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### ► **To cite this version:**

Sassan Simplicie Kambou, Adeline Valente, Philip Agnew, Domonbabele François de Sales Hien, Rakiswendé Serge Yerbanga, et al.. Non-contact detection of pyrethroids widely used in vector control by *Anopheles* mosquitoes. 2024. hal-04619904

**HAL Id: hal-04619904**

**<https://hal.science/hal-04619904>**

Preprint submitted on 3 Jul 2024

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# 1 **Non-contact detection of pyrethroids widely used in vector control by *Anopheles* mosquitoes**

2

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11

## 12 **Abstract**

13 Pyrethroids are the most widely used insecticides to control vector borne diseases including

14 malaria. Physiological resistance mechanisms to these insecticides have been well described,

15 whereas those for behavioral resistance remain overlooked. Field data suggest the presence of

16 spatial sensory detection by *Anopheles* mosquitoes of the pyrethroid molecules used in

17 insecticide-based control tools, such as long-lasting insecticide nets or insecticide residual

18 spraying, opening the way to the emergence of a wide range of behavioral adaptations among

19 malaria vectors. However, the spatial sensory detection of these molecules is controversial and

20 needs to be demonstrated. The goal of this study was to behaviorally characterize the non-

21 contact detection of three of the most common pyrethroids used for malaria vector control:

22 permethrin, deltamethrin and  $\alpha$ -cypermethrin.

23 To reach this goal, we recorded the behavior (takeoff response) of *Anopheles gambiae*

24 pyrethroid-sensitive and resistant laboratory strains, as well as field collected mosquitoes from

25 the Gambiae complex, when exposed to the headspace of bottles containing different doses of

26 the insecticides at 25 and 35°C, in order to represent a range of laboratory and field  
27 temperatures.

28 We found the proportion of laboratory susceptible and resistant female mosquitoes that took off  
29 was, in all treatments, dose and the temperature dependent. Sensitive mosquitoes were  
30 significantly more prone to take off only in the presence of  $\alpha$ -cypermethrin, whereas sensitive  
31 and resistant mosquitoes showed similar responses to permethrin and deltamethrin. Field-  
32 collected mosquitoes of the Gambiae complex were also responsive to permethrin,  
33 independently of the species identity (*An. gambiae*, *An. coluzzi* and *An. arabiensis*) or their  
34 genotypes for the *kdr* mutation, known to confer resistance to pyrethroids.

35 The observed ability of *Anopheles* spp. mosquitoes to detect insecticides without contact could  
36 favor the evolution of behavioral modifications that may allow them to avoid or reduce the  
37 adverse effect of insecticides and thus, the development of behavioral resistance.

38

39 **Key words:** Spatial detection, pyrethroids, takeoff response, *Anopheles* mosquitoes

40

#### 41 **Introduction**

42 Insecticide-based vector control remains the main component for the prevention of malaria transmission.  
43 Since the beginning of the century, large scale implementation of insecticide treated nets (ITNs) and  
44 indoor residual spraying (IRS) has resulted in a significant reduction of malaria cases [1]. Pyrethroids  
45 are the most frequently and widely used class of insecticides to control malaria vector species  
46 (*Anopheles spp.*), due to their low toxicity shown in mammals and their great efficacy in reducing  
47 malaria transmission by decreasing both human-vector contact and mosquito longevity [2]. However,  
48 the extensive use of pyrethroids to control pest insects, including in agricultural practices, has favored  
49 the selection and spread of insecticide resistance within malaria mosquito populations [3–8]. Thanks to  
50 the continuous efforts made in the last decades, researchers have been deciphering the different  
51 mechanisms behind physiological insecticide resistance in mosquitoes, such as target site resistance

52 [4,9,10], metabolic resistance [4,11] or cuticular resistance [4,12,13]. Besides physiological resistance  
53 mechanisms, the emergence of behavioral resistance –i.e., behavioral adaptations that help mosquitoes  
54 to partially or completely overcome the deleterious effects of insecticides– have been recognized and  
55 may also play an important role as a mechanism reducing the efficacy of insecticide-based vector control  
56 tools [14,15]. While less documented than physiological resistance, several studies evidenced behavioral  
57 modifications in *Anopheles* species subsequent to the implementation of insecticide-based control  
58 methods in the field, as for instance: increased mosquito outdoor host-seeking (i.e. spatial avoidance)  
59 [16–18], host shifts (i.e. trophic avoidance) [18,19] or biting daily-rhythm shifts (i.e. temporal  
60 avoidance) [20–22]. Such behavioral modifications may result from the evolution of either constitutive  
61 behavioral resistance traits (i.e., selection of genetic behavioral variants over generations) or inducible  
62 behavioral resistance traits (i.e., phenotypic plasticity within a generation) [14]. The latter implies that  
63 mosquitoes are able to detect the insecticides through their sensory system and therefore modify their  
64 behavior accordingly.

