Mosquitoes are attracted by the odour of *Plasmodium*-infected birds

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Abstract

Parasites can manipulate their hosts to increase their transmission success. Avian malaria parasites (*Plasmodium*) are thought to alter the cues such as host odour, used by host-seeking mosquitoes. Bird odour is affected by secretions from the uropygial gland and may play a role in modulating vector-host interactions. We tested the hypothesis that mosquitoes are more attracted to the uropygial secretions and/or whole-body odour (headspace) of *Plasmodium*-infected house sparrows (*Passer domesticus*) than to those of uninfected birds. We tested the attraction of nulliparous (e.g. uninfected mosquitoes without previous access to blood) *Culex pipiens* females towards these stimuli in a dual-choice olfactometer. We used Gas Chromatography-Mass Spectrometry (GC-MS) analyses to assess whether *Plasmodium* infection is associated with differences in the chemical composition of uropygial secretions. Mosquitoes were more attracted to the odours of infected than uninfected birds, regardless of sex. However, the significant interaction between infection status and the stimuli (uropygial secretion or headspace) showed that mosquitoes were more attracted to the headspace of infected birds; no differences were found in the case of uropygial secretions. The compounds in the volatile lipophilic fraction of the uropygial secretion did not differ between infected and uninfected birds. These results support the host manipulation hypothesis since avian *Plasmodium* parasites may be capable of altering their host's body odour, thereby making infected individuals more attractive to mosquitoes.

Keywords: Chemical communication, Host preference, Infectious diseases, Olfaction, Olfactometer, Wild birds

1. Introduction

The host manipulation hypothesis asserts that parasites are able to manipulate certain host traits in order to increase their transmission success (Heil, 2016). In the case of vector-borne parasites, which require a vertebrate host and an invertebrate vector to complete their life cycle, this type of manipulation could act at two different levels: parasite infection could modify insect vector traits (e.g. by increasing biting rates) or vertebrate host traits (e.g. by impeding their defensive behaviour or enhancing their attractiveness to vectors), which in turn would increase host-parasite contact rates and hence parasite transmission (Hurd, 2003; Lefèvre and Thomas, 2008; van Houte et al., 2013). This is the case for malarial parasites of the genus *Plasmodium* that infect a wide range of vertebrate species (Batista et al., 2014). For instance, Lacroix et al. (2005) found that mosquitoes were more attracted to children infected by *Plasmodium falciparum* gametocytes (the transmissible parasite stage) than children infected by trophozoites (the non-transmissible stage) or who were uninfected. In addition, these authors found that mosquitoes were less attracted to children after they were submitted to an antimalarial-medication treatment that reduced the parasite load. De Moraes et al. (2014) showed that malaria infection in mice modified the host volatile fraction of the host odour profile, thereby increasing the attraction of mosquitoes towards infected individuals.

Birds are common hosts of *Plasmodium* parasites and infected birds have been reported from all continents except Antarctica (Valkiūnas, 2005). Avian malaria has been widely used as a model system to evaluate the evolution of host-parasite-vector interactions (e.g. the host manipulation hypothesis) (Rivero and Gandon, 2018). Various

studies have analysed the potential effects of avian malaria infection in birds on mosquito attraction. For example, Cornet et al. (2013a) exposed *Plasmodium*-infected and uninfected canaries (*Serinus canaria*) to *Culex pipiens* mosquitoes and reported a higher mosquito biting rate in chronically infected birds than in uninfected birds, and in birds with acute infection. In addition, Yan et al. (2018) found that *Cx. pipiens* more often bit *Plasmodium*-infected house sparrows than individuals with an experimentally reduced intensity of *Plasmodium* infection. However, other studies have found contrasting patterns, with *Plasmodium*-uninfected birds attracting more mosquitoes than infected ones (Lalubin et al., 2012) or even an absence of any significant association between *Plasmodium* infection and vector attraction (Gutiérrez-López et al., 2019). These differences could be partially explained by the different approaches used (Gutiérrez-López et al., 2019) and highlight the need to identify the mechanisms underlying changes in the attractiveness or susceptibility to mosquitoes of *Plasmodium*-infected birds.

