

1 **Behavioural response of *Culex quinquefasciatus* and *Culex pipiens mo-***
2 ***lestus* to avian odours and its reliance on carbon dioxide**

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14 **Response of *Culex* species to avian odours and carbon dioxide**

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26 **Abstract.** How *Culex* mosquitoes select and discriminate between potential avian
27 hosts is critical for understanding the epidemiology of WNV. To this end, we studied
28 the behavioural responses of *Culex quinquefasciatus* (Say) and *Culex pipiens molestus*
29 (Forsskål), to headspace volatiles of three avian species (chicken and pigeon, sexes an-
30 alysed separately, and magpie), presented either alone or in combination with 600 ppm
31 carbon dioxide (CO₂). The attraction of *Cx. quinquefasciatus* to the headspace volatiles
32 of both sexes of chicken, as well as to that of female pigeon, in combination with CO₂
33 was significantly higher compared to the CO₂ and solvent control. While *Cx. p. moles-*
34 *tus* were attracted to headspace volatiles of chickens and magpies, they were repelled
35 by that of female pigeons. A synergistic effect between the avian volatiles and CO₂ was
36 observed for *Cx. quinquefasciatus*, whereas the addition of CO₂ had no effect on the
37 attraction of females *Cx. p. molestus*. The results of this study demonstrate that *Cx.*
38 *quinquefasciatus* and *Cx. p. molestus* are attracted to the odour of potential avian hosts.
39 Future studies aimed at identifying the bioactive volatile compounds in the headspace
40 of chickens may contribute to the potential development of effective surveillance and
41 control tools against *Culex* species.

42

43 **Key words.** Attraction, chicken, magpie, mosquitoes, pigeon, volatiles

44

45 **Introduction**

46

47 In both Europe and North America, mosquitoes of the *Culex pipiens* species complex
48 are considered as the primary bridge vectors of the West Nile Virus (WNV) (Brugman
49 *et al.*, 2018). The WNV causes sporadic outbreaks of neuroinvasive disease on a global
50 scale, with Europe and North America being most affected in recent decades (Chancey

51 *et al.*, 2015; Brugman *et al.*, 2018). Infections are predominantly asymptomatic, alt-
52 hough 20–25% of infected individuals develop mild illness known as West Nile Fever
53 (WNF), and almost 1% becomes neuroinvasive and debilitating, resulting in up to 100
54 casualties per year in the most affected regions (Chancey *et al.*, 2015). The virus is
55 maintained in a mosquito–bird–mosquito transmission cycle, with birds acting as res-
56 ervoir hosts (Chancey *et al.*, 2015). While results of blood meal analyses and field stud-
57 ies using live avian hosts have contributed to the understanding of the host range of
58 *Culex pipiens* (L.) (Apperson *et al.*, 2004; Gomes *et al.*, 2013; Börstler *et al.*, 2016;
59 Llopis *et al.*, 2016), the mechanisms underlying host selection and preference in this
60 important vector species complex remain to be elucidated. This information is crucial
61 for the development of evidence–based strategies for WNV vector monitoring and con-
62 trol.

63 The following species are recognized as members of the *Cx. pipiens* species com-
64 plex: *Cx. pipiens*, *Culex quinquefasciatus* (Say), *Culex australicus* (Dobrotworsky &
65 Drummond), and *Culex globocoxitus* (Dobrotworsky) (all: Diptera: Culicidae) (Har-
66 bach, 2011). *Culex pipiens* and *Cx. quinquefasciatus*, also referred to as the Northern
67 and Southern house mosquito, are the main vectors of WNV in Europe and North Amer-
68 ica, respectively, but occur in urban areas globally, often closely associated with hu-
69 mans (Zeller & Schuffenecker, 2004). *Culex pipiens* has two recognized forms,
70 ‘pipiens’ and ‘molestus’, which differ in behaviour and physiology as a consequence
71 of rapid ecological adaptation to rural open–air habitats and urban enclosed habitats,
72 respectively (Barr, 1981; Harbach, 2011). While ‘pipiens’ is anautogenous and predom-
73 inantly ornithophilic, ‘molestus’ is autogenous and anthropophilic (Vinogradova,
74 2000). However, both forms demonstrate considerable phenotypic plasticity in their
75 feeding behaviour (Kilpatrick *et al.*, 2006; Simpson *et al.*, 2011; Takken & Verhulst,