65 Proofs of non-contact pyrethroid sensory detection have been deduced from the avoidance response  
66 mosquitoes display against certain pyrethroid molecules, particularly the so called “volatile/volatilized  
67 pyrethroids” commonly used as spatial repellents: e.g., metofluthrin, transfluthrin or prallethrin [23–  
68 26]. However, it is less clear whether other pyrethroids commonly used in IRS and ITNs, such as  
69 permethrin, deltamethrin or alpha-cypermethrin, may elicit a behavioral response in mosquitoes before  
70 contact (i.e., from a distance). According to their vapor pressure values at 25°C: e.g., permethrin =  $1.48$   
71  $\times 10^{-8}$  mm Hg, deltamethrin =  $9.32 \times 10^{-11}$  mm Hg and cypermethrin =  $2.5 \times 10^{-9}$  mm Hg [27], these  
72 pyrethroids are considered as semi- or non-volatile molecules, and such low-volatility is not in favor of  
73 their remote detection by mosquitoes [28,29]. Nevertheless, insecticides applied for vector control in  
74 malaria endemic regions are exposed to higher temperatures, e.g., 35°C–39 °C [30–32]. Such  
75 temperatures can increase the vapor pressure values of these compounds and thus their volatility, as  
76 predicted by the Clausius-Clapeyron equation. Field studies have provided indirect evidence for the  
77 potential spatial detection of these chemical compounds in mosquitoes. For instance, meta-analysis

78 studies showed that in experimental huts, malaria vector species (*Anopheles spp.*) movement (i.e.,  
79 entrance and exiting) was affected by the presence of ITNs in the huts [33,34]. Depending on the  
80 experimental treatments, the results unveiled both: a deterrent and an attractive effect, related to the  
81 presence of ITNs in the huts, suggesting that malaria vectors might be able to detect ITNs from a distance  
82 before entering the hut. Another study using a dual-choice olfactometer assay found that knock-down  
83 resistant (*kdr*) homozygous *An. gambiae* mosquitoes, thus resistant to pyrethroids, were more attracted  
84 by a host placed behind an ITN than an untreated net, while the presence of insecticide on the net did  
85 not affect the choice of susceptible mosquitoes [35]. In other mosquito genera, a recent study evidenced  
86 an avoidance response of *Culex quinquefasciatus* and *Aedes aegypti* to permethrin, deltamethrin and  $\lambda$ -  
87 cyhalothrin, but only when individuals had been previously exposed (by contact) to the molecules before  
88 the test [36]. Overall, these results showing either an attraction or an avoidance of pyrethroid compounds  
89 by mosquitoes implies the detection of these molecules from distance. Conversely, other studies  
90 investigating *Anopheles* mosquitoes-insecticide interactions did not find any sign of remote repellency  
91 in mosquitoes prior to contact [37–42] and the characterization of the distance for the detection of  
92 insecticides used in vector control by the targeted mosquitoes remains to be clarified.

93 This study aims to determine the non-contact detection of low volatile compounds (permethrin,  
94 deltamethrin and alpha-cypermethrin) in laboratory-reared susceptible and *kdr* resistant *An. gambiae* s.s.  
95 at two different temperatures, 25 °C and 35°C, that represent a range of realistic conditions. In addition,  
96 in order to generalize the results, we tested non-contact detection in wild caught *An. gambiae*,  
97 *An. coluzzii* and *An. arabiensis* mosquitoes.

## 98 **Material and methods**

### 99 **- Mosquito rearing**

100 Two reference strains of *An. gambiae sensu stricto* were used in this study. The first is the insecticide-  
101 susceptible reference strain Kisumu (hereafter called “kis” strain), which originates from a field  
102 collection in Kenya in 1953 [43]. The second is the pyrethroid resistant strain “kdrkis”, homozygous for  
103 the *kdr* mutation. This strain was obtained by introgression of the *kdr*-west allele, harboring the L1014F

104 mutation in the voltage-gated sodium channel gene into the Kisumu genome. The *kdr*-west allele was  
105 obtained from pyrethroid resistant mosquitoes sampled in Valley du Kou, Burkina Faso [44]. Both  
106 colonies were reared at the “Vectopôle” insectary at the “Institut de Recherche pour le Développement  
107 (IRD)” in Montpellier (France) and at the “Institut de Recherche en Sciences de la Santé (IRSS)” in  
108 Bobo-Dioulasso (Burkina Faso). Adult mosquitoes were maintained under standard insectary conditions  
109 (fed with 10% of honey solution, maintained at  $27 \pm 1$  °C and 70-80% relative humidity and under a  
110 12h:12h light:dark cycle). *An. gambiae sensu lato* L1 and L2 stage larvae were collected in the field at  
111 Soumousso (11° 00' 41" N, 4° 02' 50" W) in Burkina Faso. Larvae were transferred into tubes for  
112 transportation to the insectary at the IRSS and placed under the same conditions as the laboratory strains.  
113 Adult females (fed with sucrose 10%) aged from 4 to 8 days post-emergence were used for the  
114 behavioral assays. Species identity was determined for each female by PCR [45] after behavioral tests.  
115 Genotyping for *kdr* mutations known to confer resistance to pyrethroids (*kdr* West and *kdr* East) was  
116 carried out [44,46] as well as genotyping for Ace-1(R) mutation [47] known to confer resistance to  
117 carbamates and organophosphates.

118

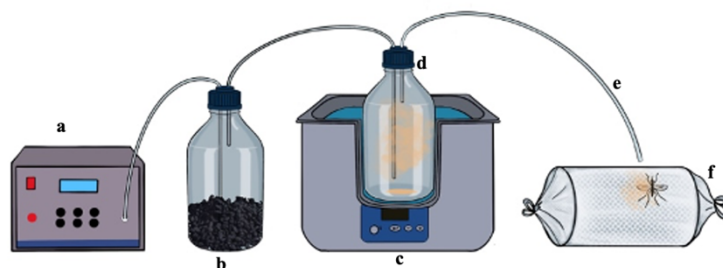
119 - **Chemical solutions used:**

120 Permethrin (Sigma-Aldrich, CAS #: 52645-53-1, product #: 45614, batch #: BCCF2182, purity: 95%),  
121 deltamethrin (Sigma-Aldrich, product #: 45423, batch #: BCCF1130, CAS #: 52918-63-5, purity:  
122 98.6%) and alpha-cypermethrin (Sigma-Aldrich, product #: 45806, batch #: BCCF16154, CAS #:  
123 67375-30-8, purity: 98.2%) stock solutions were prepared at 1mg/mL in acetone (Sigma-Aldrich,  
124 Product #: 650501, CAS #: 67-64-1, purity: 99.99%). In this study, the dose corresponds to the mass of  
125 insecticide in micrograms ( $\mu\text{g}$ ) placed into the test bottle during the experiment, as we let the acetone  
126 completely evaporate under a chemical hood from 5 min to 30 min depending on the dose. Four or five  
127 doses of pyrethroids were tested during the experiments: 4 $\mu\text{g}$ , 40 $\mu\text{g}$ , 400 $\mu\text{g}$ , 800 $\mu\text{g}$  (absent for  
128 permethrin) and 1600 $\mu\text{g}$ . Acetone was used as a negative control in the behavioral assays and it was  
129 treated in a similar manner to the insecticide samples.

