Behavioural response of *Culex quinquefasciatus* and *Culex pipiens molestus* to avian odours and its reliance on carbon dioxide

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

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Abstract. How *Culex* mosquitoes select and discriminate between potential avian hosts is critical for understanding the epidemiology of WNV. To this end, we studied the behavioural responses of *Culex quinquefasciatus* (Say) and *Culex pipiens molestus* (Forsskål), to headspace volatiles of three avian species (chicken and pigeon, sexes analysed separately, and magpie), presented either alone or in combination with 600 ppm carbon dioxide (CO_2). The attraction of *Cx. quinquefasciatus* to the headspace volatiles of both sexes of chicken, as well as to that of female pigeon, in combination with CO_2 was significantly higher compared to the CO_2 and solvent control. While *Cx. p. molestus* were attracted to headspace volatiles of chickens and magpies, they were repelled by that of female pigeons. A synergistic effect between the avian volatiles and CO_2 was observed for *Cx. quinquefasciatus,* whereas the addition of CO_2 had no effect on the attraction of females *Cx. p. molestus.* The results of this study demonstrate that *Cx. quinquefasciatus* and *Cx. p. molestus* are attracted to the odour of potential avian hosts. Future studies aimed at identifying the bioactive volatile compounds in the headspace of chickens may contribute to the potential development of effective surveillance and control tools against *Culex* species.

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

Introduction

In both Europe and North America, mosquitoes of the *Culex pipiens* species complex are considered as the primary bridge vectors of the West Nile Virus (WNV) (Brugman *et al*., 2018). The WNV causes sporadic outbreaks of neuroinvasive disease on a global scale, with Europe and North America being most affected in recent decades (Chancey *et al*., 2015; Brugman *et al*., 2018). Infections are predominantly asymptomatic, although 20–25% of infected individuals develop mild illness known as West Nile Fever (WNF), and almost 1% becomes neuroinvasive and debilitating, resulting in up to 100 casualties per year in the most affected regions (Chancey *et al*., 2015). The virus is maintained in a mosquito–bird–mosquito transmission cycle, with birds acting as reservoir hosts (Chancey *et al*., 2015). While results of blood meal analyses and field studies using live avian hosts have contributed to the understanding of the host range of *Culex pipiens* (L.) (Apperson *et al*., 2004; Gomes *et al*., 2013; Börstler *et al*., 2016; Llopis *et al*., 2016), the mechanisms underlying host selection and preference in this important vector species complex remain to be elucidated. This information is crucial for the development of evidence–based strategies for WNV vector monitoring and control.

The following species are recognized as members of the *Cx. pipiens* species complex: *Cx. pipiens*, *Culex quinquefasciatus* (Say), *Culex australicus* (Dobrotworsky & Drummond), and *Culex globocoxitus* (Dobrotworsky) (all: Diptera: Culicidae) (Harbach, 2011). *Culex pipiens* and *Cx. quinquefasciatus*, also referred to as the Northern and Southern house mosquito, are the main vectors of WNV in Europe and North America, respectively, but occur in urban areas globally, often closely associated with humans (Zeller & Schuffenecker, 2004). *Culex pipiens* has two recognized forms, ‘pipiens’ and ‘molestus’, which differ in behaviour and physiology as a consequence of rapid ecological adaptation to rural open–air habitats and urban enclosed habitats, respectively (Barr, 1981; Harbach, 2011). While ‘pipiens’ is anautogenous and predominantly ornithophilic, ‘molestus’ is autogenous and anthropophilic (Vinogradova, 2000). However, both forms demonstrate considerable phenotypic plasticity in their feeding behaviour (Kilpatrick *et al*., 2006; Simpson *et al*., 2011; Takken & Verhulst, 2013). The host preference of the cosmopolitan anautogenous *Cx. quinquefasciatus* varies according to ecotype, and has been reported as anthropophilic, mammalophilic and ornithophilic (Beier *et al*., 1990; Mboera & Takken 1999; Molaei *et al*., 2006; Takken & Verhulst, 2013).