Haematophagous insects (i.e., those that feed on blood, which is required to lay eggs) such as mosquitoes use a combination of different cues - visual cues, carbon dioxide (CO₂), temperature, moisture, and body odour - to detect and select their hosts (Eiras and Jepson, 1994; Lehane, 2005). The use of synergistic cues rather than a single independent cue may greatly facilitate host choice. The combination of CO₂ with odour probably plays the most important role in host selection (Lehane, 2005). In a field study in Tanzania, Knols et al. (1995) found that three anthropophilic (i.e. that feed on human blood) mosquito species (*Anopheles gambiae*, *Anopheles funestus* and *Culex quinquefasciatus*) based their host discrimination not only on CO₂ but also on human-specific odours including volatile compounds emitted by human skin bacteria. Indeed, *Plasmodium* infection in humans affects the volatiles of their skin odour profiles (De

Moraes et al., 2018). Similar results were reported by de Boer et al. (2017), who found that specific compounds produced by human skin bacteria (e.g. 2- and 3-methylbutanal and 3-hydroxy-2-butanone) were found in higher concentrations in *P. falciparum*-infected humans.

The uropygial gland in birds produces an oily secretion whose function, once spread over the plumage, includes waterproofing and protection against sun damage (Giraudeau et al., 2010), and anti-microbial activity against harmful bacteria that degrade feathers (Shawkey et al., 2003). In addition, uropygial secretions, whose main compounds are alkanes, ketones, aldehydes, alcohols and waxes (Campagna et al., 2012), and whose volatile and non-volatile fractions vary between species and sexes, are to a large extent responsible for birds' odour profiles (Haribal et al., 2009; Whittaker et al., 2010). Nonetheless, bird odour is also affected by breath, metabolism and the degradation of these secretions by ecto-symbionts (Maraci et al., 2018). Chemicals such as nonanal that are present in bird odour are thought to attract *Culex* mosquitoes (Syed and Leal, 2009). In addition, it has been shown that the wax ester composition in song sparrows (*Melospiza melodia*) differed between experimentally *Plasmodium*-infected and uninfected birds (Grieves et al., 2018). Variation in odour profiles and the chemical composition of uropygial secretions has been proposed as a mechanism underling mosquito attraction to *Plasmodium*-infected birds (Cornet et al., 2013b). However, despite its importance in parasite epidemiology, to our knowledge this potential mechanism remains untested.

The aim of this study was to test the prediction derived from the host manipulation hypothesis that mosquitoes are more attracted to *Plasmodium*-infected than uninfected birds due to infection-derived differences in the compounds of their uropygial gland secretions and/or in their whole-body odour (headspace odour profile).

Using a dual choice olfactometer, we counted the number of wild *Cx. pipiens* mosquitoes, as important vector of avian *Plasmodium* (Valkiūnas, 2005), attracted to stimuli obtained from wild house sparrows, *Passer domesticus*, either naturally infected by *Plasmodium* parasites or uninfected. We also analysed and compared the chemical composition of the volatile fraction of the uropygial gland secretions of infected and uninfected birds in order to detect possible differences in the compounds relative to the infection status.

2. Materials and methods

2.1. Mosquito collection and rearing

Mosquito larvae were collected in the provinces of Huelva and Seville (Andalusia, Spain) in April–May 2016 and May–September 2017. Larvae were allowed to develop in the laboratory in plastic trays with fresh water and fed ad libitum (20 ml/22 g Hobby Mikrozell; Dohse Aquaristik GmbH & Co. KG, Gelsdorf, Germany) in a climatic chamber under controlled conditions ($28^{\circ} \pm 1^{\circ}\text{C}$, 60–65% relative humidity (RH) and 12:12 h light:dark cycle). After emergence, mosquitoes were visually sexed and identified to species level (Schaffner et al., 2001). Female *Cx. pipiens* were kept in insect cages (BugDorm-43030F, 32.5 x 32.5 x 32.5 cm) and fed a 1% sugar solution ad libitum. Mosquitoes were deprived of the sugar solution 24 h before the trials began and only had access to water, which was removed from cages 1 h before the start of the trials. These mosquitoes never fed on blood, which guaranteed their lack of infection by avian malaria parasites or related haemosporidians.

2.2. Bird sampling and maintenance

A total of 80 juvenile house sparrows were captured using mist nests in August 2016 in Huelva province (San Juan del Puerto, 37°18'51''N, 6°50'27''W, Spain). Each bird was individually marked with a numbered metal ring, weighed and their wing length measured. After that, birds were blood sampled from the jugular vein with sterile needles and blood samples were kept in Eppendorf tubes. Uropygial gland secretion was extracted by gentle massaging of the gland papilla and the secretion was then immediately placed in a 2 ml gas chromatography glass vial. Blood samples and uropygial gland secretions were maintained in cold boxes in the field. In the laboratory, blood samples were centrifuged to separate plasma and cell fractions, and subsequently blood and secretion samples were stored at -20°C and -80°C, respectively. Birds were transferred to the Unit of Animal Experimentation at the Doñana Biological Station-Spanish National Research Council (EBD-CSIC, Spain), where they were maintained in pairs in cages (58.5 x 25 x 36 cm) in a vector-free room under controlled conditions with a 12:12 h light:dark cycle at 22°C ($\pm$ 1°C). Birds had ad libitum access to fresh water and a food mixture consisting of seeds and an insectivorous-eater diet mix (KIKI; GZM S.L., Alicante, Spain). All experimental procedures were approved by the CSIC Ethics Committee and Animal Health authorities, as per Spanish legislation (CEBA-EBD-12-40).