130

131 - **Behavioral assay set-up description:**

132 In this behavioral assay, we determined whether adult female *An. gambiae s.l.* detected permethrin,  
133 deltamethrin and alpha-cypermethrin without contact based on their behavioral response (i.e., takeoff).  
134 Females were exposed to the headspace of varying doses of insecticides disposed at two different  
135 temperatures: 25°C and 35 °C. A set-up including a sample bottle containing the dose of pyrethroid to  
136 be tested was placed into a water bath to adjust the headspace temperature to either 25°C or 35°C (Figure  
137 1). Headspace temperature was measured using a digital thermometer (model t110, Testo AG,  
138 Germany). A 30s continuous charcoal-purified airflow (0.48L/min) at 0.16 cm<sup>2</sup>/s was circulated by  
139 means of a stimulus controller (CS-55, Syntech, Germany), through the sample bottle and directed by  
140 polytetrafluoroethylene (PTFE) tubing towards a single female mosquito confined in a cylindrical cage  
141 (10 × 20 cm) enclosed by mosquito netting. The end of the tube was manually placed (by an operator  
142 wearing laboratory coat and nitrile gloves) at approximately 1-2 cm from the female mosquito.  
143 Mosquitoes from either a laboratory colony or field collected individuals were tested individually at  
144 room temperature (25-27±1°C).  
145 Mosquito behavioral response was recorded as a binary variable (i.e., takeoff: yes/no) during the 30s  
146 maximum period of exposure to the airflow. For each replicate, 10 individuals were tested per treatment  
147 (insecticide *versus* control at both temperatures). Three to five replicates were carried out depending on  
148 the mosquitoes involved (i.e., the two laboratory strains and field mosquitoes). Assays involving  
149 permethrin on laboratory strains were carried out in Montpellier, France. Assays involving deltamethrin  
150 and alpha-cypermethrin on laboratory and field collected mosquitoes were carried out in Bobo  
151 Dioulasso, Burkina Faso.



152

153 **Figure 1:** Schematic set-up of the behavioral assay (a: stimulus controller; b: gas washing bottle  
154 containing activated charcoal; c: water bath; d: sample bottle for insecticide or control solution; e: PTFE  
155 tubing; f: cage containing one mosquito female).

156

#### 157 - **Statistical analysis**

158 All analyses were performed in R v.4.3.1. [48]. Initial analyses found that the proportion of females  
159 taking off in the control groups differed according to the insecticide test involved: 12.6% of mosquitoes  
160 in the control group took off during the assays testing the effect of permethrin, 29.6% in the control  
161 group during the assays for deltamethrin, and 32.5% in the control group during the assays for alpha-  
162 cypermethrin (see Supplementary materials Figure S1 a,b). These differences were temporally and  
163 physically confounded with the locations in which tests were made. Consequently, the data associated  
164 with each insecticide were analyzed separately.

165 Within the dataset for each insecticide, analyses found the proportion of females responding in the  
166 control treatments varied little among treatments or replicates and did not depend on the strain,  
167 temperature or dose treatments involved (see Supplementary materials Figure S2 a-c). Consequently,  
168 the control data within each insecticide dataset were pooled together to provide a single estimate for the  
169 proportion of mosquitoes responding in the control treatments of each dataset.

170 The proportion of mosquitoes responding to the control treatments in each dataset was used as a baseline  
171 against which the proportions of mosquitoes responding in the insecticide stimulus treatments were



172 compared (see Supplementary materials Figure S2 d-f). The ratio of the latter to the former estimates  
173 the relative risk of whether mosquitoes exposed to the insecticide stimulus were more or less likely to  
174 respond than those exposed to the control stimulus. Values of relative risk were calculated for the two  
175 temperature treatments exposed to the insecticide stimulus in each replicate. In Figure 2, these data are  
176 presented in terms of  $\log(\text{relative risk})$  where values greater than zero ( $> 0$ ) indicate mosquitoes were  
177 more likely to respond to the insecticide stimulus than to the control stimulus.

178 The  $\log(\text{relative risk})$  data were analyzed by linear regression models with the  $\log(\text{proportion responding}$   
179  $\text{in the insecticide stimulus treatment})$  as the dependent variable and  $\log(\text{proportion responding in the}$   
180  $\text{matching control stimulus treatment})$  as an offset term. For each dataset, the series of models started  
181 with a fully factorial model including all the interactions among the parameters; strain, temperature and  
182 dose. Subsequent models sequentially dropped one or more parameters until the null model was reached  
183 where only the intercept is estimated. These models were ranked according to Akaike's Information  
184 Criterion, AIC [49], so as to identify the 'best' model and 'near' models describing the data nearly as  
185 equally well as the 'best' model; that is models with an AIC within a value of two of the 'best' model  
186 [51]. As we were interested in the trend of mosquito responses with dose, rather than the response  
187 associated with any particular dose, we analyzed dose as a continuous parameter on the log-scale,  
188  $\log(\text{dose})$ . Full details of the models tested can be found in the supplementary materials.

