Resistance to clothianidin reduces the efficacy of SumiShield® 50WG, a neonicotinoid formulation for indoor residual spraying, against *Anopheles gambiae*

Caroline Fouet[1], Fred A. Ashu[2,3], Marilene M. Ambadiang[2,3], Williams Tchapga[2], Charles S. Wondji[2,4] and Colince Kamdem[1]*

[1]-Department of Biological Sciences, The University of Texas at El Paso, 500 W. University Ave. El Paso, TX 79968, USA

[2]-Centre for Research in Infectious Diseases (CRID), P.O. Box 13591, Yaoundé, Cameroon

[3]-Department of Biochemistry, Faculty of Science, University of Yaoundé 1, P.O. Box 812, Yaoundé, Cameroon

[4]-Vector Biology Department, Liverpool School of Tropical Medicine (LSTM), Pembroke Place, Liverpool L3 5QA, UK

* Correspondence: gckamdemdjo@utep.edu

Abstract

Chronic exposure of mosquito larvae to pesticide residues in agricultural areas is often associated with evolution of resistance to insecticides used for vector control. This presents a concern for the efficacy of clothianidin, an agricultural neonicotinoid qualified for Indoor Residual Spraying (IRS). Using standard bioassays, we tested if reduced susceptibility to clothianidin affects the efficacy of SumiShield® 50WG, one of the two newly approved formulations, which contains 50% clothianidin. We simultaneously monitored susceptibility to clothianidin and to SumiShield® 50WG, testing adults of *Anopheles gambiae*, *An. coluzzii* and *Culex* sp collected from urban, suburban and agricultural areas of Yaoundé. We found that the level of susceptibility to the active ingredient predicted the efficacy of SumiShield® 50WG. This formulation was very potent against populations that achieved 100% mortality within 72 h of exposure to a discriminating dose of clothianidin. By contrast, mortality leveled off at 75.4 ± 3.5 % within 7 days of exposure to SumiShield® 50WG in *An. gambiae* adults collected from a farm where spraying of acetamiprid and imidacloprid is driving cross-resistance to clothianidin. These findings indicate that more potent formulations of clothianidin or different insecticides should be prioritized in areas where resistance is emerging.

Key words: *Anopheles*, clothianidin, resistance, SumiShield® 50WG, malaria, neonicotinoids.

Introduction

39 Malaria prevention in sub-Saharan Africa relies on two core interventions which used
40 chemical insecticides [1]. Indoor residual spraying (IRS) and long-lasting insecticidal nets
41 (LLINs) have contributed to a substantial reduction in the disease burden over the last
42 two decades, but the Achilles' heal of both intervention measures is the development of
43 insecticide resistance among vector populations [2,3]. A limited number of active
44 ingredients satisfy the criteria to be effectively deployed on a large scale in endemic
45 areas [4]. The spread of insecticide resistance poses a major challenge not only to the
46 efficacy of IRS and LLINs, but also to their cost-effectiveness [5]. Control programs have
47 often attempted to mitigate the negative impacts of insecticide resistance by switching
48 between active ingredients [6]. For example, some endemic countries have progressively
49 adopted more expensive alternatives such as carbamates and organophosphates for IRS
50 to mitigate mosquito resistance to pyrethroids. This shift has been responsible for a
51 decline in IRS coverage from 5% in 2010 to 2% in 2018 [7]. Sequential deployment of
52 insecticides is also associated with the development of multiple forms of phenotypic,
53 genetic and behavioral resistance that quickly become established in wild mosquito
54 populations [1]. In order to reduce the likelihood of resistance development, the WHO's
55 global plan for insecticide resistance management in malaria vectors has recommended
56 using active ingredients in rotations, combinations, mosaics and mixtures [1,8]. However,
57 for this management strategy to be successful, it is imperative to reinforce rotation
58 programs with new chemicals which remain effective against mosquito populations that
59 are currently tolerant to insecticides previously used in LLINs and IRS [4]. In order to

60 identify these alternatives, a number of agrochemicals have been tested and a few
61 candidates have been selected for use in malaria vector control [9–13].