The host preference of *Culex* mosquitoes plays a significant role in determining human risk of infection with WNV. Knowledge of vector feeding preferences, in combination with information on relative host and vector abundance, host behaviour and viral load, is crucial to identify which host species may be the most important reservoirs contributing to the spread of WNV (Kilpatrick *et al*., 2006; Rizzoli *et al*., 2015). Most *Culex* species display a preference for feeding on birds, although they may switch to humans and other mammals when birds are not available, e.g. during migration (Kilpatrick *et al*., 2006; Simpson *et al*., 2011). In Europe, blood meal analyses have identified various avian species of the orders Passeriformes, Galliformes and Columbiformes as hosts of *Culex* spp. (Brugman *et al*., 2018). Among these, magpies (*Pica pica*, Passeriformes: Corvidae), collared doves (*Streptopelia decaocto*, Columbiformes: Columbidae), chickens (*Gallus gallus domesticus*, Galliformes: Phasianidae), house sparrows (*Passer domesticus*, Passeriformes: Passeridae) and blackbirds (*Turdus merula*, Passeriformes: Turdidae) have been identified as the species most frequently bitten by *Cx. pipiens* (Roiz *et al*., 2012; Gomes *et al*., 2013; Rizzoli *et al*., 2015; Börstler *et al*., 2016). *Culex pipiens* mosquitoes in North America also feed on a range of avian species, including members of the same orders as used as hosts in Europe (Apperson *et al*., 2004; Molaei *et al*., 2006; Simpson *et al*., 2009). While most studies have focused on identifying the preferred host species of *Cx. pipiens* mosquitoes, others have also revealed that *Cx. pipiens* actively avoid some species of birds (Pickett & Woodcock, 1996; Leal *et al*., 2017), indicating that they can differentiate between potential host species.

Mosquito host selection is heavily influenced by chemical cues emanating from vertebrates (Takken & Verhulst, 2013). Initial recognition of a potential host by mosquitoes generally relies on carbon dioxide (CO_2), a cue emitted in exhaled breath of all vertebrates, which elicits activation and attraction, as well as gates the response to other host odours (Gillies, 1980). Carbon dioxide sensing in *Culex* mosquitoes is acute at the low concentrations of CO_2 found in the exhaled breath of birds (Majeed *et al*., 2017). As a result, attraction at these low levels only appears to occur over short distances (Gillies & Wilkes, 1974). Moreover, the behavioural response of *Culex* mosquitoes to CO_2 appears to be species, and context, dependent, resulting in either no attraction or a low level of attraction when CO_2 is presented alone (Gillies & Wilkes 1974; Mboera *et al*., 1998). Available data indicate that *Culex* mosquitoes use odours emanating from feathers (Allan *et al*., 2006), as well as from the uropygial glands (Russell & Hunter, 2005; Allan *et al*., 2006) for host selection. Although attraction to avian–derived odours, in the presence or absence of CO_2, has been demonstrated in *Culex* mosquitoes, responses to avian cues have received considerably less attention than responses to mammalian and especially human–derived kairomones (Bernier *et al.*, 2008; Cooperband *et al*., 2008; Allan *et al*., 2006; Syed & Leal, 2009; Majeed *et al*., 2016).

The objective of this work was to study behavioural responses of *Cx. quinquefasciatus* and *Cx. p. molestus* to odours from chickens, pigeons and magpies, in the presence and absence of CO_2, as well as to CO_2 alone.

Materials and Methods

Mosquito cultures

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

Volatile collections from birds

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

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

Captured birds were placed individually in a plastic cage (48 X 28 X 28 cm), covered with a heat–sealed oven bag (0.04 m^3) (FoodSaver® Brand, UK) for headspace collection. Charcoal filtered air was introduced into the cage via a pump (12 V, KNF–Neuberger, Freiburg, Germany) through a Teflon tube at 1 l min^{-1}. The air was then extracted from the cage, at 1 l min^{-1}, through a splitter connected to three adsorbent Teflon columns (4 x 0.4 cm i.d.), containing 40 mg Porapak Q (80/100 mesh, Alltech, Deerfield, IL, USA), for 2.5 h. Directly following the completion of the headspace volatile collection, the birds were released back into the wild or returned to the farm. Trapped volatiles were eluted with 600 µl of pentane (puriss p.a., Sigma–Aldrich Chemie GmbH, Steinheim, Germany). Before use, the columns were rinsed with 1 ml each of methanol, acetone, and pentane. The extracts were pooled by avian species and sex and then concentrated under a gentle air stream for 6–8 h to approximately half of the original volume, for use in further experiments.

