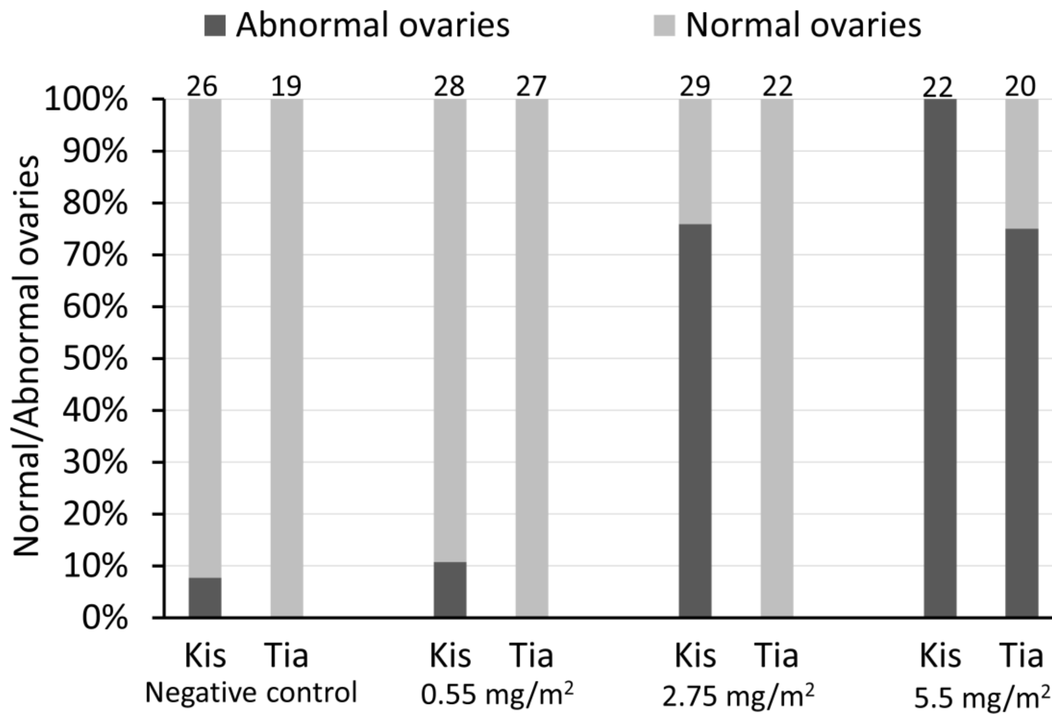

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687 **Figure 3. Effect of pyriproxyfen on egg development for two strains of *An.*
688 *gambiae*.** Proportions of normal/abnormal ovaries of mosquitoes from Tiassale and
689 Kisumu strains exposed to three different concentrations of pyriproxyfen. The number
690 over each bar corresponds to the sample size for each treatment.
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696 **Table S1. Oligonucleotide sequences used for cloning *An. gambiae* P450**
697 **genes.**
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Target	Orientation	Sequence, 5' to 3'
ompA	F	GGAATTC**CATATG**AAAAAGACAGCTATCGCG
CYP6P1	R (L)	CCGAGCAGTGACATCGGGACGGCCTGCGCTACGGTAGCGAA
CYP6P1	R	ATAAGAAT**GCGGCCGC**TCACACCTGCACGATGCGC
CYP6P2	R (L)	GGTCAAGAGCTCCATCGGGACGGCCTGCGCTACGGTAGCGAA
CYP6P2	R	ATAAGAAT**GCGGCCGC**TCAAAGCTTCTCCACCTCCAG
CYP6P4	R (L)	GCTTAACAGATCCATCGGGACGGCCTGCGCTACGGTAGCGAA
CYP6P4	R	ATAAGAAT**GCGGCCGC**CTATATCTTATCAACCTTCAG
CYP6P5	R (L)	CGTAACGGGCTCCATCGGGACGGCCTGCGCTACGGTAGCGAA
CYP6P5	R	ATAAGAAT**GCGGCCGC**CTACGCAATCTTATCCACCTTCAGG
CYP9J5	R (L)	TCAAACTGATCTCCATCGGGACGGCCTGCGCTACGGTAGCGAA
CYP9J5	R	ATAAGAAT**GCGGCCGC**TCAGTTAGCAGCTTGCTTGC
CYP6M1	R (L)	GGTTGGGAACCACATCGGGACGGCCTGCGCTACGGTAGCGAA
CYP6M1	R	ATAAGAAT**GCGGCCGC**TCAACCTTGCTTCGATTCAG