62 Clothianidin is manufactured into two new IRS formulations that have been evaluated
63 and prequalified by the World Health Organization (WHO) [4]. The two formulations are
64 SumiShield® 50WG (50% clothianidin) developed by Sumitomo Chemical and Fludora®
65 Fusion (Bayer Environmental Science) which is a mixture of 500 g/kg clothianidin and
66 62.5 g/kg deltamethrin [14,15]. There are currently 8 registered neonicotinoids, including
67 clothianidin, which have become the most widely used agricultural pesticides worldwide
68 [16,17]. Neonicotinoids target the nicotinic acetylcholine receptor (nAChR) in the insect
69 central nervous system and cause over-stimulation, which may result in paralysis and
70 death [17]. Although neonicotinoids are neurotoxic insecticides, their mode of action is
71 sufficiently distinct, thus limiting the risk of cross-resistance with other neurotoxic
72 chemicals such as pyrethroids.

73 Field trials of IRS formulations containing clothianidin have revealed long-lasting
74 insecticidal activity against different vector species on diverse surfaces [18–22]. Thus,
75 clothianidin is considered a reliable alternative to control malaria vectors in areas with
76 high pre-existing resistance to multiple insecticide classes. In preparation for rollout of
77 clothianidin formulations as part of national IRS rotation strategies, the U.S. President's
78 Malaria Initiative has conducted susceptibility testing in anopheline populations from 16
79 African countries [23]. This investigation suggested that most populations of the major
80 vectors of *Plasmodium* parasites across the continent were susceptible to filter papers

81 impregnated with SumiShield® 50WG at a diagnostic dose of 2% (w/v) clothianidin.
82 While the findings of this study are encouraging, targeted testing of populations from
83 agricultural regions where *Anopheles* mosquitoes are more likely to develop resistance
84 to neonicotinoids should be conducted alongside continental-scale surveys.

85 The role of agricultural pesticides spraying in the development of resistance to
86 insecticides used in malaria mosquito control has been widely documented [24–31]. It has
87 recently been demonstrated that *An. gambiae* larvae collected from farming areas
88 where formulations of acetamiprid and imidacloprid, two neonicotinoids, are intensively
89 used for crop protection can grow and emerge in water containing doses of
90 neonicotinoids that were lethal to susceptible strains [32]. This finding suggested that
91 larval exposure to pesticide residues is selecting for cross-resistance to several
92 neonicotinoids including clothianidin. Additionally, susceptibility tests conducted on
93 adults confirmed resistance to thiamethoxam, imidacloprid and acetamiprid in some
94 rural populations of *An. gambiae* [33,34]. Neonicotinoids are highly soluble in water and
95 can persist for months in aerobic soils, therefore making contamination of standing
96 waters, which serve as mosquito breeding sites in farming areas very likely [35–37].
97 Published information on the use of neonicotinoids across Sub-Saharan Africa is lacking,
98 but preliminary reports from Cameroon, Tanzania and Ivory Coast suggested that
99 hundreds of commercial formulations of neonicotinoids have been registered for use in
100 crop protection [38–41].

101 Agriculture is one of the main activities in sub-Saharan Africa. The widespread
102 application of neonicotinoids in crop protection could become a major challenge to the
103 efficacy of repurposed chemicals such as clothianidin. Prior to rollout of formulations
104 containing clothianidin, it is thus crucial to specifically test their efficacy against
105 *Anopheles* populations that are showing signs of resistance to the active ingredient.
106 Here we conducted intensive testing of *Anopheles* and *Culex* adult mosquito populations
107 from five locations centered on one of the largest urban farms in Cameroon, using
108 standard bioassays. Our aim was to determine if neonicotinoid tolerance observed in
109 *An. gambiae* larvae and adults could affect the efficacy of SumiShield® 50WG. We found
110 that as previously demonstrated by continent-wide surveys [23], SumiShield® 50WG is
111 very effective against clothianidin-susceptible populations of *An. coluzzii* and *An.*
112 *gambiae*. However, its efficacy is significantly reduced in *An. gambiae* adults displaying
113 low mortality to clothianidin (150 µg/ml). These findings suggest that the effectiveness
114 of clothianidin and its formulations should be monitored in depth in agricultural
115 settings, and that alternative chemicals should be considered in areas where resistance
116 to the active ingredient is emerging.

