Resistance of *Anopheles gambiae* to the new insecticide clothianidin associated with unrestricted use of agricultural neonicotinoids in Yaoundé, Cameroon

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

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

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

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

[4]LSTM Research Unit at CRID, P.O. Box 13591, Yaoundé, Cameroon

*Correspondence: colincek@crid-cam.net, kamdem_d@yahoo.com

Abstract

Background: New active ingredients, mostly repurposed from the agricultural sector, are gradually being introduced into malaria vector control programs. Prior to scaling up novel insecticides, it is vital to assess if their use in agriculture has already selected mosquito populations with reduced susceptibility in some areas. The aim of the present study was to determine if the use of neonicotinoids in agricultural pest management could drive resistance to clothianidin—a neonicotinoid recently prequalified by WHO for indoor residual spraying.

Methods: We carried out intensive sampling of *Anopheles gambiae*, *An. coluzzii* and *Culex* sp. larvae from a large suburban farm and from 4 surrounding locations belonging to the rural and urban ecosystems of Yaoundé, Cameroon. Using WHO tube tests and CDC bottle bioassays, we tested the recommended diagnostic doses of clothianidin against field-collected populations using two laboratory strains as susceptible controls. We exposed adult mosquitoes to the insecticide for 1 h and monitored mortality rates for seven consecutive days.

Results: *An. coluzzii* and *Culex* sp. mosquitoes were fully susceptible to clothianidin reaching 100% mortality by day 3. By contrast, *An. gambiae* populations revealed heterogeneous profiles ranging from susceptibility to strong resistance. Populations from the suburban farm, where neonicotinoids are routinely used, displayed the lowest mortality rate (46.5 % ± 5.65) and were capable of surviving 5X and 10X the diagnostic dose of clothianidin. When clothianidin was tested in combination with the synergist piperonyl butoxide, mortality of resistant populations increased from 46.5 % to 92.7 % implying that Cytochrome P450 enzymes play an integral role in the genetic mechanism underlying clothianidin resistance in *An. gambiae*.

Conclusions: These findings show that small clusters of resistance to clothianidin may exist around farmlands where agricultural neonicotinoids are being used. Patches of intensive agriculture should be particularly monitored in indoor residual spraying target areas.

Key words: *Anopheles gambiae*, clothianidin, resistance, cytochrome P450s, indoor residual spraying, neonicotinoids.

Introduction

Research is ongoing to diversify malaria control techniques by developing new tools that could reduce the reliance of intervention measures on chemical insecticides [1,2]. However, insecticidal interventions remain the mainstay of current prevention and control strategies. Notably, the scale-up of Indoor Residual Spraying (IRS) and Long-Lasting Insecticidal Nets (LLINs) have dramatically reduced malaria incidence and mortality in Sub-Saharan Africa over the last two decades [3]. Unfortunately, this progress might become difficult to preserve in the future if the rapid spread of insecticide resistance that has been observed among malaria vectors is not effectively managed [4,5]. For this reason, WHO continues to underscore the critical need for all countries with ongoing malaria transmission to develop and apply effective insecticide resistance management strategies [6,7].

Only a handful of insecticides are prequalified by WHO for core interventions for malaria vector control [8]. In the face of widespread insecticide resistance, control programs have had little choice but to switch between active ingredients in order to preserve the efficacy of intervention measures. This sequential deployment of insecticides is often associated with substantial increases in intervention costs as well as the development of multiple forms of resistance that are more difficult to manage [9]. For example, countries have progressively adopted more expensive alternatives such as carbamates and organophosphates for IRS to mitigate mosquito resistance to pyrethroids. This switch has been correlated with a decline in IRS coverage from a peak of 5% in 2010 to 2% in 2018 [10]. To address this issue, the WHO's global plan for insecticide resistance management in malaria vectors has recommended the deployment of active ingredients in rotations, combinations, mosaics and mixtures [6,9]. To enhance the potential effectiveness of this management strategy, a short list of new active ingredients with distinct modes of

action has been evaluated and tested for the control of mosquito vector populations that are already resistant to chemicals routinely used for IRS and LLLINs [8].

Clothianidin is a neonicotinoid manufactured into two new IRS formulations that have been evaluated and prequalified by the WHO [8]. Those are SumiShield 50WG® (50% clothianidin) developed by Sumitomo Chemical and Fludora® Fusion (Bayer Environmental Science) which is a mixture of 500 g/kg clothianidin and 62.5 g/kg deltamethrin [11,12]. Neonicotinoid pesticides were discovered in the late 1980s and have since become the most widely used class of insecticides worldwide, with large-scale applications ranging from plant protection, veterinary application, and biocides to invertebrate pest control in fish farming [13]. Currently, 8 registered neonicotinoid insecticides are available commercially worldwide [13]. These insecticides target the nicotinic acetylcholine receptor (nAChR) in the insect central nervous system and cause over-stimulation, which may result in paralysis and death. This mode of action is distinct from that of the other neurotoxic insecticides such as pyrethroids routinely used for IRS and LLLINs over the last decades. For this reason, clothianidin formulations are potentially suitable for inclusion in malaria vector control, particularly in areas with high pre-existing resistance to multiple insecticide classes. Importantly, field evaluations of SumiShield 50WG and Fludora Fusion have reported promising results, both formulations showing effective insecticidal action against different vector species as well as durable residual activity on diverse surfaces [14–17].