76 2013). The host preference of the cosmopolitan anautogenous *Cx. quinquefasciatus* var-
77 ies according to ecotype, and has been reported as anthropophilic, mammalophilic and
78 ornithophilic (Beier *et al.*, 1990; Mboera & Takken 1999; Molaei *et al.*, 2006; Takken
79 & Verhulst, 2013).

80 The host preference of *Culex* mosquitoes plays a significant role in determining hu-
81 man risk of infection with WNV. Knowledge of vector feeding preferences, in combi-
82 nation with information on relative host and vector abundance, host behaviour and viral
83 load, is crucial to identify which host species may be the most important reservoirs
84 contributing to the spread of WNV (Kilpatrick *et al.*, 2006; Rizzoli *et al.*, 2015). Most
85 *Culex* species display a preference for feeding on birds, although they may switch to
86 humans and other mammals when birds are not available, e.g. during migration (Kil-
87 patrick *et al.*, 2006; Simpson *et al.*, 2011). In Europe, blood meal analyses have identi-
88 fied various avian species of the orders Passeriformes, Galliformes and Columbiformes
89 as hosts of *Culex* spp. (Brugman *et al.*, 2018). Among these, magpies (*Pica pica*,
90 Passeriformes: Corvidae), collared doves (*Streptopelia decaocto*, Columbiformes:
91 Columbidae), chickens (*Gallus gallus domesticus*, Galliformes: Phasianidae), house
92 sparrows (*Passer domesticus*, Passeriformes: Passeridae) and blackbirds (*Turdus mer-
93 ula*, Passeriformes: Turdidae) have been identified as the species most frequently bitten
94 by *Cx. pipiens* (Roiz *et al.*, 2012; Gomes *et al.*, 2013; Rizzoli *et al.*, 2015; Börstler *et
95 al.*, 2016). *Culex pipiens* mosquitoes in North America also feed on a range of avian
96 species, including members of the same orders as used as hosts in Europe (Apperson *et
97 al.*, 2004; Molaei *et al.*, 2006; Simpson *et al.*, 2009). While most studies have focused
98 on identifying the preferred host species of *Cx. pipiens* mosquitoes, others have also
99 revealed that *Cx. pipiens* actively avoid some species of birds (Pickett & Woodcock,

100 1996; Leal *et al.*, 2017), indicating that they can differentiate between potential host
101 species.

102 Mosquito host selection is heavily influenced by chemical cues emanating from ver-
103 tebrates (Takken & Verhulst, 2013). Initial recognition of a potential host by mosqui-
104 toes generally relies on carbon dioxide (CO₂), a cue emitted in exhaled breath of all
105 vertebrates, which elicits activation and attraction, as well as gates the response to other
106 host odours (Gillies, 1980). Carbon dioxide sensing in *Culex* mosquitoes is acute at the
107 low concentrations of CO₂ found in the exhaled breath of birds (Majeed *et al.*, 2017).
108 As a result, attraction at these low levels only appears to occur over short distances
109 (Gillies & Wilkes, 1974). Moreover, the behavioural response of *Culex* mosquitoes to
110 CO₂ appears to be species, and context, dependent, resulting in either no attraction or a
111 low level of attraction when CO₂ is presented alone (Gillies & Wilkes 1974; Mboera *et*
112 *al.*, 1998). Available data indicate that *Culex* mosquitoes use odours emanating from
113 feathers (Allan *et al.*, 2006), as well as from the uropygial glands (Russell & Hunter,
114 2005; Allan *et al.*, 2006) for host selection. Although attraction to avian-derived
115 odours, in the presence or absence of CO₂, has been demonstrated in *Culex* mosquitoes,
116 responses to avian cues have received considerably less attention than responses to
117 mammalian and especially human-derived kairomones (Bernier *et al.*, 2008; Cooper-
118 band *et al.*, 2008; Allan *et al.*, 2006; Syed & Leal, 2009; Majeed *et al.*, 2016).