117 **Results**

118 **1. Evidence of clothianidin resistance in *Anopheles gambiae***

119 We used Centers for Disease Control (CDC) bottle bioassays to test a discriminating
120 dose of clothianidin against a total of 1665 wild mosquitoes belonging to three species:
121 *An. gambiae* (n = 912), *An. coluzzii* (n = 673) and *Culex* sp (n = 132) collected from 6

122 sampling sites. To validate our bioassay protocol, we first analyzed susceptibility in 554
123 individuals of two laboratory colonies: *An. gambiae* Kisumu (n = 326) and *An. coluzzii*
124 Ngousso (n = 228). Results showed that the two laboratory strains were fully susceptible
125 to clothianidin, reaching 100% mortality within 1-3 days of exposure (Fig. 2A). Among
126 field mosquitoes, *Culex* sp. populations were the most susceptible to clothianidin,
127 achieving 100% mortality approximately 24 h post exposure (Fig. 2A). A diagnostic PCR
128 confirmed that specimens collected from the two urbanized sites were 100% *An.*
129 *coluzzii*. These *An. coluzzii* populations were also susceptible to clothianidin, but 100%
130 mortality was reached between the second and the fourth day. In *An. gambiae* by
131 contrast, the overall mortality was only 58.2 ± 5.2 % in all 817 individuals tested from 4
132 locations, suggesting that some populations of this species have developed resistance to
133 clothianidin. Knockdown at 60 min was generally low, except in field populations of *An.*
134 *coluzzii* (81.8 ± 2.9%). Knockdown within 60 min of exposure ranged from 42 ± 11.0 % to
135 53.9 ± 9.9 % between lab strains and from 32.1 ± 5.0 % to 36.5 ± 12.2 % among field
136 populations (Fig. 3A). Consistent with their reduced susceptibility to clothianidin, wild
137 populations of *An. gambiae* displayed the lowest knockdown (32.1 ± 5.0 %).

138 2. **Resistance is stronger in populations from agricultural areas**

139 To examine the link between larval exposure to agricultural neonicotinoids and the
140 development of clothianidin resistance in *An. gambiae,* we compared the susceptibility
141 profile of adult populations from different collection sites. Based on the diagnostic PCR,
142 larvae collected from the farm or from one of the suburban sites (Zamengoué) were

143 100% *An. gambiae*. Samples from the two semi-rural sites, Soa and Nkolnkoumou, were
144 ~80% *An. gambiae*/20% *An. coluzzii*. All the four sites were treated as *An. gambiae*
145 habitats. Mortality rates varied along a geographic gradient, ranging from susceptibility
146 in Soa and Nkolnkoumou to resistance in samples originating from the agricultural site,
147 Nkolondom. Only 46.5 ± 5.7 % of tested individuals from the agricultural site died
148 between the first and the seventh day post-exposure (Fig. 2B). Susceptibility of adults
149 from Nkolondom was significantly low compared with other *An. gambiae* sites
150 (Wilcoxon Rank Sum test, $p < 0.05$). Knockdown at 60 min was also significantly lower
151 (20.1 ± 4.1 %) in samples from the agricultural area (Wilcoxon Rank Sum test, $p < 0.05$)
152 (Fig. 3B).

153 **3. PBO is a synergist of clothianidin**

154 Mortality observed in clothianidin-resistant populations of *An. gambiae* from the
155 agricultural site increased from 46.5 ± 5.7 % without PBO to 92.7 ± 3.7 % when adult
156 mosquitoes were pre-exposed to the synergist (Wilcoxon rank sum test, $p=0.015$) (Fig.
157 3C). This result suggested that metabolic detoxification involving cytochrome P450
158 enzymes contributes to the development of resistance to clothianidin in *An. gambiae*.

159 **4. Resistance to clothianidin reduces the efficacy of SumiShield® 50WG**

160 To determine to what extent clothianidin resistance could impact the efficacy of
161 manufactured formulations, we used WHO tube tests to estimate the susceptibility of
162 young female adult mosquitoes to SumiShield® 50WG. This formulation corresponds to
163 ~25-fold the discriminating dose of clothianidin used in CDC bottle assays. We tested

164 one *An. coluzzii* population from urban areas (Combattant), one susceptible *An.*
165 *gambiae* population (Nkolnkoumou) and resistant mosquitoes from Nkolondom. We
166 used the laboratory strain *An. coluzzii* Ngousso as negative control. As expected, the lab
167 strain was susceptible to SumiShield® 50WG, reaching 100% within 48 h of exposure to
168 the formulation (Fig. 4). Among the field populations, susceptibility to clothianidin as
169 revealed by bottle bioassays was a good predictor of the efficacy of SumiShield® 50WG.
170 Both *An. coluzzii* and *An. gambiae* populations that were fully susceptible to the active
171 ingredient were also susceptible to the IRS formulation. By contrast, *An. gambiae*
172 populations from Nkolondom that were resistant to 150 µg/ml clothianidin in bottle
173 bioassays were less susceptible to SumiShield® 50WG as well. Mortality rates against
174 filter papers impregnated with SumiShield® 50WG at a diagnostic dose of 2% (w/v)
175 clothianidin leveled off at 75.4 ± 3.5 % after 7 days of holding period. This finding
176 suggests that *Anopheles* populations with low mortality to 150 µg/ml clothianidin could
177 be challenging to control with current neonicotinoid formulations.