In preparation for rollout of clothianidin formulations as part of national IRS rotation strategies, the U.S. President's Malaria Initiative has conducted large-scale susceptibility testing of wild anopheline populations from 16 African countries [18]. This investigation revealed that populations of the most important vectors of *Plasmodium* parasites across the continent—*An. gambiae*, *An. coluzzii, An. arabiensis* and *An. funestus*—were susceptible to filter papers impregnated with SumiShield 50WG at a diagnostic dose of 2% w/v clothianidin. While the findings of this study are encouraging, continental-scale surveys should be complemented with targeted

testing of populations from agricultural regions where *Anopheles* mosquitoes are more likely to develop resistance to neonicotinoids. The association between agricultural usage of pesticides and development of insecticide resistance in *Anopheles* mosquitoes is widely documented (e.g., [19–22] reviewed in [23]). It has been hypothesized that pesticide residues contaminate aquatic habitats and contribute to the selection of resistance genes among non-target insect populations including mosquito larvae. Neonicotinoids are highly soluble in water and can persist for months in aerobic soils, therefore making contamination of mosquito breeding sites following agricultural use highly likely [24]. Published information on the usage of neonicotinoids across Sub-Saharan Africa is lacking, but preliminary reports from Cameroon, Tanzania and Ivory Coast suggest that hundreds of commercial formulations of neonicotinoids have been registered for use in crop protection [25–27]. Consequently, prior to field use of clothianidin, it is imperative to evaluate if the widespread application of neonicotinoids in agriculture is contributing to the emergence of resistant vector populations in some areas. Here we aimed to address the likelihood of clothianidin resistance driven by agricultural neonicotinoids in *Anopheles* and *Culex* mosquito populations. To do so, we assessed susceptibility in wild populations from five locations centered on one of the largest urban farms in Cameroon's political capital, Yaoundé, using laboratory bioassays. Our results reveal strong clothianidin resistance in *An. gambiae* populations that thrive in standing water within farm plots where several neonicotinoid insecticides are routinely used. This finding suggests that mosquito susceptibility and neonicotinoid usage should be particularly monitored around agricultural patches in IRS target areas.

Methods

Study sites

To investigate the effect of agricultural neonicotinoids on clothianidin susceptibility in mosquitoes, we tested populations from a large farm located in Nkolondom (3°56'43" N, 11°31'01" E), a suburban neighborhood of Yaoundé, the capital city of

144 Cameroon (Figure 1A). For comparison, we also tested mosquito populations from
145 two neighborhoods situated at the center of Yaoundé and from two rural sites at the
146 outskirts of the city [28] (Fig. 1A). Nkolondom is located in the northern part of
147 Yaoundé—approximately 7 km from the center of the city. Since 1985, artisanal
148 agriculture has gained considerable momentum in a swampy area of this locality,
149 which currently attracts at least one hundred farmers [29]. At present, this
150 suburban farm encompasses 100-200 mosaics of ~ 20m^2 adjacent plots and has
151 become the main production site of celery, amaranth, lettuce and other aromatic
152 herbs in Yaoundé (Fig. 1B). Farming activities are conducted year-round, and crops
153 are intensively watered either manually or using artisanal irrigation schemes.
154 Consequently, standing water accumulates in the fields where very high densities of
155 *An. gambiae* and *Culex sp* larvae can be collected throughout the year.
156
157 **Mosquito collection and rearing**
158 Mosquito collections took place during the long rainy season and part of the long
159 dry season: from July 2019 to January 2020 and from May 2020 to July 2020. We
160 sampled mosquito larvae across the study sites using the standard dipping method
161 [30]. Larvae were collected from standing water within the farms in Nkolondom,
162 from relatively shallow ruts in rural areas and from polluted breeding sites at the
163 center of Yaoundé city. Larvae were transported in closed containers to the CRID's
164 insectary and reared to adults under standard laboratory conditions. *An. gambiae*
165 and *An. coluzzii* are the dominant malaria vector species in Yaoundé where their
166 respective habitats have been well characterized in past surveys [28,31]. *An. coluzzii*
167 is found exclusively in the most urbanized neighborhoods while *An. gambiae* is the
168 only species present in the rural areas. Sampling was conducted to collect and test
169 one species per location. We collected and tested *Culex* populations that occur in the
170 same breeding sites as *An. gambiae* in Nkolondom to compare their susceptibility
171 profiles. For bioassay tests, we used two laboratory strains as susceptible controls:
172 *An. gambiae* Kisumu and *An. coluzzii* Ngousso. Both strains are known to be
173 susceptible to pyrethroid, carbamate, organochlorine and organophosphate
174 insecticides. We maintained all life-cycle stages of laboratory strains and wild