2.3. Molecular analyses

Genomic DNA was extracted from blood samples using a semi-automatic Maxwell kit (Maxwell[®]16 LEV system Research, Promega, Madison, WI, USA). Birds were molecularly sexed using the primers P2 (5'-TCTGCATCGCTAAATCCTTT-3')

and P8 (5'-CTCCCAAGGATGAGRAAYTG-3') following Ellegren (1996) and Griffiths et al. (1998). Detection of haemosporidian parasites of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* was performed following Hellgren et al. (2004). The presence of amplicons was verified in 1.8% agarose gels. All positive samples were sequenced in two directions in the case of *Plasmodium* and *Haemoproteus*, and in one direction in the case of *Leucocytozoon*, using the MacroGen sequencing service (MacroGen Inc. Madrid, Spain). Sequences were edited with the software Sequencher[™] v4.9. (Genes Codes Corp.[©] 1991-2009, Ann Arbor, MI 48108, USA) and parasite genera were identified by BLAST comparisons with sequences deposited in GenBank (National Center for Biotechnology Information, NCBI, USA). Birds infected only by *Plasmodium* ($n=22$) and those uninfected by any of the three parasite genera ($n=22$) were selected for the experiments. We excluded from this study birds infected by both *Haemoproteus* and *Leucocytozoon* parasites, as well as birds showing any evidence of co-infection, to avoid any potential bias in mosquito attraction.

2.4. Bird headspace sampling

During 2016, after an acclimation period of at least 15 days at the Unit of Animal Experimentation (EBD-CSIC, Spain), which was necessary to adapt the birds to captivity and minimize their stress levels, body volatiles from each individual were extracted with non-intrusive methods. To do that, we used ORBO-402 Tenax[®] TA (60/80) SPT 100/5 mg cartridges (ORBO Sigma-Aldrich Co. LLC; Tenax Buchem B.V.) connected to a borosilicate glass desiccator (5.8 L, 20 cm diameter, Mobilex GL 32, DURAN[®]). The base of the desiccator was connected to a charcoal filter, while the top was connected to the cartridge that links to the flow meter (Sable Systems Dual

Pump System and Sable System Flow Measurement System, Las Vegas, NV, USA), to deliver a flow rate of 400 ml/min. Each bird remained inside the desiccator for 15 min. Birds were not exposed for longer periods in order to minimize their stress levels. Overall, for each bird, 6 L of air were filtered through the retention cartridges. We performed the headspace extraction under dark conditions to avoid stress and birds suffered no harm during this process. Cartridges containing trapped volatiles were immediately eluted in 2 ml gas chromatography vials with 600 µl of hexane and stored at -80 °C. Following the headspace extraction, the uropygial gland secretion of each bird was extracted again. These secretions and those obtained in the field (see section 2.2) were used for different purposes due to the low volume obtained in each extraction. The sample of the secretion obtained in the field was used in the experiments in the olfactometer, while the second sample was used for Gas Chromatography-Mass Spectrometry (GC-MS) analyses (see below). Birds were blood-sampled again to confirm the infection status of the first sample. Two days after this procedure, birds were released at the site of capture without any apparent sign of harm.

2.5. Analyses of the composition of the uropygial gland secretions

The chemical composition of the uropygial gland secretions was analysed using an Agilent 7890A gas chromatograph (GC) fitted with a poly (5% diphenyl, 95% dimethylpolysiloxane) column HP5-MS (30 m length x 0.25 mm inner diameter x 0.25 µm film thickness) with helium as the carrier gas and the Agilent 5975C Triple Axis Detector mass spectrometer (MS) as the detector. We added 50 µl of hexane directly into the vial with the secretion and vortexed it. Then, we injected in the GC in splitless mode 2 µl of the supernatant containing lipids. The oven temperature program started at

80°C and was maintained for 3 min., then increased to 300°C at a rate of 5°C/min., and finally was maintained at 300°C for 35 min. The analyses of the lipophilic components of the uropygial gland secretions were made under the same analytical conditions by comparing the retention time (RT) of each compound and the presence/absence of characteristic ions in the mass spectra. In particular, we used the library NIST/EPA/NIH 2002 (NIST Mass Spectral Library, Version 2.0[®], Faircom Corporation, USA), which provides a list of potential compounds according to the reported chemical characteristics. This allowed us to identify the compounds based on a percentage of reliability. Additionally, we injected authentic commercial standards (from Sigma-Aldrich Chemical Co., St. Louis, MO, USA) under the same conditions to compare their spectra and retention times with those of the compounds identified in secretion samples analysed here. Uropygial gland secretions from seven individuals showed low quality GC-MS profiles and were thus excluded from the compositional analyses.