178 **Discussion**

179 Widespread pyrethroid resistance has been associated with a decline in efficacy of LLINs
180 and IRS in several countries [57–59]. Here we have provided evidence that the efficacy of
181 some insecticides may have started to decline in some vector populations. The efficacy
182 of SumiShield® 50WG, a formulation of clothianidin prequalified for IRS is reduced in *An.*
183 *gambiae* mosquitoes that have evolved resistance to the active ingredient.

184 We first evaluated the susceptibility of *Anopheles* and *Culex* mosquitoes to clothianidin
185 using CDC bottle bioassays. We used a protocol that differed slightly from the WHO
186 standard operating procedure for testing the susceptibility of adult mosquitoes to
187 clothianidin [48,60]. We made the choice not to use a vegetable oil surfactant as suggested
188 by the standard operating procedure because it has been demonstrated that these
189 adjuvants are synergist of neonicotinoids [34]. Additionally, at 150 µg/ml, the
190 discriminating dose used, clothianidin was soluble in ethanol when the solution was
191 allowed to rest for at least 24 h before use, and there was therefore no need to add a
192 surfactant.

193 Using a discriminating dose of 150 µg/ml in CDC bottle bioassays, we successfully
194 detected resistance to clothianidin in wild *Anopheles* mosquitoes. Knockdowns after 1 h
195 exposure to the insecticide were low and had little discriminative power. Other studies
196 assessing the susceptibility of adults *Anopheles* mosquitoes to clothianidin have
197 reported low knockdowns, which could be due to the fact that clothianidin act slowly
198 compared with other neurotoxic insecticides [17,23,50,53]. Contrary to knockdowns,
199 monitoring mortality rates for seven days provided a reliable assessment of
200 susceptibility to clothianidin in adult mosquitoes. We first observed that susceptibility
201 vary between species. *An. coluzzii* adults collected from urban areas of Yaoundé were
202 susceptible to clothianidin. As crop cultivation associated with neonicotinoid spraying is
203 less frequent in urban areas, *An. coluzzii* larvae from urbanized settings in Yaoundé are
204 presumably less exposed to neonicotinoids residues and are therefore less likely to
205 develop resistance to neonicotinoids. In *An. gambiae* however, the situation was more

206 complex, with a gradient of susceptibility to clothianidin established among suburban
207 and rural populations. Populations from a farm where neonicotinoids are used weekly
208 for crop protection were the most resistant to clothianidin (Fig. 1C). Indeed, during our
209 field survey in the Nkolondom farm, we collected empty containers of imidacloprid and
210 acetamiprid confirming their use (Fig. 1C). Dozens of formulations of the two
211 insecticides are freely sold in local stores in Yaoundé and are highly valued by farmers
212 [38,41]. The results presented in the current study are based on field surveys that were
213 conducted between 2019 and 2020. The findings have been supported by monitoring
214 that continues from 2020 to 2022 and confirmed patterns of susceptibility to
215 clothianidin observed in precedent years in *Anopheles* mosquitoes from Yaoundé and its
216 neighboring rural areas [33]. These surveys have combined larval tests and adult bioassays
217 to reveal that neonicotinoid resistance is emerging in *An. gambiae* populations from the
218 equatorial forest region of Cameroon, especially in areas where larvae are chronically
219 exposed to pesticide residues [32–34]. There is ample evidence that *An. gambiae* larvae and
220 adults from several villages around the city of Yaoundé are currently resistant to
221 imidacloprid, acetamiprid and thiamethoxam, three neonicotinoids that are among the
222 most widely used crop protection chemicals in Cameroon [32,33,38,41]. A study conducted in
223 Ivory Coast has also observed resistance to imidacloprid and acetamiprid in *An. coluzzii*
224 correlated with agricultural activities [61].

225 Intriguingly, adults of *Culex sp* whose larvae were collected from the same breeding
226 sites as *An. gambiae* in Nkolondom were fully susceptible to clothianidin. However, it is
227 well known aquatic invertebrates have variable responses and threshold of

228 susceptibility to the lethal and sublethal effects caused by neonicotinoid contaminants
229 [62]. Although *Culex* sp populations were more directly impacted by the lethal toxicity of
230 clothianidin, they likely have developed other physiological and/or behavioral
231 adjustments enabling them to adapt to neonicotinoid residues in farms [63,64].