175 mosquito populations under standard insectary conditions (25 ± 2 °C, 80% relative
176 humidity, light:dark cycles of 12:00 h each) and we provided adults with 10% sugar
177 solution.
178
179 **CDC bottle tests**
180 We tested the susceptibility of adult mosquitoes against a diagnostic dose of
181 clothianidin using CDC bottle assays according to published guidelines [34]. A recent
182 study showed that 150μg/ml clothianidin was a suitable diagnostic dose—the
183 lowest concentration of active ingredient that kills 100% of individuals of a
184 susceptible laboratory strain within 24 h of insecticide exposure [35]. To assess the
185 intensity of resistance, we also determined the mortality rates of wild mosquitoes
186 exposed to 5X and 10X the diagnostic dose. We prepared stock solutions of
187 clothianidin at 1X, 5X, and 10X the diagnostic dose using technical grade
188 clothianidin (PESTANAL®, analytical standard, Sigma-Aldrich, Dorset, United
189 Kingdom) and absolute ethanol as solvent. The solutions were conserved at 4°C in
190 the dark for 24 h before use to maximize the solubility of clothianidin. Each
191 Wheaton 250-ml bottle and its cap were coated with 1 ml of clothianidin solution
192 following the CDC protocol. For each bioassay, we used a maximum of four test
193 bottles coated with clothianidin and two control bottles coated with 1 ml of absolute
194 ethanol. Occasionally, depending on the availability of mosquitoes, we used less than
195 four test bottles and only one control. All bottles were wrapped in aluminum foil
196 and allowed to dry for 24h for complete evaporation of solvent before use. Bottles
197 were not reused but were washed three times in warm soapy water and allowed to
198 dry for 24 h before being coated.
199 We carried out susceptibility tests as follows: 20 to 25 3-5-day-old females were
200 aspired from mosquito cages and gently released into one of the test bottles where
201 they were exposed to the active ingredient or into control bottles containing ethanol
202 for 60 minutes. Following exposure, mosquitoes were transferred into a paper cup
203 and provided with 10% sugar solution. Knockdowns were scored and mortality was
204 monitored every day for seven consecutive days under standard laboratory

205 conditions. When a population displayed signals consistent with resistance, we
206 carried out repeat tests for confirmation.
207
208 **WHO tube tests**
209 We used WHO tube tests to assess the susceptibility of populations showing the
210 lowest mortality rate in CDC bottle bioassays to SumiShield 50WG, which contains
211 50% (w/w) clothianidin [36]. We carried out repeat tests of these resistant
212 populations using a susceptible field population and a susceptible lab strain as
213 negative controls. We used a diagnostic dose of 2% w/v clothianidin (13.2 mg active
214 ingredient per paper) based on guidance from Sumitomo Corporation and the US
215 President's Malaria Initiative. This dose has been successfully used to test the
216 toxicity of clothianidin towards wild anopheline populations from Sub-Saharan
217 Africa [18,37]. We prepared a stock solution by diluting 264 mg SumiShield 50WG in
218 20 ml distilled water. We impregnated Whatman filter papers (12x15cm) containing
219 13.2 mg clothianidin each using 2ml of the insecticide solution as described in [38].
220 We prepared control filter papers using 2ml of distilled water. Treated filter papers
221 were allowed to dry overnight and were kept in foil at 4°C until use. To carry out
222 bioassay tests, we aspired 20 to 25 3-5-day-old female mosquitoes from mosquito
223 cages that we introduced into each of the test tubes containing clothianidin-
224 impregnated papers. We concomitantly released 20-25 mosquitoes into each
225 control tube containing a water-impregnated paper. After 60 minutes of exposure,
226 mosquitoes were transferred to holding tubes and provided with 10% sugar
227 solution, and knockdown time was recorded. As with CDC bottle tests mortality was
228 scored every day until day 7.
229
230 **Synergist bioassays**
231 To investigate the potential role of oxidases in the genetic mechanisms underlying
232 the reduced susceptibility to clothianidin, we used CDC bottle assays to determine
233 the efficacy of the diagnostic dose in combination with the synergist PBO (4%). PBO
234 is an inhibitor of oxidases and non-specific esterases and enhances cuticular
235 penetration of the insecticide into the insect [39]. A solution of 400 µg/ml PBO was

236 prepared by diluting PBO with absolute ethanol. The solution was mixed and stored
237 in the dark at 4°C before use. Wheatman bottles and their cap were coated with 1ml
238 of the PBO solution, wrapped in aluminum foil and allowed to dry for 24h before the
239 test. 100 to 150 female mosquitoes aged between 3 and 5 days were pre-exposed
240 for 60 min to PBO-coated bottles or to control bottles coated with ethanol.
241 Mosquitoes were then gently removed from the bottles and introduced in batches of
242 20 to 25 mosquitoes into new bottles coated with 1X clothianidin solution for the
243 tests or with ethanol for the controls. After 60 minutes, mosquitoes were
244 transferred into a paper cup and provided with 10% sugar solution. Knockdowns
245 were scored and mortality was monitored every day for seven consecutive days in
246 standard laboratory conditions. We compared mortality rates with or without the
247 synergist to determine to what extent inhibiting oxydases affected the level of
248 susceptibility.
249
250 **Data analysis**
251 We used mortality rates to evaluate the efficacy of clothianidin against laboratory
252 and field mosquitoes. All tests with mortality > 20% at day 7 in controls were
253 discarded. We used Abbott's formula to correct mortality rates of tests when 5%–
254 20% individuals died between day 1 and day 7 in the corresponding controls.
255 Following the WHO guidelines on insecticide susceptibility, mosquito populations
256 were considered susceptible if mortality at day 7 was ≥ 98% and resistant if
257 mortality was less than 90%. Mortality rates between 90% and 97% indicate that
258 the presence of resistant genes in the vector population must be confirmed by
259 additional tests [36]. We used Wilcoxon test to determine if mean mortality rates
260 were significantly different among tested populations. We performed all analyses
261 using the R software [40].
262
263 **Results**
264
265 **1- Evidence of clothianidin resistance in *Anopheles gambiae***
266

