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PhD Thesis

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THE ROLE OF UROPYGIAL SECRETION AND BIRDS BODY ODOUR ON THEIR INTERACTION WITH MOSQUITOES AND PARASITES

Alazne Díez Fernández

PhD Thesis

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Universidad de Sevilla
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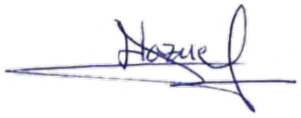
The role of uropygial secretion and birds body odour on their interaction with mosquitoes and parasites

Memoria presentada por la Licenciada en Biología, Alazne Díez Fernández, para optar al título de Doctor por la Universidad de Sevilla.



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CERTIFICAN:

Que los trabajos de investigación desarrollados en la Memoria de Tesis Doctoral "*The role of uropygial secretion and birds body odour on their interaction with mosquitoes and parasites*", son aptos para ser presentados por la Licenciada Alazne Díez Fernández ante el Tribunal que en su día se designe, para aspirar al grado de Doctor por la Universidad de Sevilla.

Y para que así conste, y en cumplimiento de las disposiciones legales vigentes, firman el presente documento en Sevilla, a 7 de Julio de 2020.

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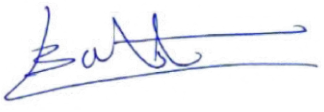

Tutor: Javier Balbontín Arenas

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Mosquito and bird sampling were carried out with all the necessary permits issued by the regional Department of the Environment (Consejería de Medio Ambiente, Junta de Andalucía). Entomological surveys and bird sampling on private lands were conducted with all the necessary permits and landowners consent.

A mi ama, a mi apa

A mi equipo

"Si he visto más lejos es porque estoy sentado

sobre los hombros de gigantes"

Isaac Newton

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Annex

ABSTRACT

Chemical signals are implied in vital proceses such as food acquisition, reproduction, predation avoidance and interspecific interactions. In blood sucking insects such as mosquitoes, chemical signals play a key role by allowing the detection of their vertebrate hosts. Besides interspecific differences, vertebrate host characteristics such as age, sex, size and infection by blood parasites, may affect the chemical signals emitted by hosts, which may in turn affect vector-host interactions and ultimately, the transmission dynamics of vector-borne diseases.

Mosquitoes transmit parasites and other pathogens causing important diseases that affect humans, wildlife, and domestic animals. This is the case of malaria parasites, which may largely affect host population dynamics. However, the mechanisms affecting the interactions between mosquitoes, parasites, and vertebrate hosts are not fully understood, especially in the case of wild non-model species. The aim of this thesis is to assess the role of different bird chemical cues (uropygial gland secretions and body odour) in mosquito attraction and the effect of host infection by avian malaria parasites on these interactions. To deal with this important question, I used a multidisciplinary approach that combines techniques from different research areas (e.g. molecular parasitology, entomology, and ornithology), and data from field and laboratory experiments under controlled conditions. As study system, I used i) two wild passerines as vertebrate hosts, the common blackbird (*Turdus merula*) and the house sparrow (*Passer domesticus*), ii) two species of mosquitoes, namely the common house mosquito (*Culex pipiens*) and the marshland mosquito (*Aedes caspius*), and iii) avian haemospodians, including mosquito-borne parasites of the genus *Plasmodium*.

In order to better understand the dynamics of host-vector-pathogen interactions, I tested the host manipulation hypothesis, which argues that parasites may modify certain characteristics of their hosts to increase their

transmission success. Thus, malaria infected birds would be more attractive to mosquitoes than uninfected ones, thereby enhancing the contact rates between parasites and insect vectors. However, the mechanisms underlying this differential attraction are still unknown. I studied the potential effect of parasite infections on the odours emitted by birds and how this may in turn affect the interactions with mosquitoes. Secretions of the uropygial gland are considered as one of the main sources of bird odour, so the effects of parasites on bird-vector interactions could be driven by their effects on the composition of these secretions. I performed different studies to determine the potential factors affecting the composition of birds' uropygial secretions, including parasite infections, and subsequently, I tested the role of secretions and body odour in mosquito attraction.