189 Generalized linear models (GLM) for binomial data from the R package 'lme4' [52] were used to analyze  
190 the data from field collected mosquitoes. Two-way models with an interaction term compared the  
191 proportions of mosquitoes responding to a stimulus treatment (insecticide vs. control) and according to  
192 (i) their species, or (ii) their *kdr*-resistance genotype. Note that mosquitoes were genotyped for species  
193 and *kdr*-resistance after being behaviorally tested and it was not always possible to resolve both  
194 genotypes for each mosquito, resulting in unequal sample sizes across treatments in (i) and (ii).

195

196

197 **Results**

198 - ***An. gambiae* laboratory susceptible (kis) and resistant (kdrkis) strains' takeoff**  
199 **response after exposure to permethrin, deltamethrin and alpha-cypermethrin**

200 **headspace**

201 In this behavioral assay, we determined whether *An. gambiae* kis and kdrkis females detected permethrin  
202 at distance based on their response (takeoff) when exposed to an airflow downstream to the treatment  
203 stimulus (i.e., the headspace of a permethrin solution after evaporation of the solvent or the control  
204 solvent after evaporation), with regards to the dose of the insecticide and the temperature of the  
205 headspace.

206 The relative risk ratio comparing the response to permethrin headspace relative to the response to the  
207 control headspace is represented in figure 2a. Depending on the insecticide, the temperature and  
208 mosquito colony, the response of mosquitoes to the insecticide headspace stimulus was significantly  
209 greater than to the control stimulus, from the dose 4 or 40 $\mu$ g (95% confidence intervals zone not crossing  
210 the 0 axe).

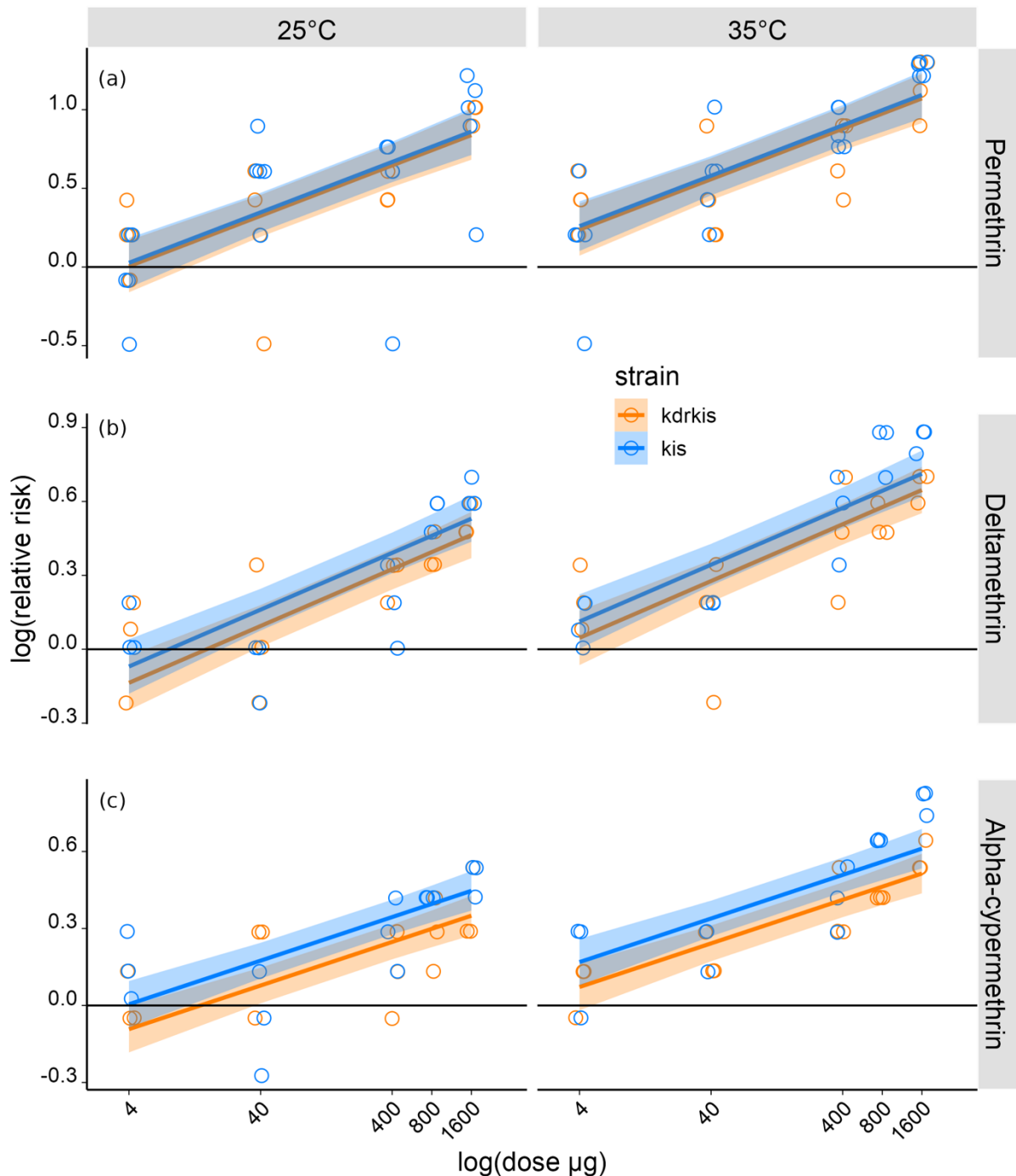
211 The proportion of susceptible and resistant female mosquitoes taking off in response to the permethrin  
212 headspace increased with temperature (25°C vs 35°C) ( $P < 0.001$ ) and dose (4; 40; 400 and 1600 $\mu$ g) ( $P$   
213  $< 0.001$ ), whereas the strain of mosquitoes had no effect ( $P=0.763$ ) (Figure 2a and Table Ia).

