Chronic exposure of mosquito larvae to pesticide residues endangers a new generation of agrochemicals repurposed for malaria prevention

Marilene M. Ambadiang[1, 2], Caroline Fouet[3], Fred A. Ashu[1, 2], Véronique Penlap-Beng[2], and Colince Kamdem[3]*

[1]*Centre for Research in Infectious Diseases (CRID), P.O. Box 13591, Yaoundé, 9 Cameroon*

[2]*Department of Biochemistry, Faculty of Science, University of Yaoundé 1, P.O. Box 11 812, Yaoundé, Cameroon*

[3]*Department of Biological Sciences, The University of Texas at El Paso, 500 W. University Ave. El Paso, TX 79968*

* Correspondence: gckamdemdjo@utep.edu

Abstract

Agrochemicals have been successfully repurposed to control mosquitoes worldwide, but pesticides used in agriculture challenge their effectiveness by contaminating surface waters and helping larval populations develop resistance. Thus, knowledge of the lethal and sublethal effects of residual pesticide exposure on mosquitoes is critical for selecting effective insecticides. Here we implemented a new experimental approach to predict the efficacy of agricultural pesticides newly repurposed for malaria vector control. We mimicked insecticide resistance selection as it occurs in contaminated aquatic habitats by rearing field-collected mosquito larvae in water containing a dose of insecticide capable of killing individuals from a susceptible strain within 24 h. We then simultaneously monitored short-term lethal toxicity within 24 h and sublethal effects for 7 days. We found that due to chronic exposure to agricultural pesticides, some mosquito populations are currently pre-adapt to resist neonicotinoids if those were used in vector control. Larvae collected from rural and

agricultural areas where neonicotinoid formulations are intensively used for insect pest management were able to survive, grow, pupate and emerge in water containing a lethal dose of acetamiprid, imidacloprid or clothianidin. These results emphasize the importance of addressing exposure of larval populations to formulations applied in agriculture prior to using agrochemicals against malaria vectors.

Keywords: pesticide, neonicotinoids, *Anopheles*, malaria, urbanization.

1. Introduction

Prevention through chemical control of vector populations has lead to a drastic reduction in malaria burden in sub-Saharan Africa over the last two decades (World Health Organization, 2022a). The prevention strategy relies heavily on repurposing agrochemicals, which provides a rapid and cost-effective approach to identify new active ingredients against vector populations (Hemingway, 2017; Hoppé et al., 2016). In order to effectively control *Anopheles* mosquito populations that have become resistant to available insecticides, dozens of agrochemicals have recently been tested against adult populations, and some promising candidates have been identified (Hoppé et al., 2016; Lees et al., 2019; N'Guessan et al., 2007; Oxborough et al., 2010; Portwood et al., 2022). However, concomitant use of the same class of agrochemicals for crop protection can provide sufficient selection pressure to engineer resistant mosquito populations before any application of the pesticide in public health (Georghiou, 1972; Georghiou et al., 1973; Lines, 1988). For example, prior to the scale up of pyrethroid-impregnated bed nets across the continent, resistance driven by crop protection spraying was already detected in some *Anopheles* populations in West Africa (Curtis et al., 1998). There is a concern history may repeat itself with the new generation of agrochemicals that are being adopted for malaria vector control (World Health Organization, 2023).

Africa's pesticide use for agriculture stood at nearly 108 thousand metric tons in 2019 (Kramer, 2022). Lax regulations allow the use of hundreds of formulations for agricultural pest management in some African countries (Akpesse, 2018; Katambo, 2018; Ngamo Tinkeu, 2018; Okolle et al., 2022; Urio et al., 2022). A large part of pesticide application occurs in small farms held by non-professional operators that are not trained and not supervised for safe and effective use of chemicals. As a result, too frequent and untargeted application provides suitable conditions for chronic exposure of mosquito larvae to pesticide residues. In tropical regions, rain and human activities create ponds in farmlands, which become ideal breeding sites for the most dangerous malaria mosquito species such as *Anopheles gambiae* and *An. coluzzii* in agricultural areas (Antonio-Nkondjio et al., 2011; Mattah et al., 2017; Zogo et al., 2019). When these breeding sites are contaminated with pesticides, chronic residual exposure pre-adapts larval populations to resist synthetic chemicals (Lines, 1988). The consequence of this adaptation is a decrease in efficacy of agrochemicals, which in turn undermines their suitability for mosquito control.

Neonicotinoids are among the most widely used pesticides in agriculture worldwide (Matsuda et al., 2020; Simon-delso et al., 2015). These chemicals are highly water-soluble and environmentally persistent, and thus may leach into surface waters and become a risk for aquatic insects (Morrissey et al., 2015; Ramadevi et al., 2022; Schaafsma et al., 2015). Two new formulations of clothianidin, a neonicotinoid, were recently approved for indoor residual spraying targeting malaria vectors, but have yet to be applied in endemic countries (Agossa et al., 2018; Kweka et al., 2018; Uragayala et al., 2018; World Health Organization, 2023). Additionally, clothianidin is normally not registered for crop protection in most African countries (Katambo, 2018; Ngamo Tinkeu, 2018). So far, agricultural spraying of other registered neonicotinoids such as acetamiprid, imidacloprid, thiamethoxam and thiacloprid

remain the main source of selection pressure that could drive resistance to clothianidin in *Anopheles* mosquitoes through cross-resistance mechanisms. This hypothesis has yet to be supported by empirical evidence, but observational studies have described suboptimal levels of neonicotinoid susceptibility in adults of at least two *Anopheles* species, populations from agricultural areas being the most tolerant (Ashu et al., 2023a; Fouet et al., 2020; Mouhamadou et al., 2019). Importantly, there is evidence that neonicotinoids used for crop protection can confer cross-resistance to clothianidin, raising concerns about the efficacy the new agrochemicals repurposed for malaria prevention (Ashu et al., 2023a, 2023b; Fouet et al., 2020).

Large African cities are the venue of dynamic coevolution between humans and disease vectors, which has sometimes reveled textbook examples of rapid adaptation of mosquitoes to synthetic chemicals (Antonio-Nkondjio et al., 2015; Kamdem et al., 2017, 2012b). In Yaoundé the capital of Cameroon, agricultural activities associated with intensive use of pesticides are pervasive in suburban and rural settings in the outskirts of the city. Mixtures containing neonicotinoids such as acetamiprid, imidacloprid, and thiamethoxam, as well as pyrethroids and fungicides are frequently sprayed on diverse crops, creating ideal conditions for contamination of surface waters and resistance selection (Ngamo Tinkeu, 2018; Okolle et al., 2022). By contrast, the center of the city provides an island where surface waters are less exposed to pesticide contamination and aquatic species are therefore less likely to develop resistance. Indeed, a survey of susceptibility in adult mosquitoes using standard testing procedures confirmed this prediction. *An. coluzzii*, the only species found in densely urbanized areas of Yaoundé remains susceptible to neonicotinoids while its sibling species *An. gambiae*, which occurs in the countryside, has developed resistance (Ashu et al., 2023a,