The overall composition of uropygial secretions of wild birds differed between sexes and age classes, while this was not the case for the infection by avian haemosporidian parasites neither the habitat type (forest *vs* urban areas). Further analyses revealed the presence of the pollutant DDE in uropygial secretions. DDE is mainly derived from the DDT used decades ago. The relative proportion of DDE was higher in older and forest-dwelling birds, and increased with bird´s body mass. Overall these results support the role of both intrinsic and extrinsic factors in between-individuals variation in the composition of uropygial gland secretions. However, these results do not provide support for the potential effect of parasite infections on the composition of bird uropygial secretions.

On the basis of the importance of the uropygial gland secretion as a source of bird odour, I evaluated the role of this secretion in the attraction of two species of mosquitoes with differential feeding patterns, namely the ornithophilic *Cx. pipiens* and the mammophilic *Ae. capius*. Both species of mosquitoes were similarly attracted to this stimulus (uropygial gland secretions + CO_2) than to the control (only CO_2) in a dual choice olfactometer, suggesting that the attraction of mosquitoes to avian hosts is not mediated by this chemical cue. Subsequently, I tested the role of *Plasmodium* infection in the attraction of *Cx. pipiens*

mosquitoes towards both uropygial secretions and bird´s body odours (headspace). *Culex pipiens* were more attracted towards the headspace of *Plasmodium* infected than uninfected birds, while no differences were found in the attraction of mosquitoes when the stimulus tested was the uropygial gland secretion. These results suggest that *Plasmodium* parasite modify bird body odour increasing the attraction of mosquitoes and therefore, increasing its capacity of transmission to new vertebrate hosts. However, these effects are not driven by changes in the volatile fraction of the uropygial gland secretion.

In sum, this thesis provides novel evidence into the complex mechanisms that drive the interactions between parasites, vectors, and vertebrates, and highlights the role of chemical cues such as the odour of birds in the attraction of mosquitoes to individuals infected by haemosporidians. These results may have important implications for the epidemiology of *Plasmodium* parasites in natural environments and open new questions for future studies on the identification of key components of bird´s odours determining mosquito attraction.

RESUMEN

Las señales químicas están implicadas en procesos vitales tan importantes como la búsqueda de alimento, reproducción y defensa frente a depredadores, entre otras, desempeñando, por tanto, un importante papel en las interacciones intra e interespecíficas. En insectos que se alimentan de sangre, como los mosquitos, el uso de pistas químicas resulta esencial para detectar a sus hospedadores vertebrados. Además de las diferencias existentes entre especies de hospedadores, ciertas características individuales como la edad, el sexo, el tamaño corporal y la infección por parásitos sanguíneos podrían afectar a las señales químicas que emiten, lo que a su vez podría afectar a las interacciones vector-hospedador y, en última instancia, a la dinámica de transmisión de enfermedades transmitidas por vectores.

Los mosquitos transmiten parásitos y otros patógenos causantes de importantes enfermedades que afectan a humanos y animales, tanto silvestres como domésticos. Este es el caso de los parásitos de la malaria, que pueden influir en gran medida en la dinámica de las poblaciones de sus hospedadores. Sin embargo, los mecanismos que afectan a las interacciones entre mosquitos, parásitos y hospedadores vertebrados siguen sin estar claros, especialmente en el caso de especies silvestres-no modelo. El objetivo de esta tesis es entender el papel de las diferentes pistas químicas que generan las aves (secreciones de la glándula uropigial y olor corporal) en la atracción de mosquitos, así como el efecto de la infección por parásitos de la malaria aviar del hospedador sobre estas interacciones. Para abordar esta importante pregunta, utilicé un enfoque multidisciplinar que combina diferentes áreas de investigación (p.e., parasitología molecular, entomología y ornitología), datos de campo y experimentos de laboratorio bajo condiciones controladas. Mi sistema de estudio incluye i) dos aves paseriformes silvestres como hospedadores vertebrados, el mirlo común (*Turdus merula*) y el gorrión común (*Passer domesticus*), ii) dos especies de mosquitos, el mosquito común (*Culex pipiens*) y el mosquito de

marismas (*Aedes caspius*), y iii) hemosporidios presentes en aves, incluidos los parásitos del género *Plasmodium* transmitidos por mosquitos.