119 The objective of this work was to study behavioural responses of *Cx. quinquefasci-*
120 *atus* and *Cx. p. molestus* to odours from chickens, pigeons and magpies, in the presence
121 and absence of CO₂, as well as to CO₂ alone.

122

123 **Materials and Methods**

124

125 *Mosquito cultures*

126 *Culex quinquefasciatus* (Thai strain) and *Cx. p. molestus* were reared under standard
127 culture conditions (27 ± 2 °C, $70 \pm 2\%$ relative humidity (RH), 12 : 12 h light : dark
128 (L:D) photoperiod. Adults of both species were kept in BugDorm cages (30 X 30 X 30
129 cm; Mega View Science, Taiwan) with ad libitum access to 10% sucrose through a
130 filter paper wick. Female *Cx. quinquefasciatus* were fed on defibrinated sheep blood
131 weekly, as described by Hill *et al.* (2009). Larvae, kept in groups of < 500 per tray,
132 were reared in plastic trays (20 X 30 X 10 cm) filled with distilled water, and fed with
133 fish food (SuperVit-8Mix-Tropical, VPG Sweden AB). Pupae were collected daily in
134 20 ml containers and transferred to the adult cages. Non–blood fed female *Cx. quinque-*
135 *fasciatus*, 4–5 days post–adult emergence, were used for the behavioural assays. *Culex*
136 *p. molestus* mosquitoes originating from a colony in the laboratory of Benaki Phyto-
137 pathological Institute, Kifissia, Greece, were reared under the same conditions as *Cx.*
138 *quinquefasciatus* colony. Due to the autogenous nature of female *Cx. p. molestus*, fe-
139 males of this species were first allowed to oviposit and then used for behavioural assays,
140 8–10 days post–adult emergence. Prior to the behavioural experiments, females of both
141 species were starved for 8 h, and only given access to water. Two hours before the
142 experiments, females were transferred to the behavioural assay room to acclimatize. All
143 behavioural experiments were conducted during the peak of host seeking in early
144 scotophase (Zeitgeber time 15 ± 2 h) (Yee & Foster, 1992).

145

146 *Volatile collections from birds*

147 Headspace volatile extracts were collected, in the field, from live domestic chickens
148 (*G. g. domesticus*), semi–wild pigeons (*Columba livia*, Columbiformes: Columbidae)
149 and wild magpies (*P. pica*) from the region of Central Macedonia, Northern Greece.

150 Permission to capture and handle birds for 24 h was provided by the Greek Ministry of
151 Environment and Energy (Department of Management of Wildlife and Hunting, Ath-
152 ens: 17-4-2013 / 129493/1072), in compliance with Directive 2010/63/EU in Europe.
153 Magpies were captured using a Multi Catch Larsen Trap (Hunting Federation of Mac-
154 edonia and Thrace), consisting of four catching compartments around its edge and a
155 compartment in the middle baited with food and water (Tsachalidis *et al.*, 2006). Pi-
156 geons were provided by a local pigeon breeder, and held in a pigeonhole during the
157 night and released during the day. Chickens were provided by an organic poultry farm.
158 In total, 13-16 individuals of each species were used. Each individual bird was sexed
159 with the exception of *P. pica*, where sex can only be determined post mortem.