98 2023b; Fouet et al., 2020). This geographic area thus provides a unique setting to dissect the
99 environmental drivers of neonicotinoid resistance in *Anopheles* mosquitoes.
100
101 The objective of the present study was to assess the interplay between residual pesticide
102 exposure and the development of resistance to new insecticides in malaria vectors. It has been
103 known for nearly a half century that exposure to agricultural pesticides can lead to the spread
104 of resistance to public health insecticides in *Anopheles* mosquitoes (Georghiou, 1972;
105 Georghiou et al., 1973). However, we still lack a holistic approach that could allow to
106 simultaneously deciphering the lethal (mortality) and sub-lethal effects (survival, growth,
107 emergence, reproduction, swimming behavior, mobility etc.) which contribute to resistance
108 selection in agricultural settings. To fill this gap, we implemented a bioassay protocol where
109 we mimicked pesticide exposure in the wild by rearing filed-collected *Anopheles* larvae in
110 water containing field-realistic doses of chemicals. Using larval populations collected from
111 urban and rural areas of Yaoundé, we compared mortality within 24 h as well as some life
112 table parameters in order to infer the role of neonicotinoid contaminants in resistance
113 selection. Our findings indicated that resistance selection in *An. gambiae* is due to chronic
114 exposure to neonicotinoids in rural and agricultural settings and translates into low mortality
115 but also high survival, growth, pupation and emergence in lethal concentrations of pesticides.
116 The present study highlights concerns regarding the efficacy of some agrochemicals
117 repurposed for malaria vector control, especially active ingredients that are concomitantly
118 used in agricultural pest management.

119 **2. Material and methods**

120 **2.1 Study sites**

121 The study was carried out in Yaoundé, the capital of Cameroon, and some of its neighboring
122 rural areas. Approval to conduct a study in the Center region (N°: 1-

140/L/MINSANTE/SG/RDPH-Ce), ethical clearance (N°: 1-141/CRERSH/2020) and research permit (N°: 000133/MINRESI/B00/C00/C10/C13) were granted by the ministry of public health and the ministry of scientific research and innovation of Cameroon. We surveyed four sites which included a farming area located in the suburbs (Nkolondom, 3°56'43" N, 11°3'01" E), two densely urbanized neighborhoods (Etoa Meki, 3°52'53" N, 11°31'40" E and Combattant, 3°52'53" N, 11°31'40" E) and another suburban site (Nkolnkoumou, 3°52'29" N, 11°23'2" E) (Figure 1). The average distance between sites was 4-5 km. The most abundant malaria vector populations in this area belong to the species complex *An. gambiae sensu lato (s.l.)* (Antonio-Nkondjio et al., 2011; Kamdem et al., 2012a, 2012b). Previous surveys have revealed that the nominal species *An. gambiae sensu stricto* (hereafter *An. gambiae*) is the only member of the complex present in the agricultural site Nkolondom while its sibling species *An. coluzzii* prefers the most urbanized areas of Yaoundé (Bamou et al., 2019; Kamdem et al., 2012b; Tene-Fossog et al., 2022). Larvae from the semi-rural neighborhood, Nkolnkoumou, are a mixture of ~ 80% *An. gambiae* and 20% *An. coluzzii* (Ashu et al., 2023a, 2023b; Fouet et al., 2020). In rural and urban settings, larvae thrive in rain-dependent puddles, which may contain high concentrations of organic pollutants in some densely urbanized areas of the city (Antonio-Nkondjio et al., 2011). In the agricultural site Nkolondom, *An. gambiae* larvae occur in waters that accumulate between furrows and ridges in farms due to rain and/or irrigation and are likely contaminated with pesticide residues (Figure 1).

2.2 Larval collection and insecticides

Typical *Anopheles gambiae s.l.* breeding sites were inspected during rainy seasons in 2022 to collect larvae using the standard dipping method (Service, 1993). Larvae were transported in plastic containers to the insectary where they were immediately tested in a controlled room (27°C, 80% relative humidity, light:dark = 12:12 h).

148 Three neonicotinoids were tested in this study: in addition to acetamiprid and imidacloprid
149 that are used in local farming activities, we also tested clothianidin, which remains an exotic
150 insecticide in Cameroon. Deltamethrin, a first-generation malaria-control insecticide, was
151 used to compare the efficacy of clothianidin. The following commercial formulations were
152 tested: acetamiprid (Aceplant 40EC, 40g/L, emulsifiable concentrate, JACO, Yaoundé,
153 Cameroon), imidacloprid (Plantima 30SC, 30g/L, concentrated suspension, JACO, Yaoundé,
154 Cameroon), clothianidin (Pestanal^{TM}, analytical standard, Sigma-Aldrich) and deltamethrin
155 (Decis 25EC, 25 g/L, emulsifiable concentrate, Bayer Cropscience SL, Valencia, Spain).
156 Stock solutions were prepared by diluting the formulation in absolute ethanol or distilled
157 water.
158
159 **2.3 Larval bioassays**
160 In order to assess the lethal and sublethal effects of each insecticide, we reared larvae
161 collected from the field in water containing a dose of the insecticide that was lethal to larvae
162 of a susceptible strain and we measured mortality as well as some life history parameters. The
163 mosquito life cycle comprises four larval stages that precede pupation and emergence of a
164 male or female adult (Service, 1993). The four stages include the 1st (L1), 2nd (L2), 3rd (L3)
165 and 4th (L4) instars and can last between one and two weeks depending on the mosquito
166 species, the feeding regime and the environmental conditions. We monitored mortality and
167 life table parameters starting from 3rd instars until emergence. 3rd instar larvae of *Anopheles*
168 are abundant in the wild, are easy to identify morphologically and typically complete
169 transformation into L4, pupation and emergence in approximately 7 days.
170 The doses tested for each insecticide were determined as follows: we exposed 3rd instar larvae
171 of the susceptible strain *An. gambiae* Kisumu to increasing concentrations of the insecticide,
172 starting from 0.001 mg/L and we selected the lowest concentration at which 100% of

173 individuals died within 24 h. Batches of 25 larvae were placed in 500 mL plastic trays filled
174 with 200 mL borehole water containing the desired concentration of insecticide and covered
175 with a net. Four replicates were tested for each insecticide dose in addition to a control
176 without insecticide (i.e. containing only water). The lowest lethal concentrations were 0.035
177 mg/L clothianidin, 0.075 mg/L imidacloprid, 0.15 mg/L acetamiprid and 1.5 mg/L
178 deltamethrin. According to studies conducted worldwide, the concentrations of neonicotinoids
179 tested fell in the upper limits of average values detected in contaminated waters and soils
180 (Morrissey et al., 2015; Ramadevi et al., 2022; Schaafsma et al., 2015). However, in some
181 agricultural areas, these concentrations can be 100 times higher (Ramadevi et al., 2022).
182
183 Larvae were collected from the field in the morning and brought to the insectary. 3rd instars
184 were sorted immediately and rinse in a tray containing borehole before being transferred into
185 test trays. Four replicates of 25 larvae were tested in 500 mL plastic trays that were filled with
186 200 mL borehole water containing the above-determined lethal dose of the insecticide. Water
187 was not changed throughout the experiment, and 10 mg of TetraMin® fish food was added to
188 each tray daily. Every 24 h, the number of L3, L4, pupae and adults was counted in each tray.
189 Larvae were considered dead if they were unable to move when touched with a dropper. Dead
190 larvae were removed from the test containers and were not replaced. Adults were also
191 removed using a mouth aspirator. Survival, growth, pupation and emergence were assessed
192 daily for 7 days, which was sufficient for 3rd instar larvae to reach the adult stage.
193
194 **2.4 Data analysis**
195 The lethal endpoint of insecticide exposure was assessed using the mortality rate at 24 h. The
196 sublethal effects of the different insecticides were addressed using survival probability as well
197 as L4 transformation rate, pupation rate and emergence rate. L4 transformation rate was