Para comprender mejor la dinámica de las interacciones entre hospedador-vector-patógeno, evalué la hipótesis de la manipulación del hospedador, la cual propone que los parásitos modifican ciertas características de sus hospedadores para aumentar su éxito de transmisión. Por lo tanto, las aves infectadas con malaria resultarían más atractivas para los mosquitos que aquellas no infectadas, aumentando así las tasas de contacto entre parásitos e insectos vectores. Sin embargo, aún se desconocen los mecanismos que subyacen a esta atracción diferencial. Estudié el efecto potencial de la infección por parásitos sobre los olores emitidos por las aves y como esto a su vez, podría afectar a las interacciones con los mosquitos. Las secreciones de la glándula uropigial son consideradas como una de las principales fuentes de olor de las aves, por lo que los efectos de los parásitos en las interacciones entre las aves y los vectores podrían estar mediados a través de cambios en la composición de estas secreciones. Realicé diferentes estudios para determinar los factores potenciales que afectan a la composición de las secreciones uropigiales de las aves, incluida la infección por parásitos y, posteriormente, evalué el papel de las secreciones y el olor corporal en la atracción de mosquitos.

La composición de las secreciones uropigiales de las aves silvestres difirió entre clases de edad y sexo, mientras que no hubo diferencias asociadas a la infección por parásitos hemosporidios ni al tipo de hábitat (bosque *vs* áreas urbanas). Análisis adicionales revelaron la presencia del contaminante DDE en las secreciones uropigiales de las aves. La degradación del DDT usado hace décadas origina DDE. La proporción relativa de DDE detectada fue mayor en aves adultas y en aquellas capturadas en bosques frente a las de ciudad, siendo superior también en aves con un mayor peso corporal. En general, estos resultados apoyan el papel de factores intrínsecos y extrínsecos en las variaciones interindividuales en la composición de las secreciones de la glándula uropigial.

Sin embargo, estos resultados no respaldan un efecto de la infección por parásitos de la malaria aviar en la composición de las secreciones de estas aves.

Teniendo en cuenta la importancia de la secreción de la glándula uropigial como fuente principal del olor en las aves, evalué el papel de esta secreción en la atracción de dos especies de mosquito con patrones de alimentación diferentes, el mosquito *Cx. pipiens* que se alimenta principalmente de aves y el mosquito *Ae. Capius* que se alimenta de mamíferos. Ambas especies de mosquitos mostraron una atracción similar hacia el estímulo (secreciones de la glándula uropigial + CO_2) y el control (solo CO_2) en un olfatómetro de doble elección, lo que sugiere que la atracción de los mosquitos hacia las aves no está mediada por la fracción volátil de esta secreción. Posteriormente, evalué el papel de la infección por *Plasmodium* en la atracción de los mosquitos *Cx. pipiens* hacia las secreciones uropigiales y olor corporal de las aves. *Culex pipiens* mostró mayor atracción hacia el olor corporal de las aves infectadas por *Plasmodium* que al de aves no infectadas, pero no se encontraron diferencias cuando el estímulo utilizado fue la secreción de la glándula uropigial. Estos resultados sugieren que los parásitos de la malaria aviar modifican el olor corporal de las aves, aumentando así la atracción de los mosquitos hacia aves infectadas y, por tanto, su capacidad de transmisión a nuevos hospedadores. No obstante, estos efectos no son producidos por cambios en la composición de la fracción volátil de la secreción de la glandula uropigial.

En resumen, esta tesis proporciona nuevas evidencia sobre los complejos mecanismos que rigen las interacciones entre parásitos, vectores y vertebrados, y destaca el papel de los compuestos químicos, como los que generan el olor de las aves, en la atracción de mosquitos hacia individuos infectados por hemosporidios. Estos resultados tienen también implicaciones importantes para la comprensión de la epidemiología de los parásitos del género *Plasmodium* en ambientes naturales, y generan nuevas preguntas para futuras investigaciones dirigidas a la identificación de compuestos clave que conforman los olores de las aves y determinan la atracción de los mosquitos.