2.6. Mosquito behavioural assays

The two dual choice (Y-shape) olfactometers used in this study were made of methacrylate and have three parts: acclimation section, flight section and two ports with vertical doors that separated the two compartments (see details in Díez-Fernández et al., 2020). The doors were closed before the start and at the end of the trials to be able to accurately count the number of mosquitoes caught in each part. The two olfactometers were used alternatively to perform the trials. Air was pumped from an open 60 L plastic bucket enriched with a continuous 180 ml/min flow of CO₂ (purity $\geq$ 99.7%) to generate airflow passing through the charcoal filter. The flow was then humidified (60% RH) and warmed ($27 \pm 1^\circ\text{C}$), before being distributed in equal parts into both ports of the

olfactometer via a flow meter (Sable Systems Dual Pump System and Sable System Flow Measurement System, Las Vegas, NV, USA) with a flow rate of 1800 ml/min.

We assessed the attraction of mosquitoes to the secretions of the uropygial gland and the headspaces of both uninfected and *Plasmodium*-infected house sparrows. During 2016, a total of 22 trials were conducted using the uropygial gland secretions of male ($n=26$) and female ($n=18$) juvenile house sparrows. The next year (2017), 18 trials were conducted using the headspace of male ($n=20$) and female ($n=16$) birds. Analyses were performed in different years due to the difficulties in collecting enough mosquito larvae from the field. In each trial, the secretion/headspace from an infected bird was used in one port and the secretion/headspace from an uninfected bird in the other. Pairs were always composed of individuals of the same sex, and the same pairs of birds were used to compare the mosquitoes' attraction to the uropygial gland secretions or to the headspace. The stimuli of infected birds were presented alternatively in each port to rule out any positional effects. In each trial, 20 (± 1) female mosquitoes (6-15 days old) were allowed to fly in complete darkness following an upwind air current towards the stimuli for 15 min. After this time, the doors separating the different compartments were closed and the number of mosquitoes trapped in each section was counted. Overall, 818 *Cx. pipiens* females were used in this study, comprising 440 and 378 mosquitoes used in the uropygial secretion and the headspace trials, respectively. Mosquitoes were only used in one trial and never re-used. The olfactometer was cleaned between consecutive trials with water and then allowed to dry; a minimum of 24 h elapsed between two consecutive trials with the same olfactometer.

2.7. Statistical analysis

Only mosquitoes that actively flew to one of the two stimuli, and were thus caught at the back of one of the two ports of the olfactometer, were considered for statistical purposes. We used a Generalized Linear Mixed Model (GLMM) with binomial distribution and logit link function to assess the attraction of mosquitoes to the uropygial gland secretions or the headspace of *Plasmodium*-infected and uninfected house sparrows. The dependent variable was defined as the number of mosquitoes that flew towards the arm where stimuli were present in relation to the total number of mosquitoes that actively flew to any port. Each bird's infection status and sex, and the stimuli (secretion or headspace) were included as fixed factors in the model. In addition, we included the interaction between the stimuli and the infection status to identify potential differences between secretions of the uropygial gland and headspace from infected and uninfected birds. The port (left or right), the trial number and the pair identity were included as random terms. Only samples from pairs that were successfully analysed for both stimuli were included in the analyses, which reduced the number of birds analysed to 36 individuals (18 pairs consisting of one infected and one uninfected bird). Only P values < 0.05 were considered as significant. Analyses were performed using SAS (SAS Institute, Inc., Cary, North Carolina, USA).