232 Bioassay tests using CDC bottles coated with the synergist PBO prior to exposure to
233 clothianidin showed a drastic increase in mortality in resistant populations. This
234 suggested that Cytochrome P450 enzymes (CYPs) play a primarily role in neonicotinoid
235 resistance in *An. gambiae*. Acetamiprid resistance in *An. gambiae* is also highly
236 dependent on metabolic detoxification mediated by CYPs [33]. More generally,
237 neonicotinoid resistance in wild populations of many crop pests, primarily those of the
238 order *Hemiptera* (aphids, whiteflies, and planthoppers), is associated with
239 overexpression of one or several CYP enzymes [65–68].

240 The spread of pyrethroid resistance has caused a decline in the effectiveness of LIINs
241 and IRS [57,58]. There is a risk that resistance will also undermine the efficacy of new
242 products such as SumiShield®® 50WG and Fludora® Fusion. Precisely, larval bioassays
243 showed that the intensity of resistance to clothianidin in *An. gambiae* collected from
244 Nkolondom is currently similar to that of deltamethrin [32]. Therefore, even without any
245 large-scale deployment of clothianidin in vector control, its efficacy may already be as
246 reduced as that of pyrethroids in some populations [69]. In our study, we have revealed
247 that the efficacy of SumiShield®® 50WG is declining in clothianidin-resistant populations.
248 We did not test Fludora® Fusion, and it remains to be determined if the dual action of

249 clothianidin and deltamethrin will result in higher efficacy against resistant populations.
250 However, a recent experiment has demonstrated that exposure of *An. gambiae* larvae
251 to sublethal doses of a mixture of different types of agrochemicals increased the
252 tolerance of adults to both clothianidin and Fludora® Fusion [70]. This suggested that any
253 formulation is at risk of losing its efficacy if clothianidin resistance spreads among
254 anopheline populations.

255 **Conclusions**

256 The current study shows that the new insecticide clothianidin may have reduced
257 efficacy in some agricultural regions of Sub-Saharan Africa due to pre-existing levels of
258 resistance among mosquito populations. These findings suggest that prior to inclusion of
259 agrochemicals in resistance management programs, their efficacy should be particularly
260 scrutinized in areas where pesticides of the same class are being used for agricultural
261 pest management.

262 **Methods**

263 **Study sites**

264 We collected mosquitoes from four suburban sites in the outskirts of Yaoundé, the
265 capital of Cameroon (Figure 1A). We also tested samples collected from two densely
266 urbanized areas of the city. Approval to conduct a study in the Center region (N°: 1-
267 140/L/MINSANTE/SG/RDPH-Ce), ethical clearance (N°: 1-141/CRERSH/2020) and
268 research permit (N°: 000133/MINRESI/B00/C00/C10/C13) were granted by the ministry
269 of public health and the ministry of scientific research and innovation of Cameroon. The

270 suburban sites included Nkolondom (3°56'43" N, 11°31'01" E), situated approximately 7
271 km west of Yaoundé. Since 1985, a swampy area in Nkolondom is exploited for intensive
272 crop cultivation [42]. In 2020, the farm which attracted at least 100 workers was
273 subdivided into 100-200 mosaics of ~20m^2 adjacent plots dedicated to the cultivation of
274 aromatic herbs, amaranth and lettuce (Fig. 1B). Standing water between ridges and
275 furrows provide mosquito breeding sites, which maintain large larval populations of *An.*
276 *gambiae* and *Culex sp* throughout the year [32–34].

277 **Mosquito populations**

278 We sampled and cumulatively tested mosquitoes from the six above-mentioned sites
279 between 2019 and 2020. *An. gambiae sensu lato* larvae were collected from standing
280 water using dippers and transported in plastic containers to the insectary where they
281 were reared to adults under standard laboratory conditions of 25–27°C, 70–90% relative
282 humidity and a 12:12 h light/dark photoperiod [43]. The two sibling species *An. gambiae*
283 *sensu stricto* (hereafter *An. gambiae*) and *An. coluzzii* are the dominant malaria vectors
284 in Yaoundé [44,45]. *An. coluzzii* is found exclusively in the most urbanized neighborhoods
285 while *An. gambiae* is the only species present in neighboring rural areas. To determine
286 which species between *An. gambiae* and *An. coluzzii* was collected from each site, we
287 genotyped 50 mosquito samples using a diagnostic PCR [46]. *Culex* sp larvae that occur in
288 the same breeding sites as *An. gambiae* in the Nkolondom farm were also sampled,
289 reared to adults and tested.