GENERAL SECTION

GENERAL INTRODUCTION

Animal communication allows individuals to make decisions based on the information they receive and process. Communication may occur between individuals of the same or different species, but also between organisms and the environment. These interactions are based on the exchange of information that visual, acoustic, tactile or olfactory (chemical) cues convey. In particular, the importance of chemical communication has largely been studied in a variety of taxa, mainly mammals (Zinkevich & Vasilieva 2001; Surov & Maltsev 2016). For the case of birds, the relative importance of olfaction with respect to other senses has received comparatively less attention. However, recent studies performed on different bird species, from passeriforms to procellariforms, support that birds may indeed use olfaction in many different contexts. For example, olfaction is involved in mate choice (Bonadonna et al. 2007), orientation (Wallraff 2004; Gagliardo 2013), antipredator behaviour (e.g. nest defence; Amo et al. 2017), and individual or species communication and recognition (Hagelin and Jones 2007). However, the importance of chemical cues may vary according to the distance from the receiver to the stimuli and may be combined with information provided by other stimuli. For example, the wandering albatrosses (*Diomedea exulans*) use olfaction to detect preys at large distances in the ocean, while they rely on vision at short distances (Nevitt et al. 2008). Likewise, by combining olfaction and vision, the oriental honey buzzard (*Pernis orientalis*) can detect the pollen from the pollen dough provided by beekeepers to bees (Yang et al. 2015).

Insects also rely on olfactory cues such as plant odours to, for example, locate food (e.g., nectar-seeking mosquitoes, Lahondère et al. 2020) or lay eggs (Städler et al. 2002). Likewise, insect groups that feed on blood such as ticks and mosquitoes use, among other cues, olfaction to locate their vertebrate hosts (Osterkamp et al. 1999; Qiu & Van Loon 2010). Mosquito odorant receptors are

located in the antennae and, to a lesser extent, in the maxillary palps and the proboscis, in hair-like structures called sensilla (Hill et al. 2009).

Blood-sucking insects, such as mosquitoes, are of special interest because different species transmit pathogens causing infectious diseases in humans, livestock, and wildlife (Harrus & Baneth 2005, Little 2014). Mosquitoes of the genera *Anopheles*, *Aedes* and *Culex* are of major importance as they are involved in the transmission of pathogens causing diseases such as malaria, dengue, chikungunya, Zika and West Nile fever (Chen et al. 1993; Shin et al. 2002; Balenghien et al. 2008, Dubrulle et al. 2009; Kimura et al. 2010; Weger-Lucarelli et al. 2016).

Avian malaria parasites of the genus *Plasmodium* and the malaria-like parasites *Haemoproteus* and *Leucocytozoon* are haemosporidians commonly infecting birds (Valkiūnas 2005). These three parasite genera share similar life cycles, but are transmitted by different insect vectors. While *Plasmodium* is transmitted by mosquitoes, *Haemoproteus* is transmitted by biting midges (Ceratopogonidae, *Culicoides* spp.) and louse flies (Hippoboscidae), and *Leucocytozoon* is transmitted by black flies (Simuliidae) (Valkiūnas 2005). Avian malaria parasites depend on these vectors to be transmitted from an infected bird to an uninfected and susceptible bird. Both insect vectors and avian hosts suffer the costs of parasite infections (Merino et al. 2000; Marzal et al. 2005; Martínez-de la Puente et al. 2010; Gutiérrez-López et al. 2019). Thus, parasites, vectors, and vertebrate hosts may be involved in a co-evolutionary arms race (Thompson 1998), where parasites may develop different strategies to increase their transmission success, while vectors and vertebrate hosts may reduce the contact rates with parasites and/or develop mechanisms (i.e. immune system or behavioural responses) to fight-off these infections (Combes 2001; de Roode et al. 2008). Therefore, to deeply understand the transmission dynamics of infectious diseases it is essential to integrate the relationships between the three protagonists, hosts, vectors, and pathogens in studies on the ecology and evolution of parasite transmission.