The relative proportion of each compound (e.g. the percentage relative to the total amount of volatile lipids in the sample) was determined as the percentage of the area of each peak in relation to the total ion current using the peak area integration capability of the Xcalibur 2.2 software (Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA). To correct for the non-independence of proportions, we performed compositional analysis based on the logit transformed proportion data by taking the natural logarithm of proportion/(1-proportion) (Aebischer, 1993). Then, we calculated the Euclidean distances between each pair of individual samples to produce a

resemblance matrix that formed the basis of the analyses. We used a two-factor permutational multivariate analysis of variance test (PERMANOVA) (Anderson, 2001; McArdle and Anderson, 2001) with 999 permutations to analyse whether the overall composition of the uropygial secretions varied between sexes and between infected and uninfected birds (with the PERMANOVA V1.0.3 add-in package (Anderson et al., 2008) in PRIMER V6.1.13 (Clarke and Gorley, 2006)). In addition, the relative proportions of the main chemical classes of compounds present in the uropygial secretions were compared between bird sexes, infection status and their interaction using GLMs with quasibinomial error distribution. Analyses were performed in R 3.4.3 (R Core Team, 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria) using the package *lme4* (Bates, D., Maechler, M., Bolker, B., Walker, S., 2014. *lme4*: Linear mixed-effects models using eigen and S4).

3. Results

The 22 *Plasmodium*-infected birds included in this study harboured the parasite lineages SGS1 ($n = 14$, belonging to *P. relictum*), GRW11 ($n = 4$, belonging to *P. relictum*, also called Rinshi-7), COLL1 ($n = 3$, also called DONANA07), and PADOM01 ($n = 1$), the last two lineages have not linked to any *Plasmodium* morphospecies. Mosquitoes were significantly more attracted to the scents of *Plasmodium*-infected birds than to the scent of uninfected birds (positive infection estimate $\pm$ SE = 0.98 ± 0.28 , degrees of freedom (d.f.) = 16, $P = 0.003$), regardless of sex (estimate $\pm$ SE = 0.00002 ± 0.26 , d.f. = 16, $P = 0.99$). The stimuli did not

significantly affect the mosquito attraction (estimate $\pm$ SE = 0.39 ± 0.26 , d.f. = 16, $P = 0.15$). However, the interaction between the infection status and the stimuli approached significance (estimate $\pm$ SE = 0.79 ± 0.39 , d.f. = 16, $P = 0.07$), because mosquitoes were more attracted to the headspace of infected than of uninfected birds (post-hoc least square mean slices test $F_{1,16} = 12.26$, $P = 0.003$), while for uropygial secretion no differences were found in relation to the infection status ($F_{1,16} = 0.45$, $P = 0.51$) (Fig.1).

3.1. Composition of uropygial gland secretions

We identified a total of 47 volatile lipophilic compounds in the uropygial gland secretions of the house sparrows (Table 1): 11 alcohols between C_{11} and C_{22} (52.05 %), five aldehydes between C_{14} and C_{18} (17.93 %), six alcohol acetates between C_{16} and C_{21} (13.82 %), 16 ketones between C_{11} and C_{19} (12.63 %), eight ethyl or methyl esters of carboxylic acids between C_{12} and C_{20} (3.56 %), and triacetin. The two main compounds were octadecanol and octadecanal.

There were no overall significant differences between the chemical profiles of infected and uninfected individuals, nor between males and females; similarly, their interaction was not significant (two-factors PERMANOVA, sex: $\text{PseudoF}_{1,33} = 0.79$, $P = 0.73$; infection: $\text{PseudoF}_{1,33} = 0.75$, $P = 0.77$; sex-infection interaction: $\text{PseudoF}_{1,33} = 0.62$, $P = 0.92$). Similar results were obtained considering only birds infected by the commonest parasite lineage SGS1. Finally, bird sex, infection status and its interaction did not affect the relative proportions of the major chemical classes of compounds (alcohols, aldehydes, alcohol acetates, ketones and ester of carboxylic acids) present in the uropygial secretions (ANOVA, in all cases, $P > 0.14$) (Table 2).

4. Discussion

According to the host manipulation hypothesis, parasites are able to modify the host phenotype in order to increase the possibilities of transmission (Heil, 2016). Here, we tested this hypothesis using a wild bird-*Plasmodium*-mosquito assemblage and found that *Cx. pipiens* vectors were more attracted to the odour of *Plasmodium*-infected hosts than to the odour of uninfected hosts. Nonetheless, of the two stimuli used, only the headspace of infected birds proved to be attractive to mosquitoes given that the uropygial gland secretions had no effects.