267 Across the 5 study sites, we used CDC bottle bioassays to test the diagnostic dose of
268 clothianidin against a total of 1665 wild mosquitoes belonging to three species: *An.*
269 *gambiae* (n = 817), *An. coluzzii* (n = 673) and *Culex* sp (n = 132). To validate the CDC
270 bottle bioassay protocol [35], we first analyzed susceptibility in 554 individuals of
271 two laboratory colonies: *An. gambiae* Kisumu (n = 326) and *An. coluzzii* Ngousso (n
272 = 228). Results showed that the two laboratory strains were fully susceptible to
273 clothianidin, reaching 100% mortality between the first and the third day post
274 exposure (Fig. 2A). Among field mosquitoes, *Culex* sp. populations were the most
275 susceptible to clothianidin, achieving 100% mortality in approximately 24 h (Fig.
276 2A). *An. coluzzii* populations from urban areas were also susceptible to the active
277 dose of clothianidin, although 100% mortality was reached only between the fourth
278 and the seventh day. In *An. gambiae* by contrast, the overall mortality was only 58.2
279 ± 5.2 % in all 817 individuals tested from 4 locations, suggesting that populations of
280 this species have developed resistance to clothianidin. As previously reported in a
281 continental survey of clothianidin susceptibility in *Anopheles* throughout 16 African
282 countries [18], knockdown at 60 min was generally low, except field populations of
283 *An. coluzzii* (81.8 ± 2.9%), Mortality within 60 min of exposure ranged from 42 ±
284 11.0 % to 53.9 ± 9.9 % for the lab strains and from 32.1 ± 5.0 % to 36.5 ± 12.2 %
285 among field populations (Fig. 3A). Consistent with their reduced susceptibility to
286 clothianidin, wild populations of *An. gambiae* displayed the lowest knockdown (32.1
287 ± 5.0 %). Across our sampling sites, *An. gambiae* populations occur mainly in rural
288 and suburban areas and are absent in the most urbanized regions [28,31]. Our
289 findings suggest that farming activities associated with the usage of agricultural
290 neonicotinoids, which are common in rural and suburban areas of Yaoundé, may be
291 contributing to the selection of clothianidin resistance in *An. gambiae* mosquitoes
292
293 **2- Association between usage of agricultural neonicotinoids and clothianidin**
294 **resistance in *An. gambiae***
295
296 To examine the link between usage of agricultural neonicotinoids and the
297 development of clothianidin resistance in *An. gambiae,* we compared susceptibility

298 profiles of populations from the suburban farm and those from the rural sites.
299 Mortality rates varied along a gradient, ranging from full susceptibility in some
300 populations from rural areas to strong resistance in samples from the Nkolondom
301 farm where only 46.5 ± 5.7 % of exposed individuals died between the first and the
302 seventh day of holding period (Fig. 2B). There was no statistically significant
303 difference in mortality among sites except between the suburban farm and each of
304 the rural sites (Wilcoxon tests, $p < 0.02$). Knockdown at 60 min in Nkolondom
305 samples was also lowest (20.1 ± 4.1 %) and significantly different from values
306 obtained with rural populations (Wilcoxon tests, $p < 0.03$) (Fig. 3B). Although these
307 observations still need to be replicated and validated across other agricultural
308 settings, our data suggest that resistance to agricultural neonicotinoids is the likely
309 driver of reduced clothianidin susceptibility observed in *An. gambiae* populations
310 from the Nkolondom farms. During our field survey in Nkolondom, we collected
311 used cans of imidacloprid and acetamiprid (Fig. 1C). We also witnessed the
312 intensive application of those products in mixtures of pesticides, which
313 corroborates observations indicating significant usage of neonicotinoids in some
314 agricultural hotspots in Cameroon (Fig. 1D) [25]. Nevertheless, we detected signals
315 consistent with resistance in a rural site without obvious evidence of agricultural
316 activities involving the use of neonicotinoids—Zamengoué, 3°56'29" N, 11°27'07"
317 E—, mortality: 72 ± 12.0 % at day 7. This finding might be due to dispersal of
318 populations from Nkolondom located about 3 km from Zamengoué or from another
319 unidentified agricultural site. This result could also suggest that other
320 environmental factors in addition to agricultural activities may contribute to the
321 selection of clothianidin-resistant vector populations. Also, we noted that *Culex* sp
322 samples collected from the breeding sites where resistant *An. gambiae* larvae thrive
323 in Nkolondom were susceptible to clothianidin. This observation lends support to
324 the view that cross-resistance to agricultural neonicotinoids is species-specific and
325 might be only one among a list of mechanisms underlying the adaptation of insect
326 populations to this class of insecticide in agricultural regions.
327

328 **3- Clothianidin resistance is strong in *An. gambiae* populations from**
329 **Nkolondom**
330
331 To assess the strength of resistance driven by agricultural neonicotinoids, we used
332 CDC bottle assays to evaluate the mortality rates of resistant populations from
333 Nkolondom against 5X to 10X the diagnostic dose of clothianidin. Results indicated
334 that only 37.5 ± 7.7 % and 77.5 ± 12.6 % of populations exposed to 5X and 10X the
335 diagnostic dose of clothianidin, respectively, died between the first and the seventh
336 day following exposure (Fig. 3C). According to WHO guidelines, when resistance is
337 confirmed at the 5X and especially at the 10X concentrations, operational failure is
338 likely [36]. To determine to what extent strong clothianidin resistance could affect
339 the efficacy of manufactured formulations, we used WHO tube tests to measure the
340 susceptibility of farm mosquitoes to SumiShield 50 WG. This formulation
341 corresponds to ~24X the diagnostic dose of clothianidin used in CDC bottle assays.
342 We used the susceptible Ngousso strain as negative controls. As expected, the lab
343 strain and the control field populations were susceptible to SumiShield 50 WG,
344 reaching 100% after 48h (Fig. 2C). By contrast, mortality rates of *An. gambiae*
345 populations from the Nkolondom farm plateaued at 75.4 ± 3.5 % after 7 days of
346 holding period. This finding suggests that resistant populations from Nkolondom
347 are currently capable of surviving very high concentrations of clothianidin used in
348 SumiShield 50 WG and could be challenging to control with the new IRS
349 formulations.
350
351 **4- Oxydases play a central role in clothianidin resistance in *An. gambiae***
352
353 To start investigating the genetic mechanisms underlying the resistance to
354 clothianidin in *An. gambiae*, especially the role of cytochrome P 450 enzymes, we
355 carried out CDC bottle assays to evaluate the toxicity of the diagnostic dose of
356 clothianidin in combination with PBO. We found that mortality among clothianidin-
357 resistant populations from the agricultural site increased from 46.5 ± 5.7 % without
358 PBO to 92.7 ± 3.7 % in presence of the synergist (Wilcoxon test: p=0.015) (Fig. 2D).

