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

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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 % $\pm$ 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

Cameroon (Figure 1A). For comparison, we also tested mosquito populations from two neighborhoods situated at the center of Yaoundé and from two rural sites at the outskirts of the city [28] (Fig. 1A). Nkolondom is located in the northern part of Yaoundé—approximately 7 km from the center of the city. Since 1985, artisanal agriculture has gained considerable momentum in a swampy area of this locality, which currently attracts at least one hundred farmers [29]. At present, this suburban farm encompasses 100-200 mosaics of $\sim 20\text{m}^2$ adjacent plots and has become the main production site of celery, amaranth, lettuce and other aromatic herbs in Yaoundé (Fig. 1B). Farming activities are conducted year-round, and crops are intensively watered either manually or using artisanal irrigation schemes. Consequently, standing water accumulates in the fields where very high densities of *An. gambiae* and *Culex sp* larvae can be collected throughout the year.

Mosquito collection and rearing

Mosquito collections took place during the long rainy season and part of the long dry season: from July 2019 to January 2020 and from May 2020 to July 2020. We sampled mosquito larvae across the study sites using the standard dipping method [30]. Larvae were collected from standing water within the farms in Nkolondom, from relatively shallow ruts in rural areas and from polluted breeding sites at the center of Yaoundé city. Larvae were transported in closed containers to the CRID's insectary and reared to adults under standard laboratory conditions. *An. gambiae* and *An. coluzzii* are the dominant malaria vector species in Yaoundé where their respective habitats have been well characterized in past surveys [28,31]. *An. coluzzii* is found exclusively in the most urbanized neighborhoods while *An. gambiae* is the only species present in the rural areas. Sampling was conducted to collect and test one species per location. We collected and tested *Culex* populations that occur in the same breeding sites as *An. gambiae* in Nkolondom to compare their susceptibility profiles. For bioassay tests, we used two laboratory strains as susceptible controls: *An. gambiae* Kisumu and *An. coluzzii* Ngousso. Both strains are known to be susceptible to pyrethroid, carbamate, organochlorine and organophosphate insecticides. We maintained all life-cycle stages of laboratory strains and wild

mosquito populations under standard insectary conditions (25 ± 2 °C, 80% relative humidity, light:dark cycles of 12:00 h each) and we provided adults with 10% sugar solution.

CDC bottle tests

We tested the susceptibility of adult mosquitoes against a diagnostic dose of clothianidin using CDC bottle assays according to published guidelines [34]. A recent study showed that 150µg/ml clothianidin was a suitable diagnostic dose—the lowest concentration of active ingredient that kills 100% of individuals of a susceptible laboratory strain within 24 h of insecticide exposure [35]. To assess the intensity of resistance, we also determined the mortality rates of wild mosquitoes exposed to 5X and 10X the diagnostic dose. We prepared stock solutions of clothianidin at 1X, 5X, and 10X the diagnostic dose using technical grade clothianidin (PESTANAL®, analytical standard, Sigma-Aldrich, Dorset, United Kingdom) and absolute ethanol as solvent. The solutions were conserved at 4°C in the dark for 24 h before use to maximize the solubility of clothianidin. Each Wheaton 250-ml bottle and its cap were coated with 1 ml of clothianidin solution following the CDC protocol. For each bioassay, we used a maximum of four test bottles coated with clothianidin and two control bottles coated with 1 ml of absolute ethanol. Occasionally, depending on the availability of mosquitoes, we used less than four test bottles and only one control. All bottles were wrapped in aluminum foil and allowed to dry for 24h for complete evaporation of solvent before use. Bottles were not reused but were washed three times in warm soapy water and allowed to dry for 24 h before being coated.

We carried out susceptibility tests as follows: 20 to 25 3-5-day-old females were aspirated from mosquito cages and gently released into one of the test bottles where they were exposed to the active ingredient or into control bottles containing ethanol for 60 minutes. Following exposure, mosquitoes were transferred into a paper cup and provided with 10% sugar solution. Knockdowns were scored and mortality was monitored every day for seven consecutive days under standard laboratory

conditions. When a population displayed signals consistent with resistance, we carried out repeat tests for confirmation.