198 defined as the percentage of L3 larvae transformed into L4. Pupation rate represented the
199 percentage of L3 that reached the pupa stage and emergence rate the percentage of L3 that
200 became adults. Line plots of mean and standard error, computed with the packages *plyr* and
201 *ggplot2* in R V. 4.2.2, were used to compare L4 transformation rate, pupation rate and
202 emergence rate at 24 h intervals. The ratio between the number of dead larvae and the initial
203 number of larvae was calculated every 24 h and provided an estimate of survival probability.
204 Kaplan Meier survival curves were plotted using the packages *ggplot 2* and *ggfortify* in R (R
205 Core Team, 2016). Larvae reaching the adult stage before the end of the test were treated as
206 censored data. Confidence intervals were computed for the four replicates, and a log-rank test
207 was used to determine if survival was significantly different between treatments.
208

209 **3. Results**

210 **3.1 Lethal toxicity in 24 h**

211 We first tested a gradient of insecticide doses on the laboratory strain *An. gambiae* Kisumu,
212 which is susceptible to common classes of insecticides used in mosquito control such as
213 pyrethroids, organophosphates, carbamates and organochlorines. The objective was to
214 determine a dose yielding 100% mortality in 24 h. We then used this dose as a discriminating
215 concentration to study the lethal and sublethal effects of neonicotinoids on field collected
216 *Anophleles* larvae. We compared the efficacy of neonicotinoids to that of deltamethrin. As
217 opposed to the susceptible strain, 100% mortality was not achieved with field populations
218 after 24 h (Figure 2). Mortality rates were very low in larvae of both *An. gambiae* and *An.*
219 *coluzzii* reared in water containing deltamethrin. Pyrethroid resistance is widespread in
220 anopheline mosquitoes, and parallel application of deltamethrin in malaria prevention and in
221 agricultural pest management has drastically reduced its efficacy against disease vectors. 24-h
222 mortality in water containing deltamethrin was typically below 30% and was not significantly

223 different between the four tested populations (Wilcoxon rank sum test, $p>0.05$). In contrast
224 with deltamethrin, susceptibility to neonicotinoids varied between species and geographic
225 areas. Consistent with findings obtained using adult bioassays (Ashu et al., 2023a; Fouet et
226 al., 2020), *An. coluzzii* larvae collected from the two urban neighborhoods, Combattant and
227 Etoa meki, were more susceptible, with mortality rates between 70-80%, except clothianidin
228 for which only about 50% of larvae were killed within 24 h. The species present in suburban
229 and rural areas of Yaoundé, *An. gambiae*, has developed strong resistance to neonicotinoids.
230 Particularly, larvae collected from the Nkolondom farm where pesticide spraying has selected
231 for resistance to acetamiprid and imidacloprid and conferred cross-resistance to clothianidin
232 (Ashu et al., 2023a; Fouet et al., 2020), displayed less than 10% mortality in water containing
233 a neonicotinoid. Mortality rate of larvae collected from the agricultural site was <10% in
234 water containing the new malaria-control chemical, clothianidin, or the first-generation
235 insecticide, deltamethrin, which suggested that both active ingredients currently have similar
236 efficacy against some *An. gambiae* populations.

237 **3.2 L4 transformation rate**

238 Some neonicotinoid insecticides act slowly and are also known to have a wide range of
239 sublethal effects in addition to acute toxicity on non-target species (Morrissey et al., 2015;
240 Schuhmann et al., 2022). Thus, beside lethal toxicity within the first 24 h of the experiment,
241 we used life table analysis to monitor some of the sublethal effects of neonicotinoids on
242 *Anopheles* larvae, starting with L4 transformation rate. The three neonicotinoids tested had
243 little effect on the ability of *An. gambiae* L3 larvae to become L4 (Figure 3A). The highest
244 transformation rate was observed in populations from the agricultural site, Nkolondom: 90 ±
245 3% in water containing acetamiprid followed by 75 ± 9% in imidacloprid in two days and 60
246 ± 3% in clothianidin after 3 days. Similarly, in Nkolnkoumou, a semi-rural site harboring ~
247 80% *An. gambiae*, transformation rates varied from 60 ± 6% for acetamiprid, to 35 ± 16%

248 and 23 ± 7% for imidacloprid and clothianidin, respectively after 2 to 4 days. Conversely,
249 even if based on 24-h mortality, *An. coluzzii* larvae showed a degree of tolerance, they were
250 strongly inhibited in water containing neonicotinoids leading to very low transformation rates
251 between 0 and 10% in both Etoa Meki and Combattant populations. In control tests,
252 transformation rates were 100% after 2 to 3 days.
253

254 **3.3 Pupation rate**

255 Pupation rate was consistent with L4 transformation and highlighted the low susceptibility of
256 agricultural and semi-rural populations of *An. gambiae* to neonicotinoids (Figure 3B). The
257 agricultural population of *An. gambiae* (Nkolondom) had the highest pupation rate, 77.5 ± 4%
258 in water containing acetamiprid, and 45 ± 11% in imidacloprid reflecting their tolerance to the
259 both neonicotinoid pesticides that are sprayed in local farms. The exotic insecticide,
260 clothianidin, at 0.035 mg/L had the most important inhibitory power among neonicotinoids,
261 but 15 to 20% of *An. gambiae* larvae were able to achieve pupation. *Anopheles coluzzii* larvae
262 had low pupation rates with less than 5% pupae obtained in all the neonicotinoids tested.
263 Notably, none of the *An. coluzzii* populations were able to pupate in clothianidin.100% pupae
264 were obtained in all the control tests after 72 h.
265

266 **3.4 Emergence rate**

267 100% of *An. gambiae* and *An. coluzzii* pupae emerged into adults in control water without
268 insecticide between the first and the sixth day in standard laboratory conditions. The total
269 emergence rate of *An. coluzzii* larvae was 0%, 2% and 4% in water containing clothianidin,
270 acetamiprid or imidacloprid, respectively after 7 days (Figure 3C). Meanwhile, at least half of
271 *An. gambiae* L3 larvae tested on average emerged between 2-7 days in acetamiprid and
272 imidacloprid. Only *An. gambiae* larvae from the agricultural site were able to emerge in

clothianidin albeit at lower rate (7 ± 3%) compared with the other neonicotinoids. However, emergence rate was also very low in deltamethrin, around 10%, indicating that for any insecticide, the ability to inhibit emergence can be compatible with strong resistance in adult mosquitoes and loss of efficacy of the insecticide. In addition to lethal toxicity in 24 h, emergence rate revealed another similarity between clothianidin, a new insecticide and deltamethrin whose effectiveness in malaria vector control has become very limited.