359 Thus, when clothianidin is combined with PBO, resistant populations almost fully
360 recovered their susceptibility, which indicates that cytochrome P450 enzymes play
361 a major role in the development of resistance in *An. gambiae*.
362
363 **Discussion**
364
365 The present study aimed to determine if the intensive use of neonicotinoid
366 insecticides in some agricultural regions in Sub-Saharan Africa could undermine the
367 efficacy of new IRS formulations containing clothianidin. Indeed, the scale up of
368 insecticides in large-scale vector control measures combined with rapid
369 urbanization and extensive agriculture have exposed *Anopheles* mosquitoes to a
370 diversity of xenobiotic compounds over the last few decades [41]. As a result,
371 current populations have evolved a myriad of genetic, behavioral and physiological
372 adaptive mechanisms, which could enhance their tolerance to new insecticides
373 [42,43]. Our findings show that this scenario is happening with clothianidin whose
374 toxicity is reduced in some regions where agricultural activities are contributing to
375 the selection of resistant *Anopheles* populations.
376
377 WHO guidelines for clothianidin resistance testing in mosquitoes are yet to be
378 established and published. Assessing clothianidin susceptibility in *Anopheles*
379 mosquitoes using conventional bioassays has been met with some technical
380 challenges so far [18,22,35,37,44]. Clothianidin is difficult to solubilize in solvents
381 commonly used in bioassays, which has made the design of standard protocols more
382 laborious than usual [18,35,37]. In addition, because this active ingredient acts
383 slowly, resistance profiles are revealed via laboratory bioassays only if exposed
384 populations are rigorously monitored during a long holding period, which can last
385 seven days or more. Despite these difficulties, the susceptibility of wild *Anopheles*
386 populations from several African countries has been successfully evaluated using
387 the recommended diagnostic doses of clothianidin [18,22,35,37]. Here we have used
388 two protocols implemented and tested previously to analyze thousands of wild
389 mosquitoes and to provide the first evidence of clothianidin resistance in

390 anopheline populations. In past studies, standard laboratory bioassays were used to
391 assess the susceptibility profile of the most important vectors of *Plasmodium*
392 parasites in Sub-Saharan Africa—*An. gambiae*, *An. coluzzii*, *An. funestus* and *An.*
393 *arabiensis* [18,22,35,37]. Resistance to clothianidin was not detected among
394 populations from 16 countries where 100% mortality was reached on average 72 h
395 following exposure in all populations tested either with WHO tube tests [18,37] or
396 with CDC bottle assays [22,35]. Our study confirms that susceptible populations of
397 *An. coluzzii* and *An. gambiae* die on average within 72 h of holding period. Of note,
398 *An. coluzzii* samples, collected from the most urbanized areas of Yaoundé where
399 populations thrive in polluted environments and are highly resistant to several
400 classes of insecticides [28,31,45,46], remained susceptible to clothianidin. A recent
401 study carried out in Ivory Coast also showed that *An. coluzzii* populations from
402 agricultural sites were susceptible to clothianidin. These data suggest that this
403 species may have not yet developed resistance mechanisms against this active
404 ingredient [22].
405
406 By contrast to previous investigations, we observed resistance to clothianidin in *An.*
407 *gambiae* with mortality rates as low as 46.5 % among populations from an
408 agricultural region. Populations from surrounding locations remained largely
409 susceptible. Overall, our findings and other previous surveys suggest that
410 clothianidin resistance is present in anopheline populations, but is likely not yet
411 widespread among species and geographic areas [18]. Furthermore, the number of
412 registered neonicotinoids and probably their usage vary between African countries
413 and among geographic areas within countries [25–27]. Such variability may also
414 lead to geographic heterogeneity in selection pressure and could partly explain why
415 resistance is not detected in some settings. The development of clothianidin
416 resistance in *An. gambiae* in an agricultural region as shown here is not very
417 surprising albeit potentially harmful to malaria control efforts. Indeed, there is a
418 long list of observations providing indirect evidence that intensive and unrestricted
419 application of agricultural pesticides contributes to the selection of malaria vector
420 populations with reduced susceptibility to some active ingredients used in public