Parasites and other pathogens drive evolution of their hosts (Hamilton & Zuk 1982) having large impacts on their population dynamics (Hudson et al. 1992; Campbell-Lendrum & Molyneux 2005) and compromising their conservation (Plowright et al. 2017). Parasites can also affect the behaviour, appearance and physiology of their hosts in a way that enhance their transmission success, a phenomenon that is known as the "host manipulation hypothesis" (Poulin 2011). For the case of vector-borne pathogens, insect vectors should be also considered in studies of host manipulation by parasites, because both insect vectors and vertebrate hosts are susceptible to being manipulated. The host manipulation hypothesis has been tested in a diversity of host-pathogen assemblages, including those with complex life cycles (Table 1). Among them, a well-studied example of parasitic manipulation is the case of Toxoplasmosis, a disease produced by the protozoan *Toxoplasma gondii*, which reduces the aversion of the intermediate host of the parasite (rodents) to the definitive host (cats), thus facilitating parasites to complete their cycle life (Hughes & Libersat 2019). Curiously, this parasite seems to increase the reckless of infected humans (Fuglewicz et al. 2017). Similarly, *Plasmodium* infection may increase the attractiveness of infected humans to mosquito vectors (Batista et al. 2014). A study on rodent malaria reported that mice infected by *Plasmodium chabaudii* attracted more *Anopheles stephensi* mosquitoes, likely due to changes in the volatile fraction of the host odour profiles, but only when levels of gametocytes were high (De Moraes et al. 2014). These results suggest that odour cues may be target stimuli affected by parasites to increase the attraction of mosquitoes towards infected vertebrate hosts. Although there exist evidence indicating that infected hosts can suffer behavioural and physiological changes (Poulin 2011; Heil 2016), the mechanisms by which parasites alter host condition are not yet well understood.

Table 1. Some examples of parasites and other pathogens that manipulate their hosts to favour their transmission success.

Pathogen/Parasite	Host	Manipulation	Effect	Reference
Hymenoepimecis sp. (Arthropoda)	*Plesiometa argyra* (Spider)	Behaviour Morphology	Spiders produce physical structures to protect parasitoids	Eberhard 2000
Ophiocordyceps unilateralis (Fungus)	Ant (tribe Camponotini)	Behaviour	Alters ant´s behaviour causing it to die in an exposed position	Evans et al. 2011
Paragordius tricuspidatus (Nematoda)	*Nemobius sylvestris* (Cricket)	Behaviour Physiology	Cricket "suicide" jumping into water. Neuronal basis modification.	Thomas et al. 2003
Cestodes (Platyhelmintes)	*Artemia parthenogenetica* (Artemia)	Behaviour Physiology	Increase photophilous and their brightness	Sánchez et al. 2007
Leucochloridium paradoxum (Platyhelmintes)	*Succinea putris* (Snail)	Behaviour	Increased exposure in illuminated and higher places	Wesołowska & Wesołowski 2014
Podocotyloides stenometra (Platyhelmintes)	*Porites spp* (Reef corals)	Physiology	Increase brightness (visibility)	Aeby 2002
Plasmodium gallinaceum (Protozoo)	*Aedes aegypti* (Mosquito)	Behaviour Physiology	Affect the threshold volume of blood for host-seeking behaviour	Koella et al. 2002
Toxoplasma gondii (Protozoo)	*Rattus norvegicus* (Rat)	Behaviour	Reduced aversion to predators	Berdoy et al. 2000
Hz-2V (Virus)	*Helicoverpa zea* (Moth)	Behaviour Physiology	Increased contact rates between males-females	Burand et al. 2005