3.5 Survival probability

Rearing field-collected larvae in water containing an agricultural pesticide affected their survival probability as revealed by Kaplan Meier survival curves (Figure 4). Larvae that survived lethal toxicity within the first 24 h of the experiment were monitored for seven days. Although *An. coluzzii* showed potential to resist the 24-h lethal effect of some of the agricultural pesticides, larvae of this species had low survival probability in neonicotinoids. By contrast, *An. gambiae* populations from agricultural and semi-rural sites displayed high survival potential in water containing lethal doses of neonicotinoids. A log-rank test revealed that regardless of the neonicotinoid that was added to water, larvae collected from Nkolondom were super-resistant and had significantly higher survival probability compared with any of the three other populations tested ($p < 0.05$). 100% survival was obtained in all control experiments without insecticide.

4. Discussion

Here we have used an innovative approach to simultaneously assess the lethal and sublethal effects of pesticide exposure in mosquito larvae. We found that monitoring some sublethal effects via persistent exposure to chemicals in a controlled aquatic environment provides critical information on the level of tolerance of larval populations. Such information could be

complementary to routine quantification of mortality in adults via standard bioassays (CDC, 2012; World Health Organization, 2022b), and combining both approaches may offer a more robust framework to better evaluate pre-existing levels of adaptation to agrochemicals in malaria mosquitoes. We conducted a laboratory experiment mimicking the selection process, which is promoting the emergence of *Anopheles* populations with increased tolerance to neonicotinoids. While analyzing mortality and life table in wild larvae reared in artificial media containing a lethal dose of the active ingredient, we observed gradients of tolerance reflecting past exposure to neonicotinoids in nature. Control tests supported the validity of our experimental approach as 3rd instar larvae unexposed to insecticides showed standard survival, growth and emergence in controlled insectary conditions.

Previous studies have suggested that resistance to neonicotinoids observed in anopheline populations from some agricultural areas could be due to chronic exposure of larvae to sublethal doses of agricultural pesticides (Ashu et al., 2023a, 2023b; Fouet et al., 2020; Mouhamadou et al., 2019). Our findings provide the first experimental support to this hypothesis. Indeed, we found that *An. gambiae* larvae had exceptional fitness in field-realistic concentrations of acetamiprid and imidacloprid, two neonicotinoids whose formulations are extensively used in farming activities in Cameroon (Fouet et al., 2020). As anopheline populations have not yet been exposed to clothianidin in sub-Saharan Africa, this exotic neonicotinoid strongly inhibited larval emergence in both *An. gambiae* and *An. coluzzii*. However, likely due to cross-resistance selected by intensive exposure to acetamiprid and imidacloprid, larvae from the agricultural site, Nkolondom, were able to survive in water contaminated with clothianidin, albeit at lower frequency.

Exposure of non-target insects to sublethal doses of neonicotinoids affect functions such as motility, behavior, growth, fecundity and survival (Morrissey et al., 2015; Řezáč et al., 2021; Shepherd et al., 2021). Our study reveals that notwithstanding these sublethal effects whose

magnitude remains unknown, *An. gambiae* populations have evolved adaptation to chronic exposure to neonicotinoid residues in contaminated aquatic habitats. *An. gambiae* larvae from rural areas around Yaoundé exhibited high survivorship, L4 transformation, pupation and emergence rate in water containing neonicotinoids contrary to urban *An. coluzzii* mosquitoes whose growth and survival were inhibited. This result corroborates susceptibility profiles of adult populations: *An. coluzzii* from Yaoundé is more susceptible to neonicotinoids while *An. gambiae* is resistant to at least four different active ingredients (Ashu et al., 2023a, 2023b; Fouet et al., 2020). Larval populations whose adults were resistant to an agrochemical (i.e. mortality against the discriminating dose < 90%) typically displayed less than 50% mortality in 24 h and more than 5% emergence in 6 days in water containing a lethal dose of the active ingredient. Testing a larger number of chemicals on diverse *Anopheles* species and populations will provide more robust guidelines on the interpretation of mortality, growth and emergence of larvae reared in pesticide-laced water. However, based on the current study and complimentary evidence from adults testing (Ashu et al., 2023a, 2023b; Fouet et al., 2020), we could establish that any agrochemical yielding less that 50% mortality after 24 h and more than 5% emergence within 6 days is likely to have limited efficacy against *Anopheles* mosquitoes.

The first evidence of insecticide resistance driven by agricultural pesticide application dates back to the 1960s. Based on observations made in El Salvador, it was clearly established that aerial spraying of carbamates and organophosphates led to the development of broad-spectrum resistance to both insecticide classes in the vector *Anopheles albimanus* (Georghiou, 1972; Georghiou et al., 1973). Since then, a number of observational studies have argued that intensive use of crop protection chemicals is a major driver of resistance to public health insecticides in anopheline mosquitoes (Chouaïbou et al., 2016; Diabate et al., 2002; Müller et al., 2008; Nwane et al., 2009; Urio et al., 2022; Yadouleton et al., 2009). However, in the

348 majority of cases, it was difficult to determine the guilty part between agricultural chemicals
349 and malaria vector control insecticides. In a review of evidence, Lines (1988) (Lines, 1988)
350 proposed a set of conditions that should be met for a crop protection chemical to be the source
351 of insecticide resistance selection in malaria vectors. Appearance of resistance in mosquitoes
352 to a particular chemical should occur before it has been used for mosquito control; there
353 should be correlation in space and time, e.g. higher levels of resistance in areas with
354 agricultural spraying and seasonality of resistance overlapping with spraying periods; the
355 cross-resistance spectrum should show that the chemicals used in agriculture may confer
356 cross-resistance to public health insecticides and that both adults and larvae can be killed by
357 the agricultural pesticides. Agricultural application of neonicotinoids fits all of the criteria in
358 Sub-Saharan Africa and provides the best example of human activities accidentally
359 engineering insecticide resistance in mosquitoes.
360 In our study, both lethal and sublethal effects point to the existence of levels of resistance that
361 could be a major obstacle to using neonicotinoids in malaria mosquito control. We noted that
362 lethal toxicity in 24 h, pupation rate and emergence rate were very similar in water containing
363 either deltamethrin or clothianidin. This finding suggested that, just like deltamethrin, the
364 efficacy of clothianidin in *An. gambiae* might already be substantially reduced. In line with
365 this prediction, it has been shown that neonicotinoid resistant mosquitoes from Nkolondom
366 were significantly less susceptible to SumiShield® 50WG, a formulation of clothianidin
367 recently approved for indoor residual spraying (Fouet et al., 2020). Moreover, a laboratory
368 experiment recently demonstrated that exposure of *Anopheles* larvae to sublethal
369 concentrations of a mixture containing several herbicides, pesticides and fungicides resulted
370 in ~ 2.5 increase in tolerance to clothianidin and Fludora® Fusion, a formulation combining
371 clothianidin and deltamethrin (Zoh et al., 2022). These studies and ours highlight the

vulnerability of some agrochemicals repurposed for malaria vector control, as resistance driven by agricultural activities could become a major thread to their efficacy.