160 Captured birds were placed individually in a plastic cage (48 X 28 X 28 cm), covered
161 with a heat-sealed oven bag (0.04 m³) (FoodSaver® Brand, UK) for headspace collec-
162 tion. Charcoal filtered air was introduced into the cage via a pump (12 V, KNF-Neu-
163 berger, Freiburg, Germany) through a Teflon tube at 1 l min⁻¹. The air was then ex-
164 tracted from the cage, at 1 l min⁻¹, through a splitter connected to three adsorbent Teflon
165 columns (4 x 0.4 cm i.d.), containing 40 mg Porapak Q (80/100 mesh, Alltech, Deer-
166 field, IL, USA), for 2.5 h. Directly following the completion of the headspace volatile
167 collection, the birds were released back into the wild or returned to the farm. Trapped
168 volatiles were eluted with 600 µl of pentane (puriss p.a., Sigma-Aldrich Chemie
169 GmbH, Steinheim, Germany). Before use, the columns were rinsed with 1 ml each of
170 methanol, acetone, and pentane. The extracts were pooled by avian species and sex and
171 then concentrated under a gentle air stream for 6-8 h to approximately half of the orig-
172 inal volume, for use in further experiments.

173

174 *Attraction of Cx. quinquefasciatus to avian volatiles*

175 Attraction of female *Cx. quinquefasciatus* to different avian headspace volatiles ex-
176 tracts, in the presence or absence of CO₂, were assessed in a flight tube bioassay, illu-
177 minated from above at 40 lux, as previously described (Majeed *et al.*, 2014). In brief, a
178 charcoal-filtered and humidified air stream (25 ± 2 °C, $65 \pm 2\%$ RH) flowed through
179 the bioassay at 30 cm s⁻¹. Where required, CO₂ was delivered in one second on-off
180 pulses at 600 ppm via a stimulus controller (SEC-2/b, Syntech) (Majeed *et al.*, 2014).
181 The consistency in amplitude and the structure of the pulsed stimuli was visualized
182 using a mini-PID (Aurora Scientific, Aurora, Ontario, Canada), and the concentration
183 of CO₂ was measured at the downwind and upwind end of the assay via a CO₂ analyser
184 (LI-820, LICOR Biosciences, Lincoln, NE, USA) (Majeed *et al.*, 2014). The pooled
185 headspace extracts of each of the avian species, divided by sex for chickens and pi-
186 geons, were diluted in half-decadic steps from the concentrated headspace volatile col-
187 lection in pentane. The diluted extract was pipetted (100 µl) onto a piece of filter paper
188 (55 mm, Munktell, Ahlstrom-Munksjo), which was suspended from a 5 cm wire coil
189 at the upwind end of the assay. The amount of headspace applied on the filter paper
190 corresponded to 0.5, 1.7, 5.2, 17 and 52 min release equivalents of the avian headspace
191 extracts. Pentane, at an equivalent amount, tested with or without CO₂, served as a con-
192 trol.

193 Two hours prior to the experiments, females were transferred in groups of three to
194 custom-made release cages (Wondwosen *et al.*, 2018). Cages were then placed in the
195 downwind end of the assay, where the insects were allowed 3 min to adapt, before the
196 butterfly valve of the cage was opened for their release. Thereafter, in the first round of
197 experiments, source contact of each mosquito (attraction) was observed for a maximum
198 of 4 minutes. Ten replicates were conducted at each of the five release rates tested (3
199 individuals per replicate per treatment, 30 individuals in total), with equal number of

200 experimental (CO₂ + extract) and control (CO₂ + pentane) flights performed separately
201 each day. A second round of experiments was conducted in order to investigate the role
202 of CO₂ on the attraction of *Cx. quinquefasciatus* to avian volatiles. For this, only the
203 release rate eliciting the highest level of attraction was tested, in the presence or absence
204 of CO₂ (5.2 min equivalents for male chicken headspace volatile extract; 17 min equiv-
205 alents for female chicken, male pigeon and magpie headspace volatile extracts; and 52
206 min equivalents for female pigeon headspace volatile extract). Treatments were pre-
207 sented in the same manner as in the first round of experiments.