421 health programs (reviewed in[23]). In Sub-Saharan Africa, resistance to pyrethroids,
422 organophosphates, carbamates and organochlorines is particularly strong among
423 populations from agricultural regions where a wide spectrum of pesticides is
424 routinely used for crop protection (e.g., [19,20,47,48]). During our field work, we
425 found evidence of usage of some formulations containing imidacloprid and
426 acetamiprid respectively, but there is a larger number of neonicotinoid formulations
427 available on local markets across Cameroon [25]. However, it is also important to
428 remind that *Culex* populations collected from standing water within farm plots in
429 Nkolondom were susceptible to clothianidin. This implies that exposure to
430 agricultural neonicotinoids does not automatically lead to resistance in mosquitoes
431 but could trigger other adaptive mechanisms that are yet to be elucidated. A direct
432 test of the association between agricultural neonicotinoids and the development of
433 resistance to clothianidin in *Anopheles* is beyond the scope of the present work and
434 should be the subject of future studies involving observational and experimental
435 approaches. In addition, one caveat of the present study is that sampling was limited
436 to only one agricultural region, and further investigation is needed to draw robust
437 conclusions about the current situation of clothianidin resistance in African
438 countries. Nonetheless, it is conceivable that in farms such as that of Nkolondom, the
439 application of neonicotinoids selects clothianidin-resistant populations, which
440 proliferate year-round in abundant breeding sites created by permanent irrigation.
441
442 Our results show that resistance to clothianidin is already strong in *An. gambiae*
443 populations from the Nkolondom farm where low mortality rates are observed even
444 with high doses of the active ingredient. Indeed, urban agriculture has been
445 intensified in the swampy area of this village since the 1980's during the economic
446 depression in Cameroon [29]. As a result, mosquito populations have likely had
447 enough time to develop such high levels of resistance. Bioassay tests using CDC
448 bottles coated with the synergist PBO in addition to clothianidin showed an increase
449 in mortality up to 90% in resistant populations. This suggests that Cytochrome P450
450 (CYPs) play a primarily role in clothianidin resistance in *Anopheles gambiae*. This
451 finding is in keeping with a large body of previous work, which revealed that

452 clothianidin resistance in wild populations of many crop pests, primarily those of
453 the order *Hemiptera* (aphids, whiteflies, and planthoppers), is associated with
454 overexpression of one or several CYP enzymes [49–52]. Nevertheless, the strength
455 of resistance observed in Nkolondom suggests that the underlying mechanisms
456 might be more complex. Based on insights gained from studying the spread of
457 pyrethroid resistance in malaria vector populations across Africa, such a high level
458 of resistance to clothianidin could involve mutations at the target site and
459 modifications of the insect cuticle in addition to the prime role played by enzymatic
460 detoxification [53].
461
462 LLINs coated with pyrethroids have been widely distributed throughout Cameroon
463 over the last decade [54]. Although no IRS campaign has been carried out in the
464 country so far, the National Malaria Control Program and PMI have identified the
465 North and Far North, as the promising targets for a future implementation of IRS
466 [55]. Most anopheline populations across the country are already highly resistant to
467 pyrethroids and sometimes to several other classes of insecticide including
468 carbamates, organophosphates and organochlorines (reviewed in [56]). In such
469 multi-resistant populations, cross-resistance to insecticides with similar modes of
470 action may contribute to the build-up of tolerance to new insecticides [57]. As
471 clothianidin has a distinct mode of action, cross-resistance to the other insecticides
472 in use in malaria vector control so far is unlikely [8,13]. However, one source of
473 concern is that CYPs enzymes, which are already overexpressed in anopheline
474 populations throughout the continent due to widespread use of pyrethroids, could
475 enhance metabolic resistance against clothianidin. Nevertheless, our data also show
476 that populations from urban areas in which overexpression of CYPs and other
477 metabolic enzymes has been previously documented remain fully susceptible to
478 clothianidin [31,45,58]. Overall, although the underlying mechanisms rely heavily
479 on detoxification enzymes of the CYPs family that are ubiquitous among multi-
480 resistant vector populations, clothianidin resistance remains clustered around the
481 hotspot of agricultural usage of neonicotinoids. We thus argue that clothianidin
482 remains a potentially effective insecticide against pyrethroid-resistant malaria

483 vector populations in Cameroon if it is not used in areas such as the Nkolondom
484 farm where the likelihood of operational failure increases. Such agricultural regions
485 should be mapped in IRS target areas prior to the rollout of clothianidin
486 formulations. Importantly, reliable bioassay protocols for clothianidin resistance
487 testing in *Anopheles* are now available [18,22,35] and could ease the scale up of
488 resistance monitoring by national malaria control programs. Mapping the sources of
489 selection pressure, which could contribute to the emergence and development of
490 resistance among vector populations in IRS target areas, is crucial to the successful
491 use of new IRS formulations.
492
493 **Conclusions**
494
495 This study shows that the novel insecticide clothianidin may have reduced efficacy
496 in some agricultural regions of Sub-Saharan Africa due to pre-existing levels of
497 resistance among target populations. The decision-making process regarding the
498 potential deployment of new active ingredients in malaria vector control programs
499 has been mainly based upon selecting compounds with a unique mode of action so
500 as to prevent cross-resistance between insecticides in current use in public health.
501 The present work suggests that before new insecticides are considered for inclusion
502 in National Malaria Control Program resistance management strategies, their
503 efficacy should be particularly monitored in areas where similar compounds are
504 being used in agriculture.
505
506

Figure legends

Figure 1: Description of the study site. (A) Map of the neighborhoods of Yaoundé where clothianidin susceptibility of *Anopheles* and *Culex* mosquitoes was evaluated. (B) A typical vegetable plot of the Nkolondom farm. (C) Used cans of imidacloprid and acetamiprid collected from the fields. (B) Application of pesticide mixtures on a vegetable plot in Nkolondom