Although our findings are based on only two *Anopheles* species sampled from a relatively small geographic area, these results confirm concerns about the efficacy of neonicotinoids against malaria vectors (Ashu et al., 2023a; Fouet et al., 2020; Hoppé et al., 2016; Mouhamadou et al., 2019; Oxborough et al., 2019). Repurposing agrochemicals has thus far provided a rapid mechanism to identifying new candidate insecticides for malaria prevention. However, the case of neonicotinoids emphasizes the crucial role of evaluating prior exposure and cross-resistance in areas where mosquito larval populations are likely to be in contact with agricultural pesticides.

Author Contributions

MA: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft; CF: Conceptualization, Formal analysis, Investigation, Methodology; FA: Investigation, Methodology; VP-B, Resources, Supervision. CK: Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Writing – review & editing.

Funding

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

Informed Consent Statement

Not applicable.

Data Availability Statement

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

Conflicts of Interest

397 The authors declare that they have no competing interests.

398

399 **References**

400

401 Agossa, F.R., Padonou, G.G., Fassinou, A.J.Y.H., Odjo, E.M., Akuoko, O.K., Salako, A.,
402 Koukpo, Z.C., Nwangwu, U.C., Akinro, B., Sezonlin, M., Akogbeto, M.C., 2018. Small-
403 scale field evaluation of the efficacy and residual effect of Fludora® Fusion (mixture of
404 clothianidin and deltamethrin) against susceptible and resistant Anopheles gambiae
405 populations from Benin, West Africa. Malar. J. 17, 484. doi:10.1186/s12936-018-2633-6
406 Akpesse, A., 2018. Neonicotinoids and their impact on ecosystem service for agriculture and
407 biodiversity in Africa [WWW Document].
408 Antonio-Nkondjio, C., Fossog, B.T., Ndo, C., Djantio, B.M., Togouet, S.Z., Awono-Ambene,
409 P., Costantini, C., Wondji, C.S., Ranson, H., 2011. Anopheles gambiae distribution and
410 insecticide resistance in the cities of Douala and Yaoundé (Cameroon): influence of
411 urban agriculture and pollution. Malar. J. 10, 154. doi:10.1186/1475-2875-10-154
412 Antonio-Nkondjio, C., Tene Fossog, B., Kopya, E., Poumachu, Y., Menze Djantio, B., Ndo,
413 C., Tchuinkam, T., Awono-Ambene, P., Wondji, C.S., 2015. Rapid evolution of
414 pyrethroid resistance prevalence in Anopheles gambiae populations from the cities of
415 Douala and Yaoundé (Cameroon). Malar. J. 14, 1–9. doi:10.1186/s12936-015-0675-6
416 Ashu, F.A., Fouet, C., Ambadiang, M.M., Penlap-Beng, V., Kamdem, C., 2023a. Enhancing
417 the efficacy of neonicotinoids against mosquitoes and overcoming resistance issues.
418 bioRxiv. doi:https://doi.org/10.1101/2023.04.18.537427
419 Ashu, F.A., Fouet, C., Ambadiang, M.M., Penlap-Beng, V., Kamdem, C., 2023b. Vegetable
420 oil surfactants are synergists that can bias neonicotinoid susceptibility testing in adult
421 mosquitoes. bioRxiv. doi:https://doi.org/10.1101/2023.04.18.537421

422 Bamou, R., Sonhafouo-Chiana, N., Navridis, K., Tchuinkam, T., Wondji, C.S., Vontas, J.,
423 Antonio-Nkondjio, C., 2019. Status of Insecticide Resistance and Its Mechanisms in
424 Anopheles gambiae and Anopheles coluzzii Populations from Forest Settings in South
425 Cameroon. Genes (Basel). 10, doi:10.3390/genes10100741. doi:10.3390/genes10100741
426 CDC, 2012. Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle
427 Bioassay, CDC Methods.
428 Chouaïbou, M.S., Fodjo, B.K., Fokou, G., Allassane, O.F., Koudou, B.G., David, J.P.,
429 Antonio-Nkondjio, C., Ranson, H., Bonfoh, B., 2016. Influence of the agrochemicals
430 used for rice and vegetable cultivation on insecticide resistance in malaria vectors in
431 southern Côte d'Ivoire. Malar. J. 15, 1–12. doi:10.1186/s12936-016-1481-5
432 Curtis, G.F., Miller, J.E., Hodjati, M.H., Kolaczinski, J.H., Kasumba, I., 1998. Can anything
433 be done to maintain the effectiveness of pyrethroid-impregnated bednets against malaria
434 vectors? Philos. Trans. R. Soc. B Biol. Sci. 353, 1769–1775. doi:10.1098/rstb.1998.0329
435 Diabate, A., Baldet, T., Chandre, F., Akogbeto, M., Guiguemde, R.T., Darriet, F., Brengues,
436 C., Guillet, P., Hemingway, J., Small, G.J., Hougard, J.M., 2002. The Role Of
437 Agricultural Use Of Insecticides In Resistance To Pyrethroids In Anopheles gambiae s . l
438 . In Burkina Faso. Am. J. Trop. Med. Hyg. 67, 617–622.
439 Fouet, C., Ashu, F.A., Ambadiang, M.M., Tchapga, W., Wondji, C.S., Kamdem, C., 2020.
440 Resistance to clothianidin reduces the efficacy of SumiShield 50WG®, a neonicotinoid
441 formulation for indoor residual spraying, against Anopheles gambiae. bioRxiv.
442 doi:https://doi.org/10.1101/2020.08.06.239509
443 Georghiou, G.P., 1972. Studies on Resistance to Carbamate and Organophosphorus
444 Insecticides in Anopheles albimanus. Am. J. Trop. Med. Hyg. 21, 797–806.
445 Georghiou, G.P., Breeland, S.G., V. Ariaratnam, 1973. Seasonal escalation of
446 organophosphorus and carbamate resistance in Anophelesalbimanus by agricultural

447 sprays. Env. Entomol 2, 369–374.
448 Hemingway, J., 2017. The way forward for vector control. Science (80-.). 358, 998–999.
449 Hoppé, M., Hueter, O.F., Bywater, A., Wege, P., Maienfisch, P., 2016. Evaluation of
450 Commercial Agrochemicals as New Tools for Malaria Vector Control. Chimia (Aarau).
451 70, 721–729. doi:10.2533/chimia.2016.721
452 Kamdem, C., Fouet, C., Etouna, J., Etoa, F.-X., Simard, F., Besansky, N.J., Costantini, C.,
453 2012a. Spatially explicit analyses of anopheline mosquitoes indoor resting density:
454 implications for malaria control. PLoS One 7, e31843.
455 doi:10.1371/journal.pone.0031843
456 Kamdem, C., Fouet, C., Gamez, S., White, B.J., 2017. Pollutants and insecticides drive local
457 adaptation in African malaria mosquitoes. Mol. Biol. Evol. 34, 1261–1275. doi:DOI:
458 10.1093/molbev/msx087
459 Kamdem, C., Tene Fossog, B., Simard, F., Etouna, J., Ndo, C., Kengne, P., Boussès, P., Etoa,
460 F.-X., Awono-Ambene, P., Fontenille, D., Antonio-Nkondjio, C., Besansky, N.J.,
461 Costantini, C., 2012b. Anthropogenic Habitat Disturbance and Ecological Divergence
462 between Incipient Species of the Malaria Mosquito Anopheles gambiae. PLoS One.
463 doi:10.1371/journal.pone.0039453
464 Katambo, M., 2018. Neonicotinoids and their impact on ecosystem services in Tanzania
465 [WWW Document].
466 Kramer, L., 2022. Pesticide use for agriculture in Africa from 2000 to 2019 [WWW
467 Document]. doi:https://www.statista.com/statistics/1291464/pesticide-use-for-
468 agriculture-in-africa/
469 Kweka, E., Mahande, A., Ouma, J., Msangi, S., Karanja, W., Temba, V., Lyaruu, L.,
470 Himeidand, Y., 2018. Novel Indoor Residual Spray Insecticide With Extended Mortality
471 Effect : A Case of SumiShield 50WG Against Wild Resistant Populations of Anopheles