WHO tube tests

We used WHO tube tests to assess the susceptibility of populations showing the lowest mortality rate in CDC bottle bioassays to SumiShield 50WG, which contains 50% (w/w) clothianidin [36]. We carried out repeat tests of these resistant populations using a susceptible field population and a susceptible lab strain as negative controls. We used a diagnostic dose of 2% w/v clothianidin (13.2 mg active ingredient per paper) based on guidance from Sumitomo Corporation and the US President's Malaria Initiative. This dose has been successfully used to test the toxicity of clothianidin towards wild anopheline populations from Sub-Saharan Africa [18,37]. We prepared a stock solution by diluting 264 mg SumiShield 50WG in 20 ml distilled water. We impregnated Whatman filter papers (12x15cm) containing 13.2 mg clothianidin each using 2ml of the insecticide solution as described in [38]. We prepared control filter papers using 2ml of distilled water. Treated filter papers were allowed to dry overnight and were kept in foil at 4°C until use. To carry out bioassay tests, we aspired 20 to 25 3-5-day-old female mosquitoes from mosquito cages that we introduced into each of the test tubes containing clothianidin-impregnated papers. We concomitantly released 20-25 mosquitoes into each control tube containing a water-impregnated paper. After 60 minutes of exposure, mosquitoes were transferred to holding tubes and provided with 10% sugar solution, and knockdown time was recorded. As with CDC bottle tests mortality was scored every day until day 7.

Synergist bioassays

To investigate the potential role of oxidases in the genetic mechanisms underlying the reduced susceptibility to clothianidin, we used CDC bottle assays to determine the efficacy of the diagnostic dose in combination with the synergist PBO (4%). PBO is an inhibitor of oxidases and non-specific esterases and enhances cuticular penetration of the insecticide into the insect [39]. A solution of 400 µg/ml PBO was

prepared by diluting PBO with absolute ethanol. The solution was mixed and stored in the dark at 4°C before use. Wheatman bottles and their cap were coated with 1ml of the PBO solution, wrapped in aluminum foil and allowed to dry for 24h before the test. 100 to 150 female mosquitoes aged between 3 and 5 days were pre-exposed for 60 min to PBO-coated bottles or to control bottles coated with ethanol. Mosquitoes were then gently removed from the bottles and introduced in batches of 20 to 25 mosquitoes into new bottles coated with 1X clothianidin solution for the tests or with ethanol for the controls. After 60 minutes, mosquitoes were transferred into a paper cup and provided with 10% sugar solution. Knockdowns were scored and mortality was monitored every day for seven consecutive days in standard laboratory conditions. We compared mortality rates with or without the synergist to determine to what extent inhibiting oxydases affected the level of susceptibility.

Data analysis

We used mortality rates to evaluate the efficacy of clothianidin against laboratory and field mosquitoes. All tests with mortality > 20% at day 7 in controls were discarded. We used Abbott's formula to correct mortality rates of tests when 5%–20% individuals died between day 1 and day 7 in the corresponding controls. Following the WHO guidelines on insecticide susceptibility, mosquito populations were considered susceptible if mortality at day 7 was $\geq 98\%$ and resistant if mortality was less than 90%. Mortality rates between 90% and 97% indicate that the presence of resistant genes in the vector population must be confirmed by additional tests [36]. We used Wilcoxon test to determine if mean mortality rates were significantly different among tested populations. We performed all analyses using the R software [40].

Results

1- Evidence of clothianidin resistance in *Anopheles gambiae*

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

2- Association between usage of agricultural neonicotinoids and clothianidin resistance in *An. gambiae*

To examine the link between usage of agricultural neonicotinoids and the development of clothianidin resistance in *An. gambiae*, we compared susceptibility

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

3- Clothianidin resistance is strong in *An. gambiae* populations from Nkolondom

To assess the strength of resistance driven by agricultural neonicotinoids, we used CDC bottle assays to evaluate the mortality rates of resistant populations from Nkolondom against 5X to 10X the diagnostic dose of clothianidin. Results indicated that only 37.5 ± 7.7 % and 77.5 ± 12.6 % of populations exposed to 5X and 10X the diagnostic dose of clothianidin, respectively, died between the first and the seventh day following exposure (Fig. 3C). According to WHO guidelines, when resistance is confirmed at the 5X and especially at the 10X concentrations, operational failure is likely [36]. To determine to what extent strong clothianidin resistance could affect the efficacy of manufactured formulations, we used WHO tube tests to measure the susceptibility of farm mosquitoes to SumiShield 50 WG. This formulation corresponds to ~24X the diagnostic dose of clothianidin used in CDC bottle assays. We used the susceptible Ngousso strain as negative controls. As expected, the lab strain and the control field populations were susceptible to SumiShield 50 WG, reaching 100% after 48h (Fig. 2C). By contrast, mortality rates of *An. gambiae* populations from the Nkolondom farm plateaued at 75.4 ± 3.5 % after 7 days of holding period. This finding suggests that resistant populations from Nkolondom are currently capable of surviving very high concentrations of clothianidin used in SumiShield 50 WG and could be challenging to control with the new IRS formulations.