214 The same experiment was performed using deltamethrin as the stimulus treatment and following the  
215 same experimental conditions except that a supplementary dose of 800 $\mu$ g was tested. The proportion of  
216 *An. gambiae* taking off in the presence of the deltamethrin headspace increased with the dose ( $P < 0.001$ )  
217 and temperature ( $P < 0.001$ ) but did not depend on the strain ( $P=0.162$ ) (Figure 2b and Table Ib).

218 The same pattern was observed with alpha-cypermethrin headspace with an increased relative risk of  
219 taking off at the higher temperature ( $P < 0.001$ ) and as insecticide dose increased ( $P < 0.001$ ) (Figure 2c,  
220 Table Ic). In contrast to permethrin and deltamethrin, alpha-cypermethrin headspace induced different  
221 responses according to mosquito strains ( $P=0.012$ ) with the resistant mosquitoes (kdrkis) being less  
222 responsive than the susceptible mosquitoes (kis) (Table Ic).

223

224



225

226 **Figure 2:** Relative risk of mosquitoes taking off when exposed to three pyrethroids headspaces vs. their  
227 response to the headspace of the control stimulus (acetone). **a)** The relative risk of *An. gambiae* kis (50  
228 females tested per category, N=800) and kdrkis (40 females tested per category, N=640) taking off  
229 depending on dose of permethrin and temperature. **b)** The relative risk of *An. gambiae* kis (30 females  
230 tested per category, N=600) and kdrkis (30 females tested per category, N=600) taking off depending

231 on dose of deltamethrin and temperature. c) The relative risk of *An. gambiae* kis (30 females tested per  
232 category, N=600) and kdrkis (30 females tested per category, N=600) taking off depending on dose of  
233 alpha-cypermethrin and temperature. The Y-axis is on a log-scale. The colored bands are the 95%  
234 confidence intervals for the linear regression, when the lower interval does not cross the Y-axis at zero  
235 it indicates the response of mosquitoes to the insecticide stimulus was significantly greater than to the  
236 control stimulus.

237

238 **Table 1:** Analysis of variance (ANOVA) based on log(relative risk) of *An. gambiae* kis and kdrkis  
239 females taking off during exposure to the test bottle headspace at different doses of pyrethroids at  
240 25°C or 35°C.

241 P < 0.05 is considered as significant. Df= degrees of freedom; Sum.Sq= sums of squares; Mean.Sq=  
242 mean square; F.value= value of F distribution; Pr..F= probability value of F the statistic.

243

244 a) Permethrin

	Df	Sum.Sq	Mean.Sq	F.value	Pr..F
Strain	1	0.009	0.009	0.091	0.763
Temperature	1	0.976	0.976	10.099	0.002
log(dose)	1	7.069	7.069	73.169	< 0.001
Residuals	68	6.570	6.097		

245 b) Deltamethrin

	Df	Sum.Sq	Mean.Sq	F.value	Pr..F
Strain	1	0.066	0.066	2.011	0.162
Temperature	1	0.500	0.500	15.251	< 0.001
log(dose)	1	2.866	2.866	87.491	< 0.001
Residuals	56	1.835	0.033		

246

247 c) Alpha-cypermethrin

	Df	Sum.Sq	Mean.Sq	F.value	Pr..F
Strain	1	0.141	1.141	6.674	0.012
Temperature	1	0.405	0.405	19.141	< 0.001
log(dose)	1	1.598	1.598	75.636	< 0.001
Residuals	56	1.183	0.021		

248

249

250 - **Field *An. gambiae* sl takeoff response to permethrin exposure**

251 We tested the takeoff responses of *An. gambiae* sl field mosquitoes exposed to the test bottle headspace  
252 containing 1600µg of permethrin at 35°C. A total of 192 individuals were exposed to either a control  
253 stimulus or an insecticide stimulus of permethrin.

254 Subsequent identification of the species tested found 48 *An. arabiensis*, 93 *An. coluzzii*, 35 *An. gambiae*  
255 *ss* (Figure 3) and 16 individuals were excluded from the analysis as their species could not be identified.

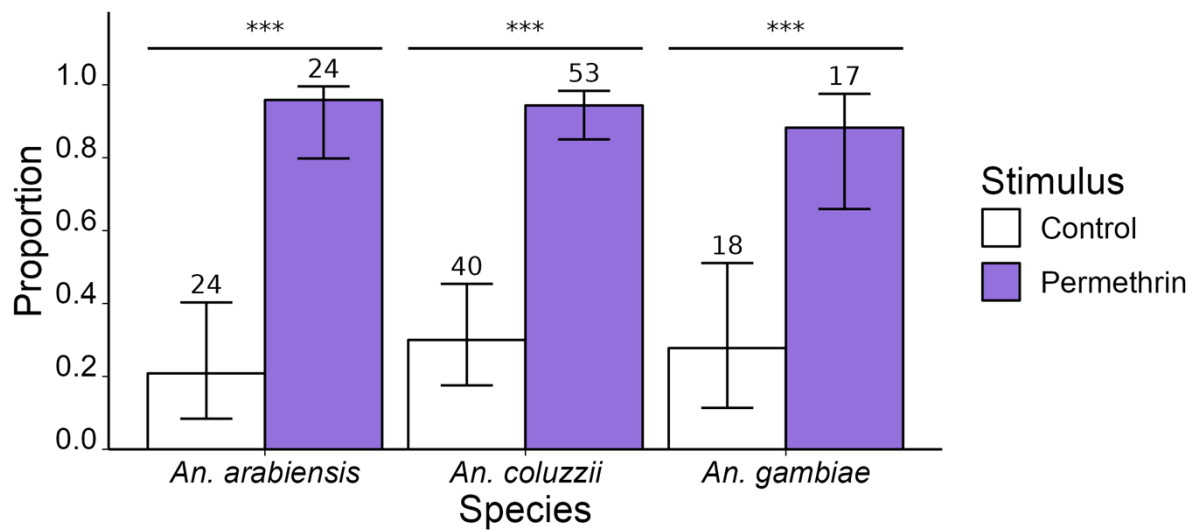
256 Field female mosquitoes were highly responsive to the presence of insecticide, with a significant  
257 increase of females taking off in the presence of permethrin headspace for the three species (Table 2).