208

209 *Attraction of Cx. p. molestus to avian volatiles*

210 Due to the high spontaneous flight activity of *Cx. p. molestus*, a Y-tube olfactometer
211 (Zaspel *et al.*, 2016) was used to assess the behavioural response of this species to the
212 different avian headspace volatiles extracts. The conditioning of the airstream was the
213 same as described for the wind tube bioassay above, with the odour stimulus, in the
214 presence or absence of CO₂, provided in one of the arms of the olfactometer. The other
215 arm contained no stimulus or pentane. Treatments and controls were provided in the
216 same manner as for *Cx. quinquefasciatus*. An attraction Preference Index (PI) was cal-
217 culated, $(T - C)/(T + C)$, where T is the number of mosquitoes associated with the test
218 odour, and C the number of mosquitoes associated with the control. The use of the term
219 attraction in this study refers to an increased number of mosquitoes that make source
220 contact within the treatment arm in the olfactometer compared with the control.

221 Mosquitoes were conditioned as described above prior to the experiments, but due
222 to their high spontaneous flight behaviour only allowed 2 minutes to make a choice.
223 Ten replicates were conducted for each treatment (3 individuals per replicate per treat-
224 ment, 30 individuals in total), with equal number of experimental (avian extracts + CO₂

225 vs pentane) and control (CO₂ vs no stimulus) flights performed separately each day.
226 The same procedure was used for assessing attraction of *Cx. p. molestus* in the absence
227 of CO₂. Again, only the release rate eliciting the highest level of attraction was tested;
228 17 min equivalents for the headspace volatile extracts of both sexes of chicken and of
229 female pigeon, and 52 min equivalents for the headspace volatile extracts of male pi-
230 geon and of magpie.

231

232 *Statistical analysis*

233 *Attraction of Cx. quinquefasciatus to avian volatiles*

234 The aim of the first round of statistical analysis was to identify release rates of avian
235 volatile extracts which elicited a significantly different response in *Cx. quinquefascia-*
236 *tus* compared to that of the pentane control. All statistical analyses were performed in
237 R (R Core Team, 2018). Binomial logistic regression was used to investigate the pro-
238 portion of mosquitoes attracted. The proportion of mosquitoes responding in each rep-
239 licate was entered into the model as the dependent variable, with release rate entered as
240 a five-level factor (0.5, 1.7, 5.2, 17 and 52 min equivalents) and test or control entered
241 as a two-level factor (avian volatile extracts or pentane control). An interaction was
242 included between the two terms to allow for differences in relative response between
243 test and control stimuli at each concentration. Terms were retained within the model as
244 critical to the experimental design and analysis regardless of statistical significance. A
245 least-squared means procedure (Lenth, 2016) was then used to identify significant dif-
246 ferences between responses to test and control stimuli at each concentration. Separate
247 models were built for each species/sex combination of avian volatiles presented.

248 Binomial logistic regression was used to test whether the proportion of *Cx. quinque-*
249 *fasciatus* attracted to avian volatiles was significantly altered by the addition of CO₂. A

250 separate model was built for each of the species/sex combination of avian volatile ex-
251 tracts tested, which were presented at their optimal release rates. The dependent varia-
252 ble was the proportion of mosquitoes in each replicate of three attracted, with stimulus
253 entered as a two-level factor (with or without CO₂). The significance of the term within
254 the model was assessed through chi-squared tests of residual deviance upon deletion.