4- Oxydases play a central role in clothianidin resistance in *An. gambiae*

To start investigating the genetic mechanisms underlying the resistance to clothianidin in *An. gambiae*, especially the role of cytochrome P 450 enzymes, we carried out CDC bottle assays to evaluate the toxicity of the diagnostic dose of clothianidin in combination with PBO. We found that mortality among clothianidin-resistant populations from the agricultural site increased from 46.5 ± 5.7 % without PBO to 92.7 ± 3.7 % in presence of the synergist (Wilcoxon test: $p=0.015$) (Fig. 2D).

Thus, when clothianidin is combined with PBO, resistant populations almost fully recovered their susceptibility, which indicates that cytochrome P450 enzymes play a major role in the development of resistance in *An. gambiae*.

Discussion

The present study aimed to determine if the intensive use of neonicotinoid insecticides in some agricultural regions in Sub-Saharan Africa could undermine the efficacy of new IRS formulations containing clothianidin. Indeed, the scale up of insecticides in large-scale vector control measures combined with rapid urbanization and extensive agriculture have exposed *Anopheles* mosquitoes to a diversity of xenobiotic compounds over the last few decades [41]. As a result, current populations have evolved a myriad of genetic, behavioral and physiological adaptive mechanisms, which could enhance their tolerance to new insecticides [42,43]. Our findings show that this scenario is happening with clothianidin whose toxicity is reduced in some regions where agricultural activities are contributing to the selection of resistant *Anopheles* populations.

WHO guidelines for clothianidin resistance testing in mosquitoes are yet to be established and published. Assessing clothianidin susceptibility in *Anopheles* mosquitoes using conventional bioassays has been met with some technical challenges so far [18,22,35,37,44]. Clothianidin is difficult to solubilize in solvents commonly used in bioassays, which has made the design of standard protocols more laborious than usual [18,35,37]. In addition, because this active ingredient acts slowly, resistance profiles are revealed via laboratory bioassays only if exposed populations are rigorously monitored during a long holding period, which can last seven days or more. Despite these difficulties, the susceptibility of wild *Anopheles* populations from several African countries has been successfully evaluated using the recommended diagnostic doses of clothianidin [18,22,35,37]. Here we have used two protocols implemented and tested previously to analyze thousands of wild mosquitoes and to provide the first evidence of clothianidin resistance in

anopheline populations. In past studies, standard laboratory bioassays were used to assess the susceptibility profile of the most important vectors of *Plasmodium* parasites in Sub-Saharan Africa—*An. gambiae*, *An. coluzzii*, *An. funestus* and *An. arabiensis* [18,22,35,37]. Resistance to clothianidin was not detected among populations from 16 countries where 100% mortality was reached on average 72 h following exposure in all populations tested either with WHO tube tests [18,37] or with CDC bottle assays [22,35]. Our study confirms that susceptible populations of *An. coluzzii* and *An. gambiae* die on average within 72 h of holding period. Of note, *An. coluzzii* samples, collected from the most urbanized areas of Yaoundé where populations thrive in polluted environments and are highly resistant to several classes of insecticides [28,31,45,46], remained susceptible to clothianidin. A recent study carried out in Ivory Coast also showed that *An. coluzzii* populations from agricultural sites were susceptible to clothianidin. These data suggest that this species may have not yet developed resistance mechanisms against this active ingredient [22].

By contrast to previous investigations, we observed resistance to clothianidin in *An. gambiae* with mortality rates as low as 46.5 % among populations from an agricultural region. Populations from surrounding locations remained largely susceptible. Overall, our findings and other previous surveys suggest that clothianidin resistance is present in anopheline populations, but is likely not yet widespread among species and geographic areas [18]. Furthermore, the number of registered neonicotinoids and probably their usage vary between African countries and among geographic areas within countries [25–27]. Such variability may also lead to geographic heterogeneity in selection pressure and could partly explain why resistance is not detected in some settings. The development of clothianidin resistance in *An. gambiae* in an agricultural region as shown here is not very surprising albeit potentially harmful to malaria control efforts. Indeed, there is a long list of observations providing indirect evidence that intensive and unrestricted application of agricultural pesticides contributes to the selection of malaria vector populations with reduced susceptibility to some active ingredients used in public