258 Genotyping of *Ace1* and *kdr* alleles evidenced the presence of *Ace1*(R) as well as East and West *kdr*  
259 resistance alleles (Supp tables 1-3). Behavioral responses were analyzed only in regards to the *kdr*  
260 genotypes as they are related to the tested insecticides. For *kdr* resistant phenotypes, individuals were  
261 sequentially categorized as 'RR' if they were homozygous '*rr*' for either *kdr*-east or *kdr*-west, and then  
262 as 'RS' if one of the two loci were heterozygous '*rs*' for resistance. Individuals homozygous '*ss*' at both  
263 loci were categorized as 'SS'. Four individuals could not be categorized due to incomplete genotype data

264 (details in supplementary material table 4). Yet, the behavioral response (Figure 4) was tested with  
265 regard to the three categorized *kdr* phenotypes RR (63 individuals), conferring resistance to pyrethroids,  
266 RS (38 individuals), conferring partial resistance [53,54] and SS (87 individuals) *kdr* phenotype which  
267 is not conferring resistance to pyrethroids. The three categories showed a significant response to  
268 exposure to insecticide (Table 3).

269

270



271

272 **Figure 3:** Proportion of field collected mosquitoes of the Gambiae complex taking-off in response to  
 273 the test bottle headspace containing the compounds. The numbers written in the bar plot correspond to  
 274 the numbers of tested individuals. Statistically significant differences in the proportion of mosquitoes  
 275 taking off are noted by asterisks ('\*\*\*' =  $p < 0.001$ ).

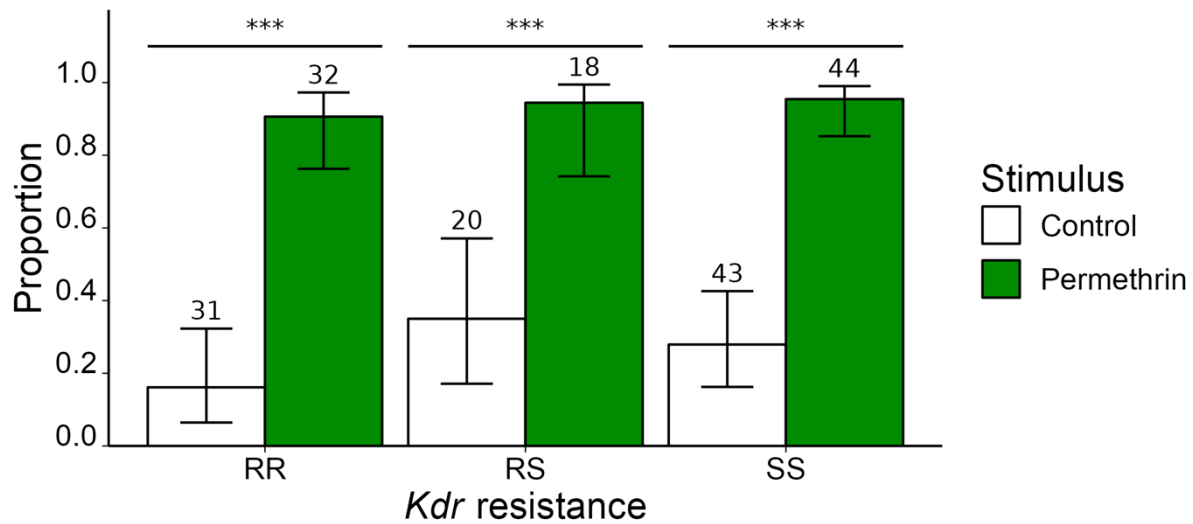
276

277 **Table 2:** Analysis of variance based on proportion of *Anopheles gambiae* *sl* mosquitoes responding to  
 278 permethrin/control headspace. Sum.Sq= sums of squares; Df= degrees of freedom; F.value= value of F  
 279 distribution.

	Sum.Sq	Df	F.values	Pr(>F)
Species	0.560	2	0.322	0.729
Stimulus	80.149	1	92.121	0.000
Species:Stimulus	1.107	2	0.636	0.541
Residuals	15.661	18		

280  $P < 0.05$  is considered as significant.

281



282

283

284 **Figure 4:** Proportion of field *An. gambiae sl* flying away in response to permethrin headspace in regards  
 285 to *kdr* genotypes. The numbers written in the bar plot correspond to the numbers of tested individuals.  
 286 Statistically significant differences of the proportion of mosquitoes taking off are noted by asterisks  
 287 ('\*\*\*' =  $p < 0.001$ ).

288

289 **Table 3:** Analysis of variance based on proportion of *Anopheles gambiae sl* mosquitoes *kdr* phenotypes  
 290 responding to permethrin/control headspaces. Sum.Sq= sums of squares; Df= degrees of freedom;  
 291 F.value= value of F distribution.