255

256 *Attraction of Cx. p. molestus to avian volatiles*

257 General linear models (Gaussian errors) were used to determine whether concentra-
258 tion of avian volatile extracts presented in the Y-tube olfactometer affected the propor-
259 tion of *Cx. p. molestus* attracted. Separate models were constructed for each species/sex
260 combination of avian volatile extracts tested, and for presentation of volatiles with CO₂.
261 The attraction PI (-1 – +1) for each replicate was entered as the dependent variable,
262 with volatile concentration as a factor (with CO₂: 0, 0.5, 1.7, 5.2, 17, and 52 min equiv-
263 alents). One sample t-tests were used to identify volatile concentrations which were
264 significantly attractive or repellent, by comparing the attraction preference index to 0.

265 Binomial logistic regression was used to test whether the addition of CO₂ to the
266 optimal concentration of avian odour affected mosquito responses in the Y-tube olfac-
267 tometer. The dependent variable was the number of mosquitoes in each group of three
268 attracted to the test arm versus the control arm. Presence or absence of CO₂ was entered
269 into the model as a two-level factor, the significance of which was assessed through
270 changes in residual deviance following deletion from the model.

271

272 **Results**

273

274 *Attraction of Cx. quinquefasciatus to avian volatiles*

275 In the presence of 600 ppm CO₂, female *Cx. quinquefasciatus* were attracted (made
276 source contact) in response to the headspace volatile extracts of both sexes of chickens
277 and female pigeons, but not that of male pigeons and magpies. Significant source con-
278 tact was observed at amounts of the chicken headspace extract corresponding to 5.2 (Z
279 = 3.1; $P < 0.01$; Fig. 1) and 17 ($Z = 2.3$; $P < 0.05$; Fig. 1) min release from male chick-
280 ens, and 5.2 ($Z = 2.0$; $P < 0.05$; Fig. 1), 17 ($Z = 2.9$; $P < 0.01$; Fig. 1) and 52 ($Z = 2.1$;
281 $P < 0.05$; Fig. 1) min release from female chickens. However, only the highest amount
282 of the headspace of female pigeons elicited significant source contact ($Z = 2.5$; $P <$
283 0.05; Fig. 1).

284 The presence of 600 ppm CO₂ significantly increased the attraction of host-seeking
285 *Cx. quinquefasciatus* to chicken and pigeon volatiles, but not to the volatiles of magpies
286 ($X^2 = 2.5$; $df = 1$; NS ; Fig. 2). In particular, CO₂ significantly increased the attraction of
287 the mosquitoes to the odour of both male ($X^2 = 14.0$; $df = 1$; $P < 0.001$; Fig. 2) and
288 female chickens ($X^2 = 7.8$; $df = 1$; $P < 0.01$; Fig. 2), as well as to the odour of both male
289 ($X^2 = 6.9$; $df = 1$; $P < 0.01$; Fig. 2) and female ($X^2 = 7.6$; $df = 1$; $P < 0.01$; Fig. 2)
290 pigeons.

291

292 *Attraction of Cx. p. molestus to avian volatiles*

293 In the presence of CO₂, a significant overall effect of concentration on the attraction
294 of *Cx. p. molestus* to female chicken volatiles ($F = 2.9$; $df = 5, 54$; $P < 0.05$; Fig. 3b)
295 was observed. In contrast, no overall significant effect on attraction of *Cx. p. molestus*
296 was found to that of male chicken volatiles ($F = 1.7$; $df = 5, 54$; $P = 0.148$; Fig. 3a), as
297 well as of male pigeon ($F = 0.2$; $df = 5, 54$; $P = 0.9414$; Fig. 3c), of female pigeon (F
298 = 0.4; $df = 5, 54$; $P = 0.8696$; Fig. 3d) and of magpie volatiles ($F = 2.1$; $df = 5, 54$; $P =$
299 0.087; Fig. 3e). However, considered individually, female *Cx. p. molestus* were

300 significantly attracted at amounts of the chicken headspace extracts corresponding to
301 17 min release from both male ($t = 3.4$; $df = 9$; $P < 0.01$; Fig. 3a) and female chickens
302 ($t = 2.9$; $df = 9$; $P < 0.05$; Fig. 3b), and 52 min release from magpie ($t = 2.9$; $df = 9$; P
303 < 0.05 ; Fig. 3e). In contrast, female *Cx. p. molestus* were indifferent to the headspace
304 of male and female pigeons, except when stimulated by the headspace volatile extract
305 of female pigeons at amounts corresponding to 52 min release, at which they chose to
306 enter the control arm over the treatment arm ($t = 2.8$; $df = 9$; $P < 0.01$; Fig. 3d).