290 **Clothianidin susceptibility testing**

291 The susceptibility of adult mosquitoes that emerged from field-collected larvae was
292 tested against clothianidin using CDC bottle assays [47]. The bioassay procedure followed
293 a modified version of the WHO standard operating procedure for testing the
294 susceptibility of adult mosquitoes to clothianidin [48,49]. Precisely, we did not used the
295 vegetable oil surfactant, Mero® (1% v/v), as adjuvant. A recent study showed that some
296 vegetable oil surfactants are synergists of neonicotinoids, which when used in
297 susceptibility testing lead to an overestimation of the insecticidal activity of the active
298 ingredient [34]. Instead, mortality was evaluated against the active ingredient alone
299 dissolved in ethanol using a discriminating dose (i.e., the lowest dose at which 100% of
300 adults from a susceptible population die) of 150 µg/ml as determined by a previous
301 study [50]. We prepared stock solutions using a technical-grade formulation of clothianidin
302 (PESTANAL®, analytical standard, Sigma-Aldrich, Dorset, United Kingdom) and absolute
303 ethanol as solvent. The solutions were conserved at 4°C in the dark for at least 24 h
304 before use to maximize solubility of clothianidin. Each Wheaton 250-ml bottle and its
305 cap were coated with 1 ml of a solution containing 150 µg/ml clothianidin in ethanol
306 following the CDC guidelines [47]. For each bioassay, we used four test bottles coated with
307 clothianidin and two control bottles coated with 1 ml of absolute ethanol. All bottles
308 were wrapped in aluminum foil and allowed to dry for 24 h for complete evaporation of
309 solvent before use. Coated bottles were not reused and were washed three times in
310 warm soapy water and allowed to dry for 24 h between experiments. 20 to 25 3-5-day-
311 old females were aspired from mosquito cages and released into one of the test bottles
312 where they were exposed to the active ingredient or into control bottles containing

313 ethanol for 1 h. After the exposure period, mosquitoes were transferred into a paper
314 cup and provided with 10% sugar solution. Knockdowns were scored immediately and
315 mortality was monitored every 24 h for seven consecutive days. We used adults from
316 two susceptible strains as controls: *An. gambiae* Kisumu and *An. coluzzii* Ngousso. Both
317 strains are known to be susceptible to pyrethroid, carbamate, organochlorine and
318 organophosphate insecticides.

319 **Synergist bioassay**

320 We used CDC bottle assays to test if the synergist piperonyl butoxide (PBO) could
321 enhance the potency of clothianidin. PBO is an inhibitor of oxidases and non-specific
322 esterases [51]. A solution of 400 µg/ml PBO was prepared by diluting PBO with absolute
323 ethanol. The solution was mixed and stored in the dark at 4°C before use. Wheaton
324 bottles and their cap were coated with 1 ml of the PBO solution, wrapped in aluminum
325 foil and allowed to dry for 24 h before the test. 100 to 150 female mosquitoes aged
326 between 3 and 5 days were pre-exposed for 1 h to PBO-coated bottles or to control
327 bottles coated with ethanol. Mosquitoes were then removed from the bottles and
328 batches of 20 to 25 individuals were introduced into new bottles coated with 150 µg/ml
329 clothianidin or with ethanol. After 1 h, mosquitoes were transferred into a paper cup
330 and provided with 10% sugar solution. Knockdowns were scored and mortality was
331 monitored every 24 h for seven consecutive days in standard laboratory conditions. We
332 compared mortality rates with or without the synergist to determine if oxydase
333 inhibition by PBO affected the level of susceptibility.

334 **Efficacy of SumiShield® 50WG**

335 We used WHO tube tests to assess the efficacy of SumiShield® 50WG against adult
336 mosquitoes displaying a gradient of tolerance to clothianidin [52]. We used a
337 discriminating dose of 2% w/v clothianidin (13.2 mg active ingredient per paper)
338 following the manufacturer's recommendation [23,53]. We prepared a stock solution by
339 diluting 264 mg SumiShield® 50WG in 20 ml distilled water. We impregnated Whatman
340 filter papers (12 x 15 cm) containing 13.2 mg clothianidin each using 2 ml of the
341 insecticide solution as described in [54]. Control filter papers were impregnated using 2 ml
342 of distilled water. Treated filter papers were allowed to dry overnight and were kept in
343 foil at 4°C until use. To carry out bioassay tests, we aspired 20 to 25 3-5-day-old female
344 mosquitoes from mosquito cages that we introduced into each of the test tubes
345 containing clothianidin-impregnated papers. We concomitantly released 20-25
346 mosquitoes into each control tube. After 1 h, mosquitoes were transferred into holding
347 tubes and provided with 10% sugar solution, and knockdown time was recorded. As
348 with CDC bottle tests mortality was scored every day until day 7.