	Sum.Sq	Df	F.values	Pr(>F)
Kdr resistance	2.455	2	1.446	0.262
Stimulus	91.182	1	107.397	< 0.001
Interaction	0.242	2	0.142	0.868
Residuals	15.282	18		

292  $P < 0.05$  is considered as significant.

293

294 **Discussion**

295 This study provides evidence that female malaria vector mosquitoes are able to detect the most common  
296 pyrethroid molecules currently used in vector control (i.e., permethrin, deltamethrin and alpha-  
297 cypermethrin) without direct contact, regardless of the species or their *kdr* genotype. The response to  
298 exposure from a distance to the different insecticide headspaces was significantly driven by two  
299 parameters: compound dose and temperature.

300 We found a positive relationship between the dose of insecticide placed in a test bottle and the risk that  
301 a mosquito would take off relative to those in a matching control treatment. Depending on the nature of  
302 the insecticide and the temperature, mosquitoes displayed a sudden locomotor response (takeoff) even  
303 when exposed at the lowest doses tested. In this experiment, the amount of insecticide carried by the  
304 airflow reaching the mosquito was not quantified, thus it is difficult to extrapolate to the real conditions  
305 found during vector control implementation. Commercially available ITNs utilize greater amounts of  
306 insecticide compared to our study. The average surface of mosquito nets available on the market is  
307 approximately 17 m<sup>2</sup>, and for instance, Olyset<sup>®</sup> contains 1000mg/m<sup>2</sup> of permethrin, Permanet<sup>®</sup> 2.0  
308 contains 56mg/m<sup>2</sup> of deltamethrin, and 200 mg/m<sup>2</sup> of alpha-cypermethrin are used for the Interceptor<sup>®</sup>.  
309 Yet, ITN's are designed for a slow release of the compounds by incorporating them within the fibers,  
310 and the actual quantity volatilized at a given time is expected to be much lower than the total content  
311 values. Very few studies have measured the quantity of pyrethroid released from ITNs. One of the few  
312 studies available detected 0.021µg/m<sup>3</sup> of cyfluthrin in the close environment of a treated net [55].  
313 Another study, based on volatile collections inside deltamethrin impregnated nets reported that  
314 deltamethrin concentration vary between 0.041 µg/m<sup>3</sup> and 0.261 µg/m<sup>3</sup> in breathing zone and depending  
315 on whether the room air conditions were static or dynamic [56]. Regarding IRS, the recommended doses  
316 of insecticides spread on indoor walls are in between 20 and 30 mg/m<sup>2</sup> [57]. In this case, insecticides  
317 are not captured within the fibers, which suggests that a greater amount of insecticide will be  
318 bioavailable and potentially able to volatilize compared to those found on nets. Unfortunately, there are  
319 no studies, to our knowledge, that have quantified the insecticides present in the air after IRS  
320 implementation. The comparison between the quantity of volatilized insecticide in our experiments and  
321 the quantity present in natural conditions issued from common insecticide methods (ITN's and IRS)



322 remains to be investigated. In any case, the goal of this study was to find evidence for the possible  
323 detection of these insecticides by malaria mosquitoes without contact, and the dose-dependent  
324 behavioral response provides it.

325 The observed behavioral response to insecticide headspace exposure was also temperature-dependent.  
326 Higher response rates were observed when the insecticides were exposed at a temperature of 35°C,  
327 compared to 25°C. Despite the expected low volatility of the tested insecticides, increasing  
328 environmental temperature may facilitate the release of a higher number of molecules, as vapor pressure  
329 increases with temperature as estimated by the Clausius-Clapeyron equation. It is worth noting that the  
330 mosquitoes in the study were placed at laboratory temperature (25±1 °C) but exposed to an airflow  
331 passing through a water bath at either 25°C or 35°C. This higher temperature condition could potentially  
332 influence not only the volatility of the insecticide but also the mosquito's behavior itself. Interestingly,  
333 the probability of a behavioral response to the control airflow remained consistent across different  
334 temperatures (Figure S2). This means that temperature of the airflow alone did not significantly impact  
335 mosquito behavior. Instead, the insecticide-dependent behavioral responses may relate to the  
336 temperature of the mosquito itself after experiencing for some time warm air coming from the airflow.  
337 This aligns with an emerging body of research highlighting the effect of temperature on mosquito  
338 olfaction [58]. The two hypothesis of an increased volatility and a higher olfactory sensitivity of the  
339 mosquito at 35°C compared to 25°C are not mutually exclusive and remain to be investigated.

340 The irritancy effect of pyrethroids after contact has been previously shown to be significantly diminished  
341 in *kdr*-resistant mosquitoes [40,59]. In this experiment, the knock-down mutation did not modify the  
342 response rates of mosquitoes exposed to permethrin. However, insecticide-susceptible mosquitoes were  
343 significantly more prone to take flight when exposed to alpha-cypermethrin than their *kdr* resistant  
344 counterparts. Similar trends were observed when exposed to deltamethrin, though the results did not  
345 reach statistical significance. These findings suggest two phenomena: i) a differential behavioral effect  
346 depending on the insecticide used, which could be the result of insecticide-specific neurotoxic effects  
347 (i.e., sodium-channel hyper activation) on mosquitoes carrying a specific *kdr* mutation (L1014F, in this  
348 study); and ii) a sensory receptor-dependent detection mechanism which is not related to the insecticide

349 neurotoxic effect. Interestingly, recent studies in a phylogenetically distant mosquito species, *Aedes*  
350 *aegypti*, have shown that repellency to pyrethrum (i.e., a natural extract containing different pyrethrins,  
351 from which pyrethroids are inspired) and to some pyrethroids is given by a dual mechanism: through a  
352 specific olfactory receptor present in the antennae of mosquitoes and also by the activation of sodium  
353 channels [60–62]. These potential mechanisms, and their relative contributions, should also be  
354 investigated in *Anopheles* mosquitoes.