307 No significant effect of the addition of CO₂ was found on the attraction of *Cx. p.*
308 *molestus* to the optimal release rates of male ($X^2 < 0.0001$; $df = 1$; $P > 0.05$) or female
309 chicken volatiles ($X^2 = 0.03$; $df = 1$; $P > 0.05$). Similarly, there was no effect of the
310 addition of CO₂ on the attraction of *Cx. p. molestus* to male ($X^2 = 0.15$; $df = 1$; $P > 0.05$)
311 or to female pigeon volatiles ($X^2 = 0.17$; $df = 1$; $P > 0.05$) as well as to magpie volatiles
312 ($X^2 = 0.008$; $df = 1$; $P > 0.05$).

313

314 **Discussion**

315

316 *Culex p. molestus* and several ecotypes of *Cx. quinquefasciatus* are anthropophilic in
317 their host preference, but still feed on various avian species. This study confirms and
318 extends previous observations that the two *Culex* species are attracted to, and capable
319 of, discriminating between odours emanating from potential avian hosts. Interestingly,
320 the importance of CO₂ in mediating responses to hosts appears to be evident for *Cx.*
321 *quinquefasciatus* and not *Cx. p. molestus*, suggesting that they have developed different
322 strategies for locating their avian hosts.

323 *Culex quinquefasciatus* and *Cx. p. molestus* were both attracted to the headspace

324 volatiles of chicken, as demonstrated previously by Alan *et al.* (2006) for *Cx. quinque-*
325 *fasciatus*. *Culex quinquefasciatus* were attracted to the headspace of female pigeon but
326 showed no significant attraction to the volatiles of magpie. This may be due to a lack
327 of adaptation, as the geographical range of magpies and *Cx. quinquefasciatus* do not
328 overlap (Eguchi, 2016). *Culex p. molestus* actively chose the control between the head-
329 space volatiles of pigeon and the control, in the two-choice bioassay, whereas they
330 were attracted to the headspace volatiles of magpie, as also demonstrated by Rizzoli *et*
331 *al.* (2015). The observed behavioural preference of both *Culex* species is in line with
332 previous blood meal analyses from field collected specimens. For both *Cx. quinquefas-*
333 *ciatus* and *Cx. p. molestus*, a significant proportion of field caught females has been
334 shown to have blood fed on chickens (Garcia-Rejon *et al.*, 2010; Gomes *et al.*, 2013;
335 Martínez-de la Puente *et al.*, 2016; Rizzoli *et al.*, 2015). The pigeon, *C. l. domestica*,
336 is an introduced species in the Americas (Johnston, 1988), and is therefore rarely iden-
337 tified in blood meal analyses of *Culex* species (Molaei *et al.*, 2006). However, the
338 closely related mourning dove (*Zenaida macroura*, Columbiformes: Columbidae) is
339 one of the most frequent vertebrate hosts identified for *Cx. quinquefasciatus* (Molaei *et*
340 *al.*, 2006; Garcia-Rejon *et al.*, 2010). In contrast, *Cx. p. molestus* appear to actively
341 avoid feeding on pigeons (Rizzoli *et al.*, 2015; Martínez-de la Puente *et al.*, 2016),
342 whereas it has often been shown to feed on magpies (Gomes *et al.*, 2013; Rizzoli *et al.*,
343 2015).