It has been suggested that infection by vector-borne pathogens is associated with changes in host odour profiles, which potentially affect the host selection by different species of vectors (Prugnolle et al., 2009), thereby entailing important consequences for pathogen transmission. Strong evidence supports the host manipulation hypothesis in certain malaria parasites, with infected individuals (especially those infected by the transmissible stage of the parasites) being more attractive to mosquitoes (Ferguson and Read, 2004; Lacroix et al., 2005; Cornet et al., 2013a, 2013b). Robinson et al. (2018) have shown that *Plasmodium*-infected children are more attractive to *Anopheles* mosquitoes, and that this enhanced attraction is related to greater production of certain aldehydes (e.g. heptanal, octanal, nonanal, (E)-2-octenal and (E)-2-decenal) on their skins. Furthermore, when De Moraes et al. (2014) manipulated and added compounds that were typically present in the odour of infected mice (e.g. hexanoic acid, 2- and 3-methyl butanoic acid and tridecane) to uninfected mice, mosquitoes were more attracted to the latter individuals, which is further evidence for the odour-mediated attraction of mosquitoes towards infected individuals. Our results support this possibility and represent, to the best of our knowledge, the first evidence of how *Plasmodium*-driven effects on bird odours influence vector attraction. Our results thus provide further

understanding of the mechanisms behind the higher biting rates of mosquitoes of birds with chronic *Plasmodium* infections (Cornet et al., 2013a, 2013b). Contrary to previous studies in which authors used immobilized (Cornet et al., 2013a, 2013b) or pairs of free-moving individuals (Yan et al., 2018), we only used the odour stimuli of birds, thereby avoiding the potential interference of additional host cues (e.g. temperature) or anti-mosquito behaviour, which may affect host selection by mosquitoes (Darbro and Harrington, 2007). Our results, however, contrast with those of Lalubin et al. (2012), who also used a dual choice olfactometer and showed that *Cx. pipiens* were less attracted to *Plasmodium*-infected great tits (*Parus major*) than to uninfected individuals. These discrepancies between studies may be due to the different approaches followed, since Lalubin et al. (2012) used live animals instead of only their odour stimuli, as in our case. Our study focussed on uninfected mosquitoes, but further research is necessary to understand how parasites infecting vectors may affect their host's selection and its epidemiological implications (Gandon, 2018). Nonetheless, Cornet et al. (2013b) found that both uninfected and avian *Plasmodium*-infected mosquitoes fed more frequently on *Plasmodium*-infected birds than on uninfected ones, supporting the role of avian infection status as a driver of host selection by mosquitoes, as we found here.

The uropygial gland secretions of birds have traditionally been suggested to play a key role in vector-host interactions and to potentially explain *Plasmodium*-mediated increases in mosquito attraction (see Cornet et al., 2013a). For example, more mosquitoes were captured in traps baited with American crow (*Corvus brachyrhynchos*) uropygial gland secretions, but only when traps were located five metres above ground level (Russell and Hunter, 2005). More recently, Díez-Fernández et al. (2020) found that *Cx. pipiens* and *Aedes caspius* mosquitoes did not differ in their attraction to the uropygial gland secretions of uninfected birds and to a control stimulus. This is also the

case of other vector species, as reported in a study by Martínez-de la Puente et al. (2011) who found no relationship between birds' uropygial gland secretions and attraction for *Culicoides* and blackflies. We found that mosquitoes were more attracted towards the headspace of *Plasmodium*-infected birds but this effect was not apparent when only using uropygial secretions. In accordance with the results of our behavioural tests, we found no significant differences in the composition of the volatile lipophilic components in the uropygial secretions of infected and uninfected house sparrows. Although the mechanisms are unclear, these findings suggest that infection by avian malaria parasites could trigger changes in the headspace of birds that are related to mosquito attraction, and not necessarily related to the chemical composition of the uropygial secretion. Contrasting results were found by Grieves et al. (2018) when they analysed the non-volatile wax ester composition from the uropygial secretions of four experimental groups of song sparrows. They experimentally infected birds with *Plasmodium* sp. and detected variations in the wax composition in infected birds and in birds resistant to the infection. It is important to note that we analysed the volatile fraction of the compounds, unlike Grieves et al. (2018) who analysed the waxes which may represent the non-volatile fraction of secretions, but may contain compounds that attract mosquitoes. Overall, our results suggest that the headspace of birds may not merely reflect the composition of uropygial gland secretions but may also be affected by other factors such as the bird's microbiota that degrade the uropygial secretion present in feathers (Ross et al., 2019). This could also differ between *Plasmodium*-infected and uninfected birds, or even among individuals of different age. This information demonstrates the necessity to develop further analyses to identify potential differences in the chemical composition of headspace of birds, instead of only focussing on the uropygial gland secretions. In addition, further studies should be conducted on adult

birds, as the composition of uropygial gland secretions may differ between bird ages. Malarial parasites could modify the odour profile of infected humans by stimulating the release of mosquito attractants (e.g. isoprenoid precursors) (Emami et al., 2017; De Moraes et al., 2018). In birds, a potential candidate in this process could be nonanal, which may play a key role in *Cx. pipiens*-bird interactions (Syed and Leal, 2009). This possibility merits further investigation.