355 Expanding our investigation, we examined three major field-collected vector species of the Gambiae  
356 complex in Burkina Faso: *An. gambiae*, *An. coluzzii*, and *An. arabiensis*. Remarkably, all three species  
357 exhibited takeoff responses to permethrin, irrespective of their *kdr* resistance status. This suggests that  
358 the ability to detect this particular insecticide, and potentially other pyrethroids, may be a common  
359 characteristic within this complex of species and could be prevalent among most malaria vector  
360 populations in sub-Saharan Africa. However, to confirm the occurrence of this phenomenon, further  
361 research is required in distant Gambiae populations and in other major malaria vectors, such as *An.*  
362 *funestus* or *An. stephensi*.

363

## 364 **Conclusion**

365 This study not only provides evidence that malaria mosquitoes are able to detect insecticides without  
366 the need to get into direct contact, but unravels some of the variables that may influence such detection.  
367 The results contrast with previous experimental studies concluding the absence of distant detection by  
368 mosquitoes. The discrepancies between these results and our observations may be due to different  
369 experimental set up and/or how insecticide was presented to the mosquitoes. Nonetheless, the results of  
370 this study highlight that dose and temperature may be critical parameters for the spatial detection of  
371 insecticides. Such experimental evidence supporting the non-contact detection of permethrin,  
372 deltamethrin, and alpha-cypermethrin by malaria vectors deserves further exploration in natural  
373 conditions. The presence of non-contact detection in several species of the Gambiae complex opens the  
374 possibility to the potential evolution of a large range of behavioral adaptations against insecticides,

375 which may impede or reduce the efficacy of vector control interventions and lead to the evolution of  
376 behavioral resistance.

377

### 378 Acknowledgments

379 We thank Carole Ginibre and Bethsabée Scheid for mosquito rearing and Vectopole facility  
380 management.

381

### 382 Author contributions

383 Conceptualization: CP, AC, DC; Data curation: SSK, PA, DC; Formal analysis: SSK, PA, AC, DC;

384 Funding acquisition: CP, AC, DC; Investigation: SSK, AV; Methodology: SSK, AV, PA, AC, DC;

385 Project administration: DFSH, RSY, AC, DC; Visualization: SSK, DC; Validation: NM; Supervision:

386 RSY, KRD, CP, AC, DC; Writing - original draft: SSK, PA, AC, DC; Writing – review and editing:

387 SSK, AV, PA, DFSH, RSY, NM, KRD, CP, AC, DC.

388

### 389 Funding

390 This study was financed by the French National Research Agency – ANR (<https://anr.fr>), project

391 INDEEd (ANR-21-CE35-0021-01) granted to DC. The “Institut de Recherche pour le Développement”

392 through the program ARTS provided funding for SSK doctoral studies.

393

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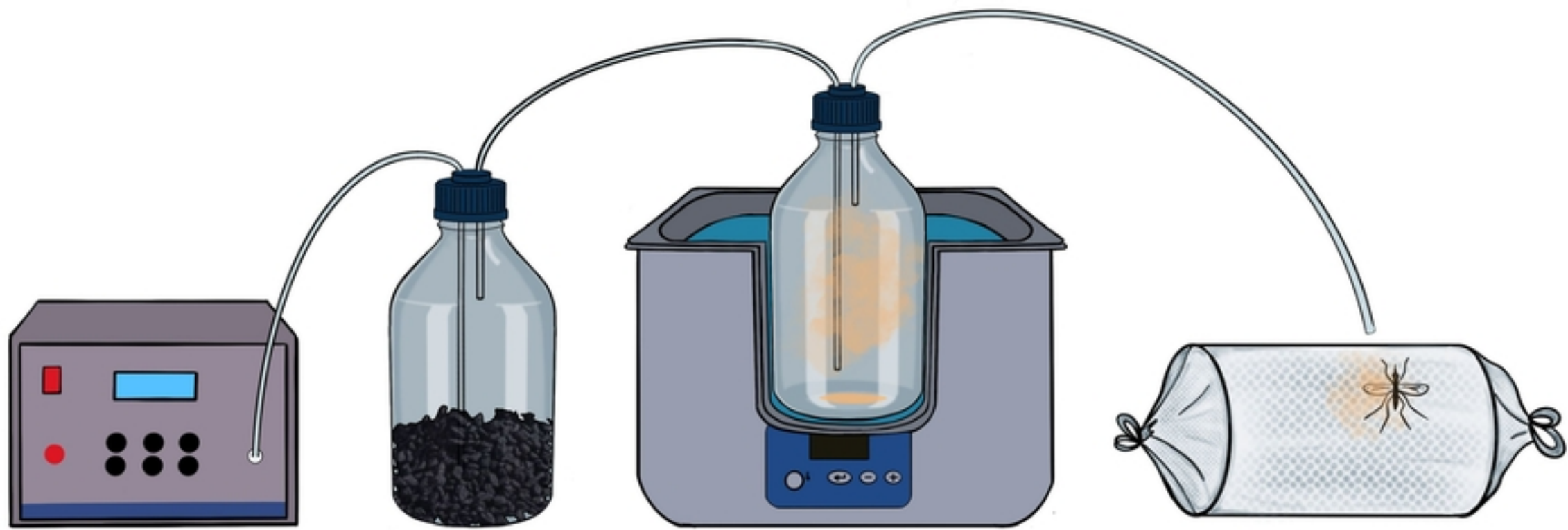
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25°C

35°C