R (L): reverse "linker" primer complementary to the first 21 bases of the P450 cDNA joined to the last bases of ompA sequence

*Nde*I (CATATG) and *Not*I (GCGGCCGC) restiction sites highligthed in bold

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701 **Table S2. Accession numbers for P450 clones**

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Gene	Reference*	Single nucleotide polymorphism**
CYP6P1	AY028785	1 synonymous, 0 missense
CYP6P2	XM_312047 (AGAP002869)	2 synonymous, 1 missense c.241 G>A; V81I
CYP6P4	XM_312048 (AGAP002867)	0 synonymous, 0 missense
CYP6P5	XM_312049 (AGAP002866)	22 synonymous, 1 missense c.1369 C>T; R457W
CYP9J5	XM_551896 (AGAP012296)	1 synonymous, 0 missense
CYP6M1	AY062208.1	23 synonymous, 8 missense c.103 G>A V35I c.232 A>C M78L c.640 G>A V214M c.645 A>C E215D c.720 T>G F240L c.1041 C>G D347E c.1201 T>G S401A c.1264 G>C A422P

*Genebank accession code for complete cds, in brackets vectorbase code ** Polymorphisms detected in our individuals

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706 **Table S3. P450 yields and CPR activity.**
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P450	N[a]	Expression (nmol/L)[b]	Membrane content nmol P450/mg[c]	Membrane content nmol cyt c/min/mg[d]
CYP6M2 (1)	1	nt	0.054	65
CYP6P3 (1)	1	nt	0.067	620
CYP6M2 (2)	1	nt	0.606	57
CY6P3 (2)	2	38.8 ± 3.2	0.18 ± 0.02	188.5 ± 51.0
CYP6P2	4	23.1 ± 1.9	0.21 ± 0.02	232.3 ± 76.1
CYP6P4	6	67.9 ± 15.3	0.61 ± 0.14	258.1 ± 100.4
CYP6P5	6	10.7 ± 3.1	0.10 ± 0.02	165.1 ± 71.9
CYP9J5	4	26.0 ± 7.5	0.25 ± 0.07	281.3 ± 113.2
CYP6Z2	2	122.4 ± 24.5	0.92 ± 0.02	245.4 ± 33.4

[a] Number of independent membranes used for yield calculations.
[b] nmol of P450 isolated membrane preparation per litre of bacterial cultures; nt, not tested
[c] P450 concentration in membrane preparation as nmol of P450 per mg of total protein.
[d] CPR activity in membrane preparation measured as nmol of cytochrome c reduced per min per mg of total protein
Measurements are expressed as means ± standard deviation.
(1) single plasmid, (2) double plasmid

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716 **Table S4 Mortality and sterilisation cause by a short-time exposure to two**
717 **type of LLINs on Tiassale *An. gambiae* mosquitoes**. Mortality and
718 sterilisation on Tiassale multi-resistant mosquitoes after 30 sec exposure to two
719 different types of LLINs containing PPF. n bloodfed accounts for the number of
720 surviving mosquitoes that fed on blood and survived for five days until ovary
721 dissections.

LLIN	n	Mortality % (95%CI)	n bloodfed	Abnormal	% Sterilised
Untreated	39	2.56 (0.13-15.1)	38	0	0
PPF	31	22.6 (10.3-41.5)	24	21	87.5
Olyset Duo	38	15.8 (6.59-31.9)	38	6	15.8

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