472 arabiensis in Northern Tanzania. Glob. Heal. Sci. Pract. 6, 758–765.
473 Lees, R., Praulins, G., Davies, R., Brown, F., Parsons, G., White, A., Ranson, H., Small, G.,
474 Malone, D., 2019. A testing cascade to identify repurposed insecticides for next-
475 generation vector control tools: Screening a panel of chemistries with novel modes of
476 action against a malaria vector. Gates Open Res. 3, 1–25.
477 doi:10.12688/gatesopenres.12957.2
478 Lines, J.D., 1988. Do agricultural insecticides select for insecticide resistance in mosquitoes?
479 A look at the evidence. Parasitol. Today 4. doi:10.1016/0169-4758(88)90083-X
480 Matsuda, K., Ihara, M., Sattelle, D.B., 2020. Neonicotinoid insecticides: Molecular targets,
481 resistance, and toxicity. Annu. Rev. Pharmacol. Toxicol. 60, 241–255.
482 doi:10.1146/annurev-pharmtox-010818-021747
483 Mattah, P.A.D., Futagbi, G., Amekudzi, L.K., Mattah, M.M., De Souza, D.K., Kartey-
484 Attipoe, W.D., Bimi, L., Wilson, M.D., 2017. Diversity in breeding sites and distribution
485 of Anopheles mosquitoes in selected urban areas of southern Ghana. Parasites and
486 Vectors 10, 1–15. doi:10.1186/s13071-016-1941-3
487 Morrissey, C.A., Mineau, P., Devries, J.H., Sanchez-Bayo, F., Liess, M., Cavallaro, M.C.,
488 Liber, K., 2015. Neonicotinoid contamination of global surface waters and associated
489 risk to aquatic invertebrates: A review. Environ. Int. 74, 291–303.
490 doi:10.1016/j.envint.2014.10.024
491 Mouhamadou, C.S., Souza, S.S. De, Fodjo, B.K., Zoh, M.G., Bli, N.K., Koudou, B.G., 2019.
492 Evidence of insecticide resistance selection in wild Anopheles coluzzii mosquitoes due
493 to agricultural pesticide use. Infect. Dis. Poverty 8, 1–8.
494 Müller, P., Chouaïbou, M., Pignatelli, P., Etang, J., Walker, E.D., Donnelly, M.J., Simard, F.,
495 Ranson, H., 2008. Pyrethroid tolerance is associated with elevated expression of
496 antioxidants and agricultural practice in Anopheles arabiensis sampled from an area of

497 cotton fields in Northern Cameroon. Mol. Ecol. 17, 1145–1155. doi:10.1111/j.1365-
498 294X.2007.03617.x
499 N'Guessan, R., Boko, P., Akogbeto, M., Yates, A., Rowland, M., 2007. Chlorfenapyr : A
500 pyrrole insecticide for the control of pyrethroid or DDT resistant Anopheles gambiae (
501 Diptera : Culicidae) mosquitoes. Acta Trop. 102, 69–78.
502 doi:10.1016/j.actatropica.2007.03.003
503 Ngamo Tinkeu, L., 2018. Current situation on the registration and use of neonicotinoids in
504 Cameroon [WWW Document].
505 Nwane, P., Etang, J., Chouaibou, M., Toto, J.C., Kerah-hinzoumbé, C., Mimpfoundi, R.,
506 Awono-ambene, H.P., Simard, F., 2009. Trends in DDT and pyrethroid resistance in
507 Anopheles gambiae s.s. populations from urban and agro-industrial settings in southern
508 Cameroon. BMC Infect. Dis. 9, doi:10.1186/1471-2334-9-163. doi:10.1186/1471-2334-
509 9-163
510 Okolle, N.J., Monono, E.Y., Tabikam, Amungwa Ivan Mambo, Stephania Kinge Magwell,
511 P.F.R., 2022. Insecticide Use and Application in Cameroon, in: Insecticides. IntechOpen.
512 Oxborough, R.M., Kitau, J., Matowo, J., Mndeme, R., Feston, E., Boko, P., A., O., Metonnou,
513 C.G., Irish, S., N'Guessan, R., Mosha, F.W., Rowland, M., 2010. Evaluation of indoor
514 residual spraying with the pyrrole insecticide chlorfenapyr against pyrethroid-susceptible
515 Anopheles arabiensis and pyrethroid-resistant Culex quinquefasciatus mosquitoes. Trans.
516 R. Soc. Trop. Med. Hyg. 104, 639–645. doi:10.1016/j.trstmh.2010.07.008
517 Oxborough, R.M., Seyoum, A., Yihdego, Y., Dabire, R., Gnanguenon, V., Wat, F., Agossa,
518 F.R., Yohannes, G., Coleman, S., Samdi, L.M., Diop, A., Faye, O., Magesa, S.,
519 Manjurano, A., Okia, M., Alyko, E., Masendu, H., Baber, I., Sovi, A., Rakotoson, J.,
520 Varela, K., Abong, B., Lucas, B., Fornadel, C., Dengela, D., 2019. Susceptibility testing
521 of Anopheles malaria vectors with the neonicotinoid insecticide clothianidin; results