Attraction of Cx. quinquefasciatus to avian volatiles

Attraction of female *Cx. quinquefasciatus* to different avian headspace volatiles extracts, in the presence or absence of CO_2, were assessed in a flight tube bioassay, illuminated from above at 40 lux, as previously described (Majeed *et al.*, 2014). In brief, a charcoal–filtered and humidified air stream (25 ± 2 °C, 65 ± 2% RH) flowed through the bioassay at 30 cm s^{-1}. Where required, CO_2 was delivered in one second on–off pulses at 600 ppm via a stimulus controller (SEC-2/b, Syntech) (Majeed *et al.*, 2014). The consistency in amplitude and the structure of the pulsed stimuli was visualized using a mini–PID (Aurora Scientific, Aurora, Ontario, Canada), and the concentration of CO_2 was measured at the downwind and upwind end of the assay via a CO_2 analyser (LI–820, LICOR Biosciences, Lincoln, NE, USA) (Majeed *et al.*, 2014). The pooled headspace extracts of each of the avian species, divided by sex for chickens and pigeons, were diluted in half–decadic steps from the concentrated headspace volatile collection in pentane. The diluted extract was pipetted (100 µl) onto a piece of filter paper (55 mm, Munktell, Ahlstrom–Munksjo), which was suspended from a 5 cm wire coil at the upwind end of the assay. The amount of headspace applied on the filter paper corresponded to 0.5, 1.7, 5.2, 17 and 52 min release equivalents of the avian headspace extracts. Pentane, at an equivalent amount, tested with or without CO_2, served as a control.

Two hours prior to the experiments, females were transferred in groups of three to custom-made release cages (Wondwosen *et al.*, 2018). Cages were then placed in the downwind end of the assay, where the insects were allowed 3 min to adapt, before the butterfly valve of the cage was opened for their release. Thereafter, in the first round of experiments, source contact of each mosquito (attraction) was observed for a maximum of 4 minutes. Ten replicates were conducted at each of the five release rates tested (3 individuals per replicate per treatment, 30 individuals in total), with equal number of experimental (CO_2 + extract) and control (CO_2 + pentane) flights performed separately each day. A second round of experiments was conducted in order to investigate the role of CO_2 on the attraction of *Cx. quinquefasciatus* to avian volatiles. For this, only the release rate eliciting the highest level of attraction was tested, in the presence or absence of CO_2 (5.2 min equivalents for male chicken headspace volatile extract; 17 min equivalents for female chicken, male pigeon and magpie headspace volatile extracts; and 52 min equivalents for female pigeon headspace volatile extract). Treatments were presented in the same manner as in the first round of experiments.

Attraction of Cx. p. molestus to avian volatiles

Due to the high spontaneous flight activity of *Cx. p. molestus*, a Y–tube olfactometer (Zaspel *et al*., 2016) was used to assess the behavioural response of this species to the different avian headspace volatiles extracts. The conditioning of the airstream was the same as described for the wind tube bioassay above, with the odour stimulus, in the presence or absence of CO_2, provided in one of the arms of the olfactometer. The other arm contained no stimulus or pentane. Treatments and controls were provided in the same manner as for *Cx. quinquefasciatus*. An attraction Preference Index (PI) was calculated, (T – C)/(T + C), where T is the number of mosquitoes associated with the test odour, and C the number of mosquitoes associated with the control. The use of the term attraction in this study refers to an increased number of mosquitoes that make source contact within the treatment arm in the olfactometer compared with the control.

Mosquitoes were conditioned as described above prior to the experiments, but due to their high spontaneous flight behaviour only allowed 2 minutes to make a choice. Ten replicates were conducted for each treatment (3 individuals per replicate per treatment, 30 individuals in total), with equal number of experimental (avian extracts + CO_2

vs pentane) and control (CO_2 vs no stimulus) flights performed separately each day. The same procedure was used for assessing attraction of *Cx. p. molestus* in the absence of CO_2. Again, only the release rate eliciting the highest level of attraction was tested; 17 min equivalents for the headspace volatile extracts of both sexes of chicken and of female pigeon, and 52 min equivalents for the headspace volatile extracts of male pigeon and of magpie.