To summarize, this study provides clear support for the host manipulation hypothesis in a natural host-parasite assemblage. The results reported here provide evidence for the existence of differences between the odours of *Plasmodium*-infected and uninfected birds, which lead to a differential attraction of mosquito vectors. However, we also show that the volatile compounds in uropygial gland secretions alone are not directly responsible for this differential attraction and that there are probably additional factors that modify the resultant body odour of infected individuals.

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Figure legend

Fig. 1. Mean ($\pm$ S.E.) number of *Culex pipiens* mosquitoes that actively flew towards two different stimuli, i.e, uropygial gland secretion (left) and headspace from the whole body odour (right), of *Plasmodium*-infected and uninfected house sparrows (*Passer domesticus*).

Table 1. Mean relative proportion ($\pm$ S.D.) of volatile compounds found in the uropygial gland secretion of *Plasmodium*-infected and uninfected male and female house sparrows (*Passer domesticus*). The number of individuals presenting each compound is shown in brackets.

RT	Compounds	Males (n=21)				Females (n=16)			
		Uninfected (n=10)		Infected (n=11)		Uninfected (n=8)		Infected (n=8)	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Ketones									
13.2	2-Undecanone	ND		ND		0.34 (2)	0.77	0.33 (1)	0.93
15.8	2-Dodecanone	0.59 (4)	1.19	1.22 (4)	2.09	0.90 (4)	1.08	2.53 (3)	4.72
17.2	6,10-dimethyl-2-undecanone	ND		ND		0.02 (1)	0.06	ND	
17.7	2-Tridecanone	ND		ND		0.01 (1)	0.03	ND	
18.3	5-Tridecanone	3.87 (4)	10.57	0.94 (4)	1.82	0.99 (4)	1.12	2.14 (4)	2.85
20.1	Unidentified ketone	ND		ND		0.45 (1)	1.27	ND	
20.7	2-Tetradecanone	0.82 (3)	2.19	1.23 (3)	2.49	1.27 (4)	1.40	1.17 (4)	1.33
23.0	2-Pentadecanone	0.34 (3)	0.67	1.16 (4)	2.43	0.47 (3)	0.66	1.04 (4)	1.18
23.6	4-Hexadecanone	ND		ND		0.03 (1)	0.08	ND	
24.0	Unidentified ketone	ND		0.20 (1)	0.68	0.03 (1)	0.08	0.05 (1)	0.14
25.2	2-Hexadecanone	0.17 (2)	0.45	0.31 (3)	0.79	0.62 (4)	0.78	0.76 (4)	0.90
25.6	Unidentified ketone	ND		ND		0.09 (1)	0.26	ND	
27.3	2-Heptadecanone	1.45 (5)	2.34	0.36 (4)	0.67	0.12 (1)	0.33	0.28 (1)	0.79
28.3	3-Octadecanone	ND		ND		0.01 (1)	0.03	ND	
29.0	1,13-Tetradecadien-3-one	4.83 (6)	4.91	7.01 (8)	5.64	5.40 (5)	5.48	6.41 (4)	8.55
29.3	2-Nonadecanone	0.22 (2)	0.55	0.17 (1)	0.56	ND		0.15 (2)	0.30
Alcohols									
15.3	Undecanol	ND		ND		0.02 (1)	0.06	ND	