522 from 16 African countries, in preparation for indoor residual spraying with new
523 insecticide formulations. Malar. J. 18, https://doi.org/10.1186/s12936-019-2888-6.
524 doi:10.1186/s12936-019-2888-6
525 Portwood, N.M., Mbewe, N.J., Tungu, P.K., Mlay, G., Small, G., Snetselaar, J., Kristan, M.,
526 Levy, P., Walker, T., Kirby, M.J., Kisinza, W., Mosha, F.W., Rowland, M., Messenger,
527 L.A., 2022. Multi-centre discriminating concentration determination of broflanilide and
528 potential for cross- resistance to other public health insecticides in Anopheles vector
529 populations. Sci. Rep. 1–10. doi:10.1038/s41598-022-26990-6
530 R Core Team, 2016. R: A language and environment for statistical computing.
531 Ramadevi, R., Ramachandraiah, C., Reddy, G.V.S., 2022. A Review on Contamination of
532 Soil and Water by Neonicotinoid Pesticides and Trends it's in Soil and Water Samples
533 with Chromatographic Analytical Techniques. Orient. J. Chem. 38, 259–267.
534 doi:10.13005/ojc/380205
535 Řezáč, M., Gloríková, N., Wilder, S.M., Heneberg, P., 2021. The sublethal effects of
536 neonicotinoids on spiders are independent of their nutritional status. Sci. Rep. 11, 1–11.
537 doi:10.1038/s41598-021-87935-z
538 Schaafsma, A., Limay-Rios, V., Baute, T., Smith, J., Xue, Y., 2015. Neonicotinoid insecticide
539 residues in surface water and soil associated with commercial maize (corn) fields in
540 Southwestern Ontario. PLoS One 10, 1–21. doi:10.1371/journal.pone.0118139
541 Schuhmann, A., Schmid, A.P., Manzer, S., Schulte, J., Scheiner, R., 2022. Interaction of
542 Insecticides and Fungicides in Bees. Front. Insect Sci. 1, 1–14.
543 doi:10.3389/finsc.2021.808335
544 Service, M.W., 1993. Mosquito ecology: field sampling methods, 2nd ed. Springer,
545 Netherlands. doi:10.1007/978-94-015-8113-4
546 Shepherd, S., Lima, M.A.P., Oliveira, E.E., Sharkh, S.M., Aonuma, H., Jackson, C.W.,

547 Newland, P.L., 2021. Sublethal neonicotinoid exposure attenuates the effects of
548 electromagnetic fields on honey bee flight and learning. Environ. Adv. 4, 100051.
549 doi:10.1016/j.envadv.2021.100051
550 Simon-delso, N., Amaral-rogers, V., Belzunces, L., Bonmatin, J.M., Chagnon, M., Downs, C.,
551 Furlan, L., Gibbons, D.W., Giorio, C., Girolami, V., 2015. Systemic insecticides
552 (neonicotinoids and fipronil): trends , uses , mode of action and metabolites. Environ.
553 Sci. Pollut. Res. 22, 5–34. doi:10.1007/s11356-014-3470-y
554 Tene-Fossog, B., Fotso-Toguem, Y.G., Amvongo-Adjia, N., Ranson, H., Wondji, C.S., 2022.
555 Temporal variation of high-level pyrethroid resistance in the major malaria vector
556 Anopheles gambiae s.l. in Yaoundé, Cameroon, is mediated by target-site and metabolic
557 resistance. Med. Vet. Entomol. 36, 247–259. doi:10.1111/mve.12577
558 Uragayala, S., Kamaraju, R., Tiwari, S.N., Sreedharan, S., Ghosh, S.K., Valecha, N., 2018.
559 Village-scale (Phase III) evaluation of the efficacy and residual activity of SumiShield
560 50® WG (Clothianidin 50 %, w/w) for indoor spraying for the control of pyrethroid-
561 resistant Anopheles culicifacies Giles in Karnataka state , India. Trop. Med. Int. Heal.
562 23, 605–615. doi:10.1111/tmi.13056
563 Urio, N.H., Pinda, P.G., Ngonzi, A.J., Muyaga, L.L., Msugupakulya, B.J., Finda, M.,
564 Matanila, G.S., Mponzi, W., Ngowo, H.S., Kahamba, N.F., Nkya, T.E., Okumu, F.O.,
565 2022. Effects of agricultural pesticides on the susceptibility and fitness of malaria vectors
566 in rural south-eastern Tanzania. Parasites and Vectors 15, 1–14. doi:10.1186/s13071-
567 022-05318-3
568 World Health Organization, 2023. List of WHO Prequalified Vector Control Products [WWW
569 Document]. URL https://extranet.who.int/pqweb/vector-control-products/prequalified-
570 product-list
571 World Health Organization, 2022a. World malaria report 2022. Geneva.

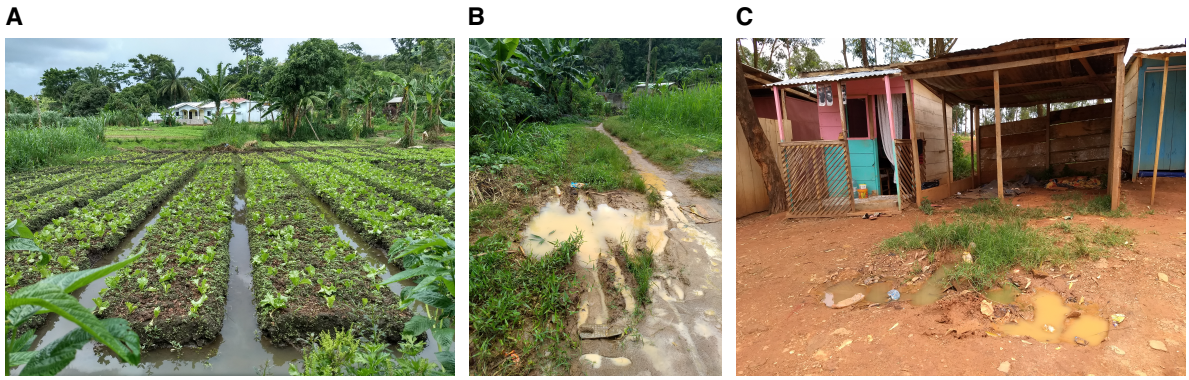
World Health Organization, 2022b. Standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO tube tests, World Health Organization.

Yadouleton, A.W.M., Asidi, A., Djouaka, R.F., Brama, J., Agossou, C.D., Akogbeto, M.C., 2009. Development of vegetable farming: A cause of the emergence of insecticide resistance in populations of Anopheles gambiae in urban areas of Benin. Malar. J. 8, 1–8. doi:10.1186/1475-2875-8-103

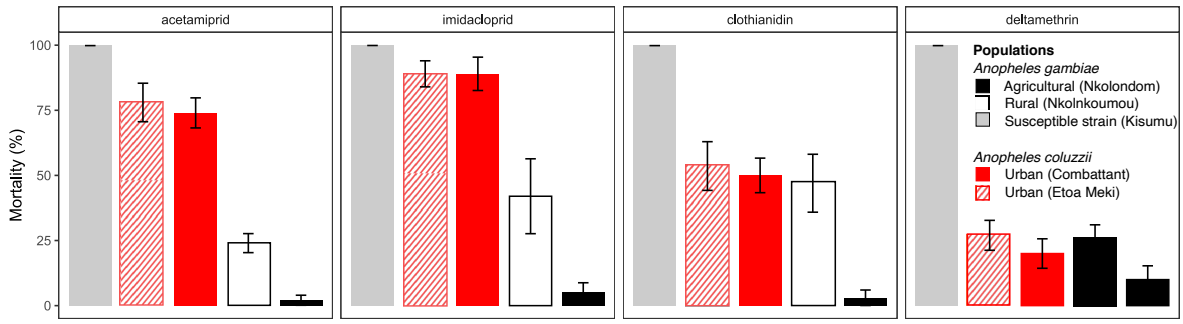
Zogo, B., Koffi, A.A., Alou, L.P.A., Fournet, F., Dahounto, A., Dabiré, R.K., Baba-Moussa, L., Moiroux, N., Pennetier, C., 2019. Identification and characterization of Anopheles spp. breeding habitats in the Korhogo area in northern Côte d'Ivoire: A study prior to a Bti-based larviciding intervention. Parasites and Vectors 12, 1–10. doi:10.1186/s13071-019-3404-0