344 Carbon dioxide significantly synergised the attraction of *Cx. quinquefasciatus* to
345 the headspace volatiles of chicken and pigeon, whereas it had no effect on the behav-
346 ioural response of *Cx. p. molestus*. While it may be argued that a higher level of CO₂
347 would increase the behavioural response of *Cx. p. molestus*, the concentration used was
348 ecologically relevant, and equivalent to that emitted by a chicken (Constantini *et al.*,

349 1996). We argue that *Culex* species have developed different strategies, which differ-
350 entially rely on CO₂, for host attraction and location. This is supported by previous
351 studies demonstrating either a synergistic action of CO₂ (Kline *et al.*, 1990; Mboera,
352 2000; Alan *et al.*, 2006) or a lack of reliance on CO₂ (Takken & Kline, 1989; Kemme
353 *et al.*, 1993; Essen *et al.*, 1994) on the behavioural response of *Culex* species to avian
354 odour and odorants.

355 The demonstrated behavioural responses of *Cx quinquefasciatus* and *Cx. p. molestus*
356 *tus* to avian odours present novel aspects to be analysed in future experiments. The
357 observed discrimination between headspace volatiles of potential avian hosts merits
358 further analysis of the underlying mechanism, in which both host attractants and non-
359 host volatiles are likely to play a role, as shown for other blood feeding (Gikonyo *et al.*,
360 2002; Jaleta *et al.*, 2016). While a few behavioural active volatile compounds have been
361 identified, and shown to affect host seeking in *Culex* mosquitoes (Allan *et al.*, 2006;
362 Bernier *et al.*, 2008; Cooperband *et al.*, 2008; Syed & Leal, 2009; Majeed *et al.*, 2016),
363 we are, however, far from understanding the role of avian volatiles in regulating host
364 selection of these disease vectors. Through ongoing electrophysiological, chemical and
365 behavioural analyses, we aim to identify blends of avian volatiles, initially from chick-
366 ens, that can be used in effective and environmentally-friendly disease surveillance and
367 control tools for *Culex* mosquitoes. Such tools are sorely needed to define a standard-
368 ized and unbiased method for assessing mosquito abundance, or a control tool that can
369 be used for mass trapping to reduce transmission of WNV.

370

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382

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541 **Figure Legends**

542 **Figure 1:** Average number of female *Cx. quinquefasciatus* attracted to avian headspace
543 odours. Attraction (source contact) of female mosquitoes, was assessed in a flight tube
544 assay, to headspace volatiles of male and female chickens, of male and female pigeons,
545 as well as of magpies, over increasing release rates, combined with pulsed CO₂ (filled
546 bars; n = 30 female mosquitoes), and to pulsed CO₂ combined with pentane (control)
547 (open bars; n = 30 female mosquitoes). Asterisks indicate significant differences among
548 treatments and control (Binomial logistic regression and a least-squared means proce-
549 dure to identify significant differences; **P* < 0.05, ***P* < 0.01, ****P* < 0.001; ns indi-
550 cates not significant).

551

552 **Figure 2:** Average number of female *Culex quinquefasciatus* attracted to avian vola-
553 tiles, combined with or without CO₂. Asterisks indicate significant differences among
554 treatments with CO₂ (dark blue bars) and without CO₂ (light blue bars) (binomial lo-
555 gistic regression; **P* < 0.05, ***P* < 0.01, ****P* < 0.001; ns indicates not significant).

556

557 **Figure 3:** Behavioural response of female *Culex pipiens molestus* to avian headspace
558 volatiles. Preference, indicated by an attraction preference index, of female mosquitoes,
559 to headspace volatiles of male and female chickens, of male and female pigeons, as
560 well as of magpies (n = 30 female mosquitoes), over increasing release rates, combined
561 with CO₂ (green squares), and to a pentane control, as assessed in a Y-tube bioassay.
562 Control (no stimulus vs CO₂) is shown as black squares. Error bars represent standard
563 errors of the mean. (General linear models (Gaussian errors); **P* < 0.05, ***P* < 0.01,
564 ****P* < 0.001).