349 **Data analysis**

350 We used mortality rates to evaluate the efficacy of clothianidin against laboratory and
351 field mosquitoes. All tests with mortality > 20% at day 7 in controls were discarded. We
352 used Abbott's formula to correct mortality rates of tests when 5–20% individuals died
353 between day 1 and day 7 in the corresponding controls [55]. Following the WHO guidelines
354 on insecticide susceptibility, mosquito populations were considered susceptible if

355 mortality at day 7 was ≥ 98% and resistant if mortality was less than 90%. Mortality
356 rates between 90% and 97% indicate that the presence of resistant genes in the vector
357 population must be confirmed by additional tests [52]. We used a Wilcoxon rank sum test
358 to determine if mean mortality rates were significantly different among tested
359 populations. We performed all analyses using the R software V 4.2.2 [56].

360 **Author Contributions**

361 CF: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original
362 draft; FA: Investigation, Methodology; MA: Investigation, Methodology; WT:
363 Investigation, Methodology; CW: Resources, Supervision. CK: Conceptualization, Formal
364 analysis, Funding acquisition, Investigation, Project administration, Writing – review &
365 editing.

366 **Funding**

367 This study was supported by a National Institutes of Health grant (R01AI150529) to CK.
368 The funders had no role in study design, data collection and analysis, decision to publish,
369 or preparation of the manuscript.

370 **Data Availability Statement**

371 The data for this study have been presented within this article.

372 **Conflicts of Interest**

373 The authors declare no competing interests.

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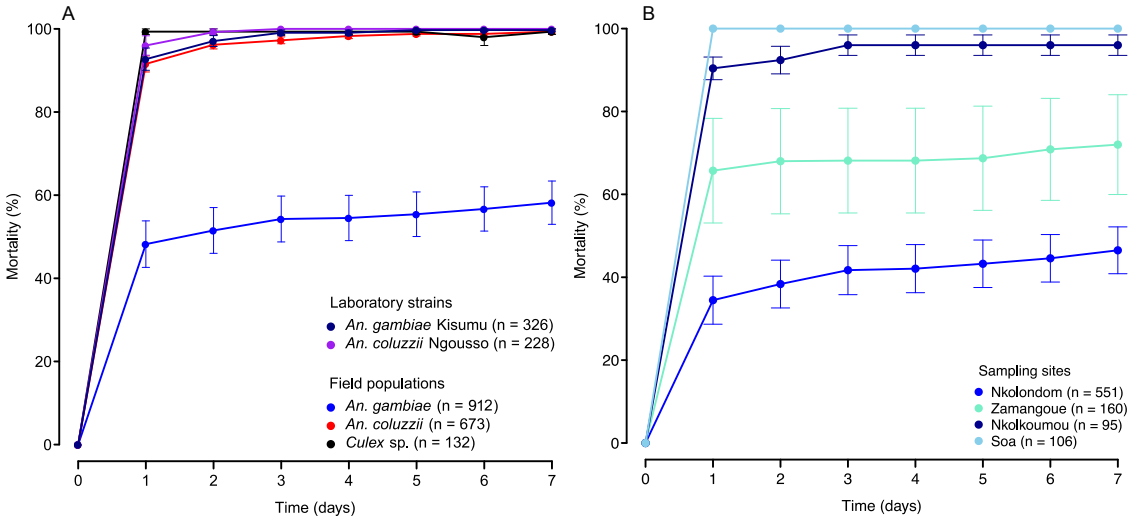
Figure legends

Figure 1: Description of the study site. (A) Map of the sampling locations where *Anopheles* and *Culex* mosquitoes were collected to evaluate susceptibility to clothianidin. (B) Picture of a larval breeding site in Nkolondom. (C) Examples of empty containers found in farms indicating the use of imidacloprid and acetamiprid formulations.



588

589 **Figure 2**: Baseline susceptibility of laboratory strains and field mosquito populations to
590 clothianidin monitored for 7 days using CDC bottle bioassays. (A) Mortality values of
591 *Anopheles* and *Culex* female adults exposed to 150 µg/ml clothianidin. (B) Gradient of
592 susceptibility revealed in wild populations of *An. gambiae*. Error bars represent the
593 standard error of the mean and (n) the number of individuals tested.