Zoh, M.G., Tutagata, J., Fodjo, B.K., Mouhamadou, C.S., Sadia, C.G., McBeath, J., Schmitt, F., Horstmann, S., David, J.P., Reynaud, S., 2022. Exposure of Anopheles gambiae larvae to a sub-lethal dose of an agrochemical mixture induces tolerance to adulticides used in vector control management. Aquat. Toxicol. 248. doi:10.1016/j.aquatox.2022.106181

Figure legends



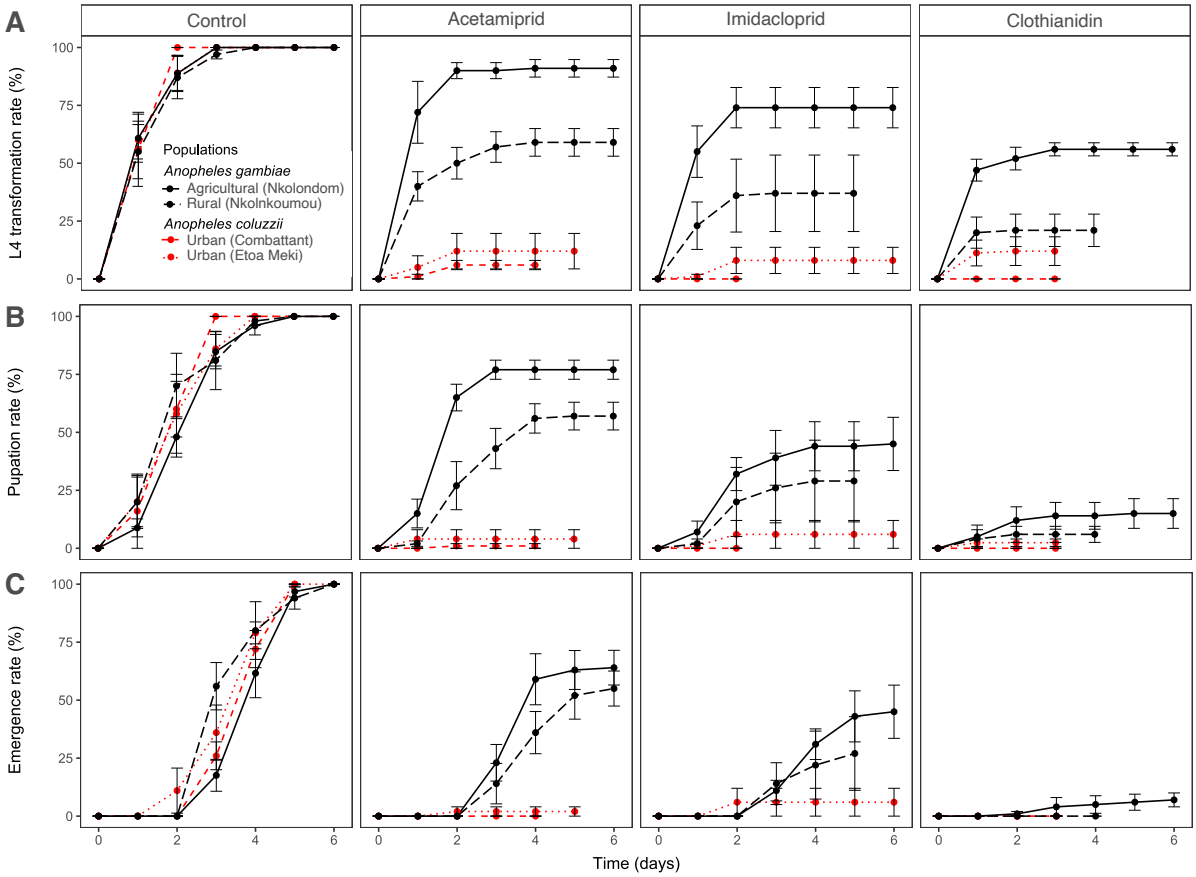
591 **Figure 1:** Examples of breeding sites where wild mosquito larvae were sampled. *An. gambiae*
592 larvae were collected from furrows between ridges in the agricultural site (A) or from puddles
593 created by human activities in Nkolnkoumou (suburban area) (B). *An. coluzzii* larvae were
594 sampled from human-made puddles in densely urbanized settings (C).



595

596 **Figure 2:** 24-h mortality of mosquito larvae reared in water containing a lethal dose of
597 agrochemical. 100% of larvae of the susceptible strain *An. gambiae* Kisumu were killed
598 within 24 h. Wild populations showed varying levels of tolerance to four insecticides.
599 Mortality values were lowest in larvae collected from the agricultural area (Nkolondom).
600 Error bars represent the standard error of the mean.

601



602

603 **Figure 3:** Sublethal effects of three neonicotinoids on life table parameters of *Anopheles*

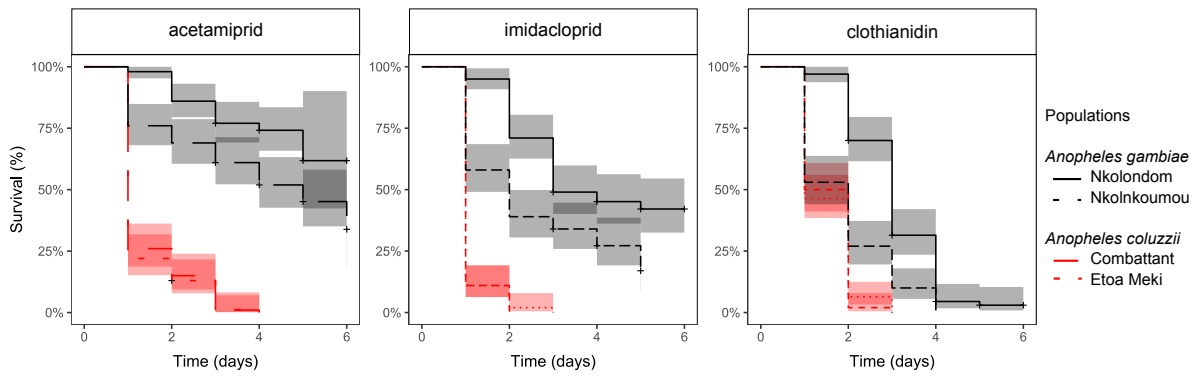
604 larvae. Rate of transformation of 3rd instars (L3) into 4th instars (L4) (A), pupation rate (B)

605 and emergence rate (C). Larvae that survived 24-h lethal toxicity were monitored for 7 days

606 under standard laboratory conditions. Controls were larvae reared in water without

607 insecticide. Vertical bars represent the standard error of the mean.

608



609

610 **Figure 4:** Kaplan Meier survival curves (lines) with 95% confidence intervals (colored bands)
611 of 3rd instar larvae reared in water containing a lethal dose of neonicotinoid. Survival
612 probabilities of four wild populations were compared under controlled laboratory conditions.
613 (+) indicates emergence that occurred before the end of the experiment and was treated as
614 censored data.
615
616
617
618
619
620