health programs (reviewed in[23]). In Sub-Saharan Africa, resistance to pyrethroids, organophosphates, carbamates and organochlorines is particularly strong among populations from agricultural regions where a wide spectrum of pesticides is routinely used for crop protection (e.g., [19,20,47,48]). During our field work, we found evidence of usage of some formulations containing imidacloprid and acetamiprid respectively, but there is a larger number of neonicotinoid formulations available on local markets across Cameroon [25]. However, it is also important to remind that *Culex* populations collected from standing water within farm plots in Nkolondom were susceptible to clothianidin. This implies that exposure to agricultural neonicotinoids does not automatically lead to resistance in mosquitoes but could trigger other adaptive mechanisms that are yet to be elucidated. A direct test of the association between agricultural neonicotinoids and the development of resistance to clothianidin in *Anopheles* is beyond the scope of the present work and should be the subject of future studies involving observational and experimental approaches. In addition, one caveat of the present study is that sampling was limited to only one agricultural region, and further investigation is needed to draw robust conclusions about the current situation of clothianidin resistance in African countries. Nonetheless, it is conceivable that in farms such as that of Nkolondom, the application of neonicotinoids selects clothianidin-resistant populations, which proliferate year-round in abundant breeding sites created by permanent irrigation.

Our results show that resistance to clothianidin is already strong in *An. gambiae* populations from the Nkolondom farm where low mortality rates are observed even with high doses of the active ingredient. Indeed, urban agriculture has been intensified in the swampy area of this village since the 1980's during the economic depression in Cameroon [29]. As a result, mosquito populations have likely had enough time to develop such high levels of resistance. Bioassay tests using CDC bottles coated with the synergist PBO in addition to clothianidin showed an increase in mortality up to 90% in resistant populations. This suggests that Cytochrome P450 (CYPs) play a primary role in clothianidin resistance in *Anopheles gambiae*. This finding is in keeping with a large body of previous work, which revealed that

clothianidin resistance in wild populations of many crop pests, primarily those of the order *Hemiptera* (aphids, whiteflies, and planthoppers), is associated with overexpression of one or several CYP enzymes [49–52]. Nevertheless, the strength of resistance observed in Nkolondom suggests that the underlying mechanisms might be more complex. Based on insights gained from studying the spread of pyrethroid resistance in malaria vector populations across Africa, such a high level of resistance to clothianidin could involve mutations at the target site and modifications of the insect cuticle in addition to the prime role played by enzymatic detoxification [53].

LLINs coated with pyrethroids have been widely distributed throughout Cameroon over the last decade [54]. Although no IRS campaign has been carried out in the country so far, the National Malaria Control Program and PMI have identified the North and Far North, as the promising targets for a future implementation of IRS [55]. Most anopheline populations across the country are already highly resistant to pyrethroids and sometimes to several other classes of insecticide including carbamates, organophosphates and organochlorines (reviewed in [56]). In such multi-resistant populations, cross-resistance to insecticides with similar modes of action may contribute to the build-up of tolerance to new insecticides [57]. As clothianidin has a distinct mode of action, cross-resistance to the other insecticides in use in malaria vector control so far is unlikely [8,13]. However, one source of concern is that CYPs enzymes, which are already overexpressed in anopheline populations throughout the continent due to widespread use of pyrethroids, could enhance metabolic resistance against clothianidin. Nevertheless, our data also show that populations from urban areas in which overexpression of CYPs and other metabolic enzymes has been previously documented remain fully susceptible to clothianidin [31,45,58]. Overall, although the underlying mechanisms rely heavily on detoxification enzymes of the CYPs family that are ubiquitous among multi-resistant vector populations, clothianidin resistance remains clustered around the hotspot of agricultural usage of neonicotinoids. We thus argue that clothianidin remains a potentially effective insecticide against pyrethroid-resistant malaria

vector populations in Cameroon if it is not used in areas such as the Nkolondom farm where the likelihood of operational failure increases. Such agricultural regions should be mapped in IRS target areas prior to the rollout of clothianidin formulations. Importantly, reliable bioassay protocols for clothianidin resistance testing in *Anopheles* are now available [18,22,35] and could ease the scale up of resistance monitoring by national malaria control programs. Mapping the sources of selection pressure, which could contribute to the emergence and development of resistance among vector populations in IRS target areas, is crucial to the successful use of new IRS formulations.

Conclusions

This study shows that the novel insecticide clothianidin may have reduced efficacy in some agricultural regions of Sub-Saharan Africa due to pre-existing levels of resistance among target populations. The decision-making process regarding the potential deployment of new active ingredients in malaria vector control programs has been mainly based upon selecting compounds with a unique mode of action so as to prevent cross-resistance between insecticides in current use in public health. The present work suggests that before new insecticides are considered for inclusion in National Malaria Control Program resistance management strategies, their efficacy should be particularly monitored in areas where similar compounds are being used in agriculture.

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.

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

Authors' contributions

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

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Not applicable.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Regional Ethics Committee for Human health Research of Ministry of Public Health Cameroon (1-141/CRERSHC/2020).

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