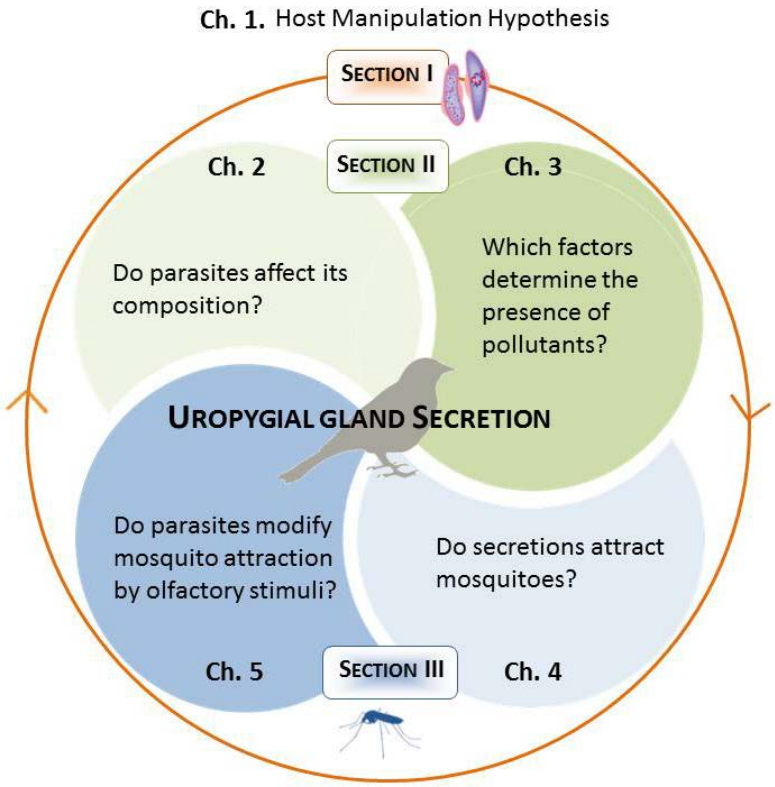
Birds, mosquitoes, and *Plasmodium* represent excellent models to test the host-manipulation hypothesis (Rivero & Gandon 2018). Previous studies indicate that infected wild birds attract more *Culex pipiens* mosquitoes than individuals treated with an antimalarial drug (Yan et al. 2018). As in the case of other vertebrate-malaria models (e.g. rodents, De Moraes et al. 2014), variation in body odour profiles due to parasite infection has been invoked as the mechanism underlying changes in vector attraction. In particular, the effect of infection on the composition of the secretions of the uropygial gland has been proposed as the mechanism likely explaining the increased attraction of mosquitoes to *Plasmodium*-infected birds (Cornet et al. 2013).

The uropygial gland (also called preen gland) is a holocrine gland located dorsally at the base of the tail of birds (Clark 2004). With the exception of some species within the orders Piciformes, Psittaciformes, Struthioniformes and Columbiformes, the uropygial gland is present in most bird species, having a variety of shapes and sizes (Johnston 1988). Nonetheless, the typical morphology of the gland consists of two lobes and a papilla that facilitates the release of the secretion that birds spread with the bill over their plumage. The uropygial gland produces a complex and variable secretion composed predominantelly of alkanes, ketones, aldehydes, alcohols (volatile fraction) and waxes (non-volatile fraction) (Campagna et al. 2012). The chemical composition of the uropygial gland secretion varies according to bird characteristics, such as species (Haribal et al. 2009), sexes (Amo et al. 2012) and age classes (Shaw et al. 2011). In addition, intraspecific variations may occur between populations (Whittaker et al. 2010), seasons (Reneerkens et al. 2002) and diet types (Thomas et al. 2010).

The uropygial gland secretion has several functions, most notably waterproofing and protection of plumage against sun damage (Giraudeau et al. 2010), but also anti-microbial activity against feather degrading bacteria (Shawkey et al. 2003). In addition, the secretion may function as a potential mechanism of removal of toxic substances such as pesticides (e.g. in seabirds, Yamashita et al. 2007). Moreover, secretions of the uropygial gland may be

involved in intra and interspecific communication (Moreno-Rueda 2017). Together with other factors, including feather-skin microbiota and intestinal microbiota, genetic components, the secretions of the uropygial gland are considered to a large extent responsible for birds' odour (Haribal et al. 2009). Thus, bird odour would be the result of the oxidation and/or reduction of the compounds of the secretion (Wisthaler & Weschler 2010). For example, Mardon et al. (2011) suggested that some short-chain odorants present in feathers of blue petrels (*Halobaena caerulea*) are part of the degradation of the compounds from the non-volatile fraction of the uropygial secretion. However, in spite of their potential relevance in the interactions of birds with parasites and vectors, the role of secretions of the uropygial gland and bird odours in the transmission of avian malaria has not been studied yet (Fig. 1).