Statistical analysis

Attraction of Cx. quinquefasciatus to avian volatiles

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

Binomial logistic regression was used to test whether the proportion of *Cx. quinquefasciatus* attracted to avian volatiles was significantly altered by the addition of CO_2. A separate model was built for each of the species/sex combination of avian volatile extracts tested, which were presented at their optimal release rates. The dependent variable was the proportion of mosquitoes in each replicate of three attracted, with stimulus entered as a two–level factor (with or without CO_2). The significance of the term within the model was assessed through chi–squared tests of residual deviance upon deletion.

Attraction of Cx. p. molestus to avian volatiles

General linear models (Gaussian errors) were used to determine whether concentration of avian volatile extracts presented in the Y–tube olfactometer affected the proportion of *Cx. p. molestus* attracted. Separate models were constructed for each species/sex combination of avian volatile extracts tested, and for presentation of volatiles with CO_2. The attraction PI (-1 – +1) for each replicate was entered as the dependent variable, with volatile concentration as a factor (with CO_2: 0, 0.5, 1.7, 5.2, 17, and 52 min equivalents). One sample t–tests were used to identify volatile concentrations which were significantly attractive or repellent, by comparing the attraction preference index to 0.

Binomial logistic regression was used to test whether the addition of CO_2 to the optimal concentration of avian odour affected mosquito responses in the Y–tube olfactometer. The dependent variable was the number of mosquitoes in each group of three attracted to the test arm versus the control arm. Presence or absence of CO_2 was entered into the model as a two–level factor, the significance of which was assessed through changes in residual deviance following deletion from the model.

Results

Attraction of Cx. quinquefasciatus to avian volatiles

In the presence of 600 ppm CO_2, female *Cx. quinquefasciatus* were attracted (made source contact) in response to the headspace volatile extracts of both sexes of chickens and female pigeons, but not that of male pigeons and magpies. Significant source contact was observed at amounts of the chicken headspace extract corresponding to 5.2 ($Z = 3.1$; $P < 0.01$; Fig. 1) and 17 ($Z = 2.3$; $P < 0.05$; Fig. 1) min release from male chickens, and 5.2 ($Z = 2.0$; $P < 0.05$; Fig. 1), 17 ($Z = 2.9$; $P < 0.01$; Fig. 1) and 52 ($Z = 2.1$; $P < 0.05$; Fig. 1) min release from female chickens. However, only the highest amount of the headspace of female pigeons elicited significant source contact ($Z = 2.5$; $P < 0.05$; Fig. 1).

The presence of 600 ppm CO_2 significantly increased the attraction of host–seeking *Cx. quinquefasciatus* to chicken and pigeon volatiles, but not to the volatiles of magpies ($X^2 = 2.5$; df = 1; *NS;* Fig. 2). In particular, CO_2 significantly increased the attraction of the mosquitoes to the odour of both male ($X^2 = 14.0$; df = 1; $P < 0.001$; Fig. 2) and female chickens ($X^2 = 7.8$; df = 1; $P < 0.01$; Fig. 2), as well as to the odour of both male ($X^2 = 6.9$; df = 1; $P < 0.01$; Fig. 2) and female ($X^2 = 7.6$; df = 1; $P < 0.01$; Fig. 2) pigeons.