17.8	Dodecanol	ND	ND		0.13 (1)	0.36	ND	
18.2	Unidentified alcohol	ND	ND		0.02 (1)	0.04	ND	
22.9	Tridecanol	ND	0.14 (2)	0.38	1.00 (1)	2.82	0.17 (1)	0.47
23.2	Tetradecanol	ND	0.04 (1)	0.15	0.28 (2)	0.53	0.15 (1)	0.43
24.8	Pentadecanol	0.96 (3)	1.59	1.10 (3)	1.99	3.84 (3)	0.97 (2)	1.97
26.9	Hexadecanol	2.48 (4)	3.86	5.11 (7)	4.87	2.69 (3)	2.36 (4)	3.61
28.9	Heptadecanol	4.08 (5)	4.57	5.69 (7)	5.51	8.25 (5)	6.24 (5)	7.22
30.8	Octadecanol	43.58 (9)	18.13	40.99 (10)	25.96	41.10 (6)	35.45 (8)	15.40
32.6	Eicosanol	0.29 (2)	0.72	ND	ND		0.09 (1)	0.26
34.3	Docosanol	ND	ND		ND		1.00 (1)	2.82
Aldehydes								
21.1	Tetradecanal	0.29 (2)	0.67	ND		0.15 (2)	0.31	0.20 (2)
23.4	Pentadecanal	0.05 (1)	0.16	ND		0.10 (2)	0.21	0.06 (1)
25.6	Hexadecanal	1.06 (4)	1.54	9.52 (3)	30.03	0.38 (3)	0.59	0.27 (1)
27.6	Heptadecanal	0.21 (2)	0.45	0.21 (2)	0.54	0.29 (2)	0.65	0.19 (1)
29.5	Octadecanal	20.28 (9)	30.30	8.18 (8)	8.53	18.64 (8)	33.59	11.64 (6)
Esters of carboxylic acids								
22.2	Tridecanoic acid, 3-methyl-, methyl ester	ND		0.32 (3)	0.78	0.45 (2)	1.22	0.43 (2)
23.7	Decanoic acid, ethyl-, 3-methyl ester	3.18 (2)	9.45	0.38 (1)	1.25	ND		ND
24.4	Pentadecanoic acid, methyl ester	ND		0.05 (2)	0.12	0.01 (1)	0.01	0.32 (2)
25.8	Pentadecanoic acid, ethyl ester	ND		0.54 (1)	1.81	ND		ND
26.5	Pentadecanoic acid, 14-methyl-, methyl ester	ND		0.31 (3)	0.87	ND		0.64 (3)
27.8	Hexadecanoic acid, ethyl ester	0.18 (1)	0.56	1.05 (3)	1.88	0.33 (2)	0.85	ND
29.7	Heptadecanoic acid, ethyl ester	1.35 (4)	2.01	1.20 (4)	2.22	0.06 (1)	0.18	0.38 (1)
31.5	Octadecanoic acid, ethyl ester	0.22 (2)	0.49	0.76 (4)	1.23	1.23 (3)	2.09	0.85 (3)
Alcohol acetates								
25.4	Tetradecanol, acetate	0.05 (1)	0.15	0.32 (3)	0.69	0.60 (3)	0.92	0.69 (3)

27.4	Pentadecanol, acetate	1.42 (6)	2.18	1.24 (4)	2.38	1.96 (5)	1.74	2.98 (5)	3.49
29.4	Hexadecanol, acetate	1.96 (6)	2.97	2.27 (7)	3.45	2.11 (5)	1.92	6.44 (6)	7.10
31.2	Heptadecanol, acetate	3.46 (7)	4.60	4.13 (5)	7.02	3.54 (6)	2.67	7.13 (6)	5.77
32.9	Octadecanol, acetate	2.96 (6)	4.14	3.62 (5)	5.17	2.07 (4)	2.35	8.02 (5)	12.04
33.6	Nonadecanol, acetate	ND		0.20 (1)	0.67	ND		0.24 (1)	0.67
Others									
14.6	1,2,3-Propanetriol, triacetate (=triacetin)	ND		ND		0.01 (1)	0.02	ND	

RT, retention time; ND, not detected.

Table 2. Mean relative proportion ($\pm$ S.D.) of the major classes of volatile compounds found in the uropygial gland secretion of infected and uninfected male and female house sparrows (*Passer domesticus*). Results from ANOVA tests (estimate, standard error (S.E.), and *P* value) on the effects of bird sex, infection status by *Plasmodium* and their interaction on the relative proportion of these compounds are shown.

Classes of compounds	Male		Female		Sex			Infection status			Interaction Sex - Infection status		
	Uninfected	Infected	Uninfected	Infected	Est.	S.E.	P	Est.	S.E.	P	Est	S.E.	P
Alcohols	51.38 (22.94)	53.08 (31.14)	57.31 (24.46)	46.43 (22.18)	-0.11	0.23	0.64	-0.21	0.25	0.41	0.24	0.33	0.47
Aldehydes	21.89 (29.93)	17.91 (28.56)	19.57 (33.17)	12.36 (20.30)	0.14	0.86	0.87	-0.54	1.03	0.60	0.29	1.30	0.82
Alcohol acetates	9.50 (13.51)	11.78 (16.16)	10.28 (7.99)	23.71 (22.10)	-0.08	0.63	0.90	0.84	0.55	0.14	-0.62	0.79	0.44
Ketones	12.30 (10.54)	12.60 (10.81)	10.75 (7.88)	14.86 (12.85)	0.15	0.48	0.75	0.37	0.48	0.45	-0.34	0.64	0.60
Ester of carboxilic acids	4.92 (9.02)	4.63 (7.40)	2.08 (2.10)	2.63 (3.78)	0.89	0.92	0.34	0.24	1.06	0.81	-0.31	1.24	0.81