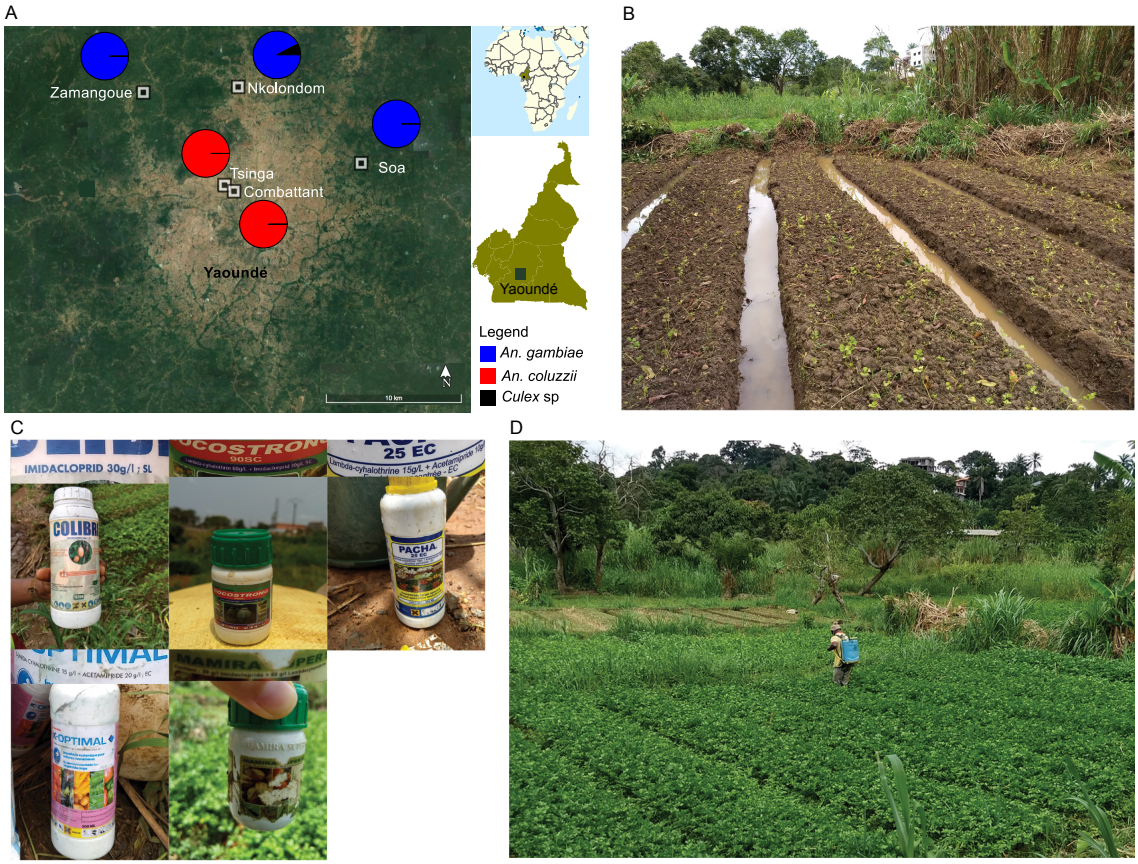
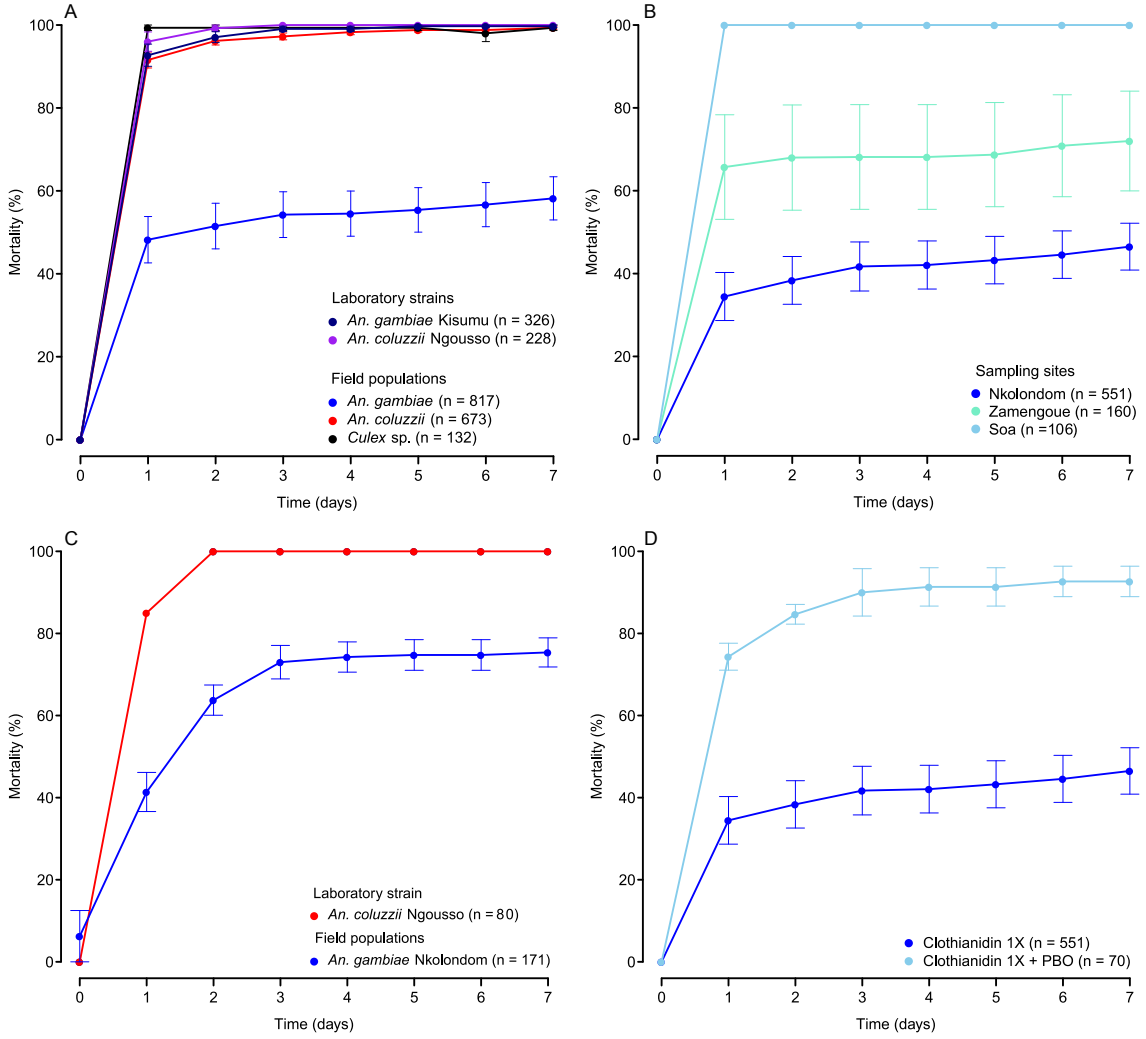
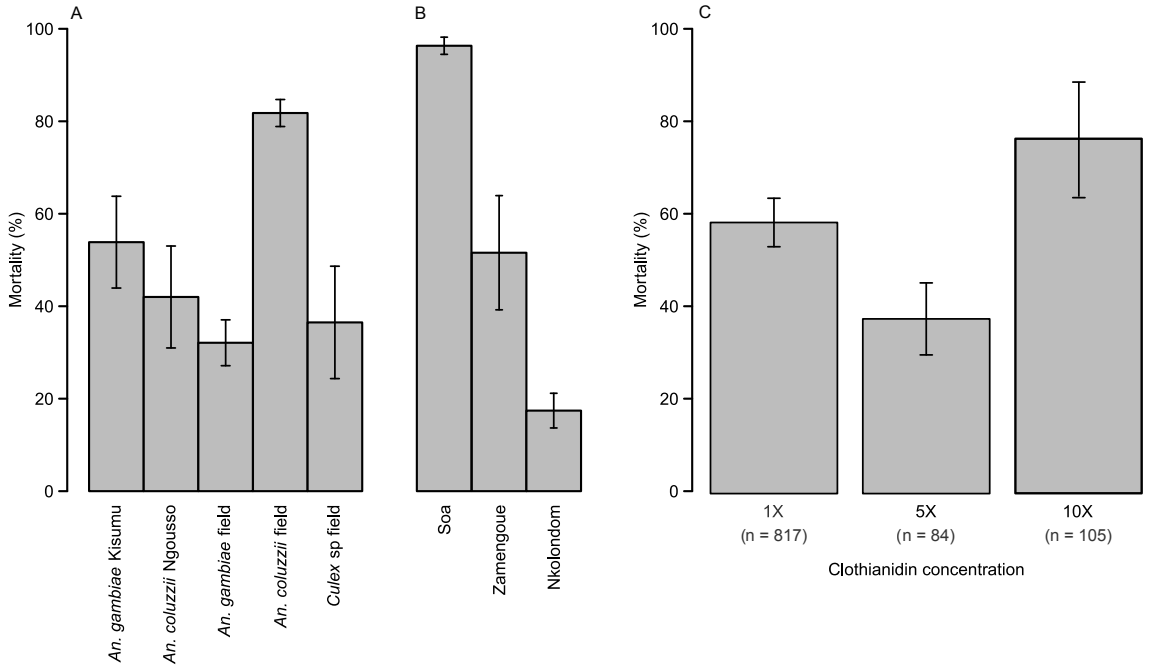