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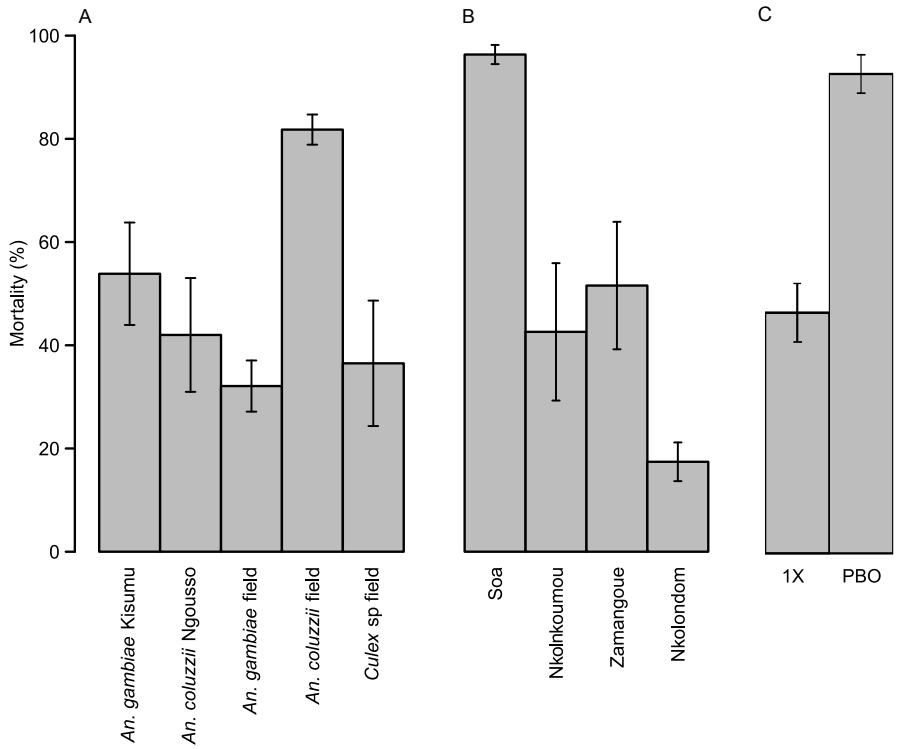

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599 **Figure 3**: Knockdowns values and synergistic effect of PBO. Knockdowns after 1 h
600 exposure to 150 µg/ml clothianidin were compared between *Anopheles* and *Culex*
601 species (A) and between field populations of *An. gambiae* (B). Standard test (1X) and
602 synergist bioassay (PBO) showing increase in mortality due to PBO (C). Standard errors
603 of the mean are shown as vertical bars.

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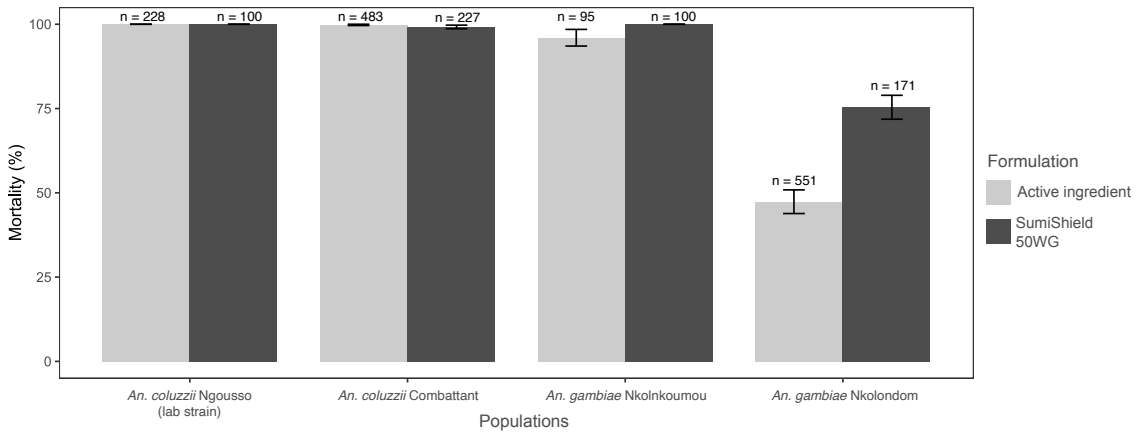

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606 **Figure 4**: Relationship between susceptibility to clothianidin as revealed by CDC bottle

607 bioassays and efficacy of SumiShield® 50WG evaluated with WHO tube tests.

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