Attraction of Cx. p. molestus to avian volatiles

In the presence of CO_2, a significant overall effect of concentration on the attraction of *Cx. p. molestus* to female chicken volatiles ($F = 2.9$; df = 5, 54; $P < 0.05$; Fig. 3b) was observed. In contrast, no overall significant effect on attraction of *Cx. p. molestus* was found to that of male chicken volatiles ($F = 1.7$; df = 5, 54; $P = 0.148$; Fig. 3a), as well as of male pigeon ($F = 0.2$; df = 5, 54; $P = 0.9414$; Fig. 3c), of female pigeon ($F = 0.4$; df = 5, 54; $P = 0.8696$; Fig. 3d) and of magpie volatiles ($F = 2.1$; df = 5, 54; $P = 0.087$; Fig. 3e). However, considered individually, female *Cx. p. molestus* were significantly attracted at amounts of the chicken headspace extracts corresponding to 17 min release from both male ($t = 3.4$; df = 9; $P < 0.01$; Fig. 3a) and female chickens ($t = 2.9$; df = 9; $P < 0.05$; Fig. 3b), and 52 min release from magpie ($t = 2.9$; df = 9; $P < 0.05$; Fig. 3e). In contrast, female *Cx. p. molestus* were indifferent to the headspace of male and female pigeons, except when stimulated by the headspace volatile extract of female pigeons at amounts corresponding to 52 min release, at which they chose to enter the control arm over the treatment arm ($t = 2.8$; df = 9; $P < 0.01$; Fig. 3d).

No significant effect of the addition of CO_2 was found on the attraction of *Cx. p. molestus* to the optimal release rates of male ($X^2 < 0.0001$; df = 1; $P > 0.05$) or female chicken volatiles ($X^2 = 0.03$; df = 1; $P > 0.05$). Similarly, there was no effect of the addition of CO_2 on the attraction of *Cx. p. molestus* to male ($X^2 = 0.15$; df = 1; $P > 0.05$) or to female pigeon volatiles ($X^2 = 0.17$; df = 1; $P > 0.05$) as well as to magpie volatiles ($X^2 = 0.008$; df = 1; $P > 0.05$).

Discussion

Culex p. molestus and several ecotypes of *Cx. quinquefasciatus* are anthropophilic in their host preference, but still feed on various avian species. This study confirms and extends previous observations that the two *Culex* species are attracted to, and capable of, discriminating between odours emanating from potential avian hosts. Interestingly, the importance of CO_2 in mediating responses to hosts appears to be evident for *Cx. quinquefasciatus* and not *Cx. p. molestus*, suggesting that they have developed different strategies for locating their avian hosts.

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

Carbon dioxide significantly synergised the attraction of *Cx. quinquefasciatus* to the headspace volatiles of chicken and pigeon, whereas it had no effect on the behavioural response of *Cx. p. molestus*. While it may be argued that a higher level of CO_2 would increase the behavioural response of *Cx. p. molestus*, the concentration used was ecologically relevant, and equivalent to that emitted by a chicken (Constantini *et al*., 1996). We argue that *Culex* species have developed different strategies, which differentially rely on CO_2, for host attraction and location. This is supported by previous studies demonstrating either a synergistic action of CO_2 (Kline *et al*., 1990; Mboera, 2000; Alan *et al*., 2006) or a lack of reliance on CO_2 (Takken & Kline, 1989; Kemme *et al*., 1993; Essen *et al*., 1994) on the behavioural response of *Culex* species to avian odour and odorants.

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

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Figure Legends

Figure 1: Average number of female *Cx. quinquefasciatus* attracted to avian headspace odours. Attraction (source contact) of female mosquitoes, was assessed in a flight tube assay, to headspace volatiles of male and female chickens, of male and female pigeons, as well as of magpies, over increasing release rates, combined with pulsed CO_2 (filled bars; n = 30 female mosquitoes), and to pulsed CO_2 combined with pentane (control) (open bars; n = 30 female mosquitoes). Asterisks indicate significant differences among treatments and control (Binomial logistic regression and a least–squared means procedure to identify significant differences; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns indicates not significant).

Figure 2: Average number of female *Culex quinquefasciatus* attracted to avian volatiles, combined with or without CO_2. Asterisks indicate significant differences among treatments with CO_2 (dark blue bars) and without CO_2 (light blue bars) (binomial logistic regression; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns indicates not significant).

Figure 3: Behavioural response of female *Culex pipiens molestus* to avian headspace volatiles. Preference, indicated by an attraction preference index, of female mosquitoes, to headspace volatiles of male and female chickens, of male and female pigeons, as well as of magpies (n = 30 female mosquitoes), over increasing release rates, combined with CO_2 (green squares), and to a pentane control, as assessed in a Y–tube bioassay. Control (no stimulus vs CO_2) is shown as black squares. Error bars represent standard errors of the mean. (General linear models (Gaussian errors); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).