Figure 2: Susceptibility to clothianidin in laboratory strains and field populations. (A) Mortality of *Anopheles* and *Culex* populations exposed to 150µg/ml clothianidin in CDC bottles. (B) A gradient of clothianidin susceptibility in wild populations of *An. gambiae*. (C) Susceptibility of a laboratory strain and of Nkolondom populations to SumiShield 50WG evaluated with WHO tube tests. (D) Dramatic increase in clothianidin toxicity in Nkolondom populations in the presence of the synergist PBO.



526 Figure 3: Knockdowns after 60 min exposure and susceptibility to increasing doses
527 of clothianidin. (A) Knockdowns in *Anopheles* and *Culex* populations in CDC bottle
528 bioassays. (B) Knockdowns in *An. gambiae* field populations. (C) Mortality of
529 mosquitoes from the Nkolondom farm exposed to 1X, 5X and 10X the active dose of
530 clothianidin in CDC bottles. Standard errors are shown..



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545 **Authors' contributions**

546 CF, CK and CSW designed the study. CF, WT, MA, FA and CK carried out
547 entomological collections and laboratory tests. CF and CK analyzed the data and
548 drafted the manuscript, which was revised by co-authors. All authors read and
549 approved the final manuscript.

550

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554

555 **Competing interests**

556 The authors declare that they have no competing interests.

557

558 **Availability of data and materials**

559 Not applicable.

560

561 **Consent for publication**

562 Not applicable.

563

564 **Ethics approval and consent to participate**

565 The study protocol was reviewed and approved by the Regional Ethics Committee
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568

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