Fig. 1. Schematic representation of the interactions between uropygial gland secretion, vectors, and vector-borne pathogens analysed in this thesis. This thesis addresses the host manipulation hypothesis as a mechanism used by parasites to enhance their transmission success by altering their hosts´traits (Section I, Chapter 1). In Section II, Chapter 2 explores the relationship between parasite infection and the composition of the uropygial gland secretion, while Chapter 3 deals with the endogenous and exogenous factors that may be related to the presence of pollutants in the secretion. In Section III, Chapter 4 assesses whether the secretion may act as an attractive cue for two different species of mosquitoes, while Chapter 5 returns to the host manipulation hypothesis described in the first chapter, to address whether parasites may alter odour stimuli to increase the contact rates between birds and vectors, thus increasing parasite transmission.



STUDY MODELS

Vertebrate hosts

In this thesis, I used two widespread passerines as avian models, namely the house sparrow (*Passer domesticus* Linnaeus, 1758) and the common blackbird (*Turdus merula* Linnaeus, 1758) (Fig. 2 A-B). The house sparrow is a resident, group living species that is commonly present in cities. Their body length is 14-16cm and the body mass ranges from 24 to 29g. Adults present clear sexual differences in their plumage, but these are inexistent in juveniles until the first moult (Svensson et al. 2010). Blackbirds are common in urban, suburban and rural areas, with resident populations across the Iberian Peninsula and high numbers of individuals wintering in Spain (Aparicio 2011). The body length is about 23-29cm and body mass usually ranges from 75 to 100g. Adults are sexually dimorphic in their beak and plumage coloration, but no clear differences exist in juveniles (Svensson et al. 2010). Their diet is almost omnivore, and includes insects and oligochaetes (predominantly earthworms *Lumbricus terrestris*) and a high proportion of fruits in autumn and winter (Guitián et al. 2000). These bird species were selected as study models because they are common hosts of mosquitoes (Hatchwell et al. 2001; Muñoz et al. 2012; Ferraguti et al. 2018) being naturally infected by different mosquito-borne pathogens in Southern Spain (see below). These birds may play an important role in the transmission of flaviviruses such as West Nile virus and Usutu virus in the area (Ferraguti et al. 2016; Martínez-de la Puente et al. 2018), which naturally circulate between birds and mosquitoes and may occasionally affect humans and/or horses. In addition, both bird species are commonly infected by *Plasmodium* and the related haemosporidians *Haemoproteus* and *Leucocytozoon* (MalAvi database, Bensch et al. 2009).

Insect vectors

I used mosquitoes of two genera; *Aedes* (comprising the marshland mosquito *Aedes* (*Ochlerotatus) caspius* and *Aedes vittatus*, see Annex 1) and *Culex* (the common house mosquito *Culex pipiens*) (Fig. 2 C-D). Females of these species, the only blood-sucking sex, show a relatively opportunistic feeding behaviour, being able to feed on blood from both mammals and birds. In spite of that, birds dominate the diet of *Cx. pipiens*, while the *Ae. caspius* and *Ae. vittatus* usually feed on mammal blood (Martínez-de La Puente et al. 2001; Muñoz et al. 2012). *Culex pipiens* is a well-known competent vector of avian malaria (Valkiūnas 2005, Gutiérrez-López et al. 2020). By contrast, although avian *Plasmodium* has been molecularly detected in *Ae. caspius* (Ferraguti et al. 2013), further experimental studies provide support for the incapacity of these parasites to develop in mosquito saliva (Gutiérrez-López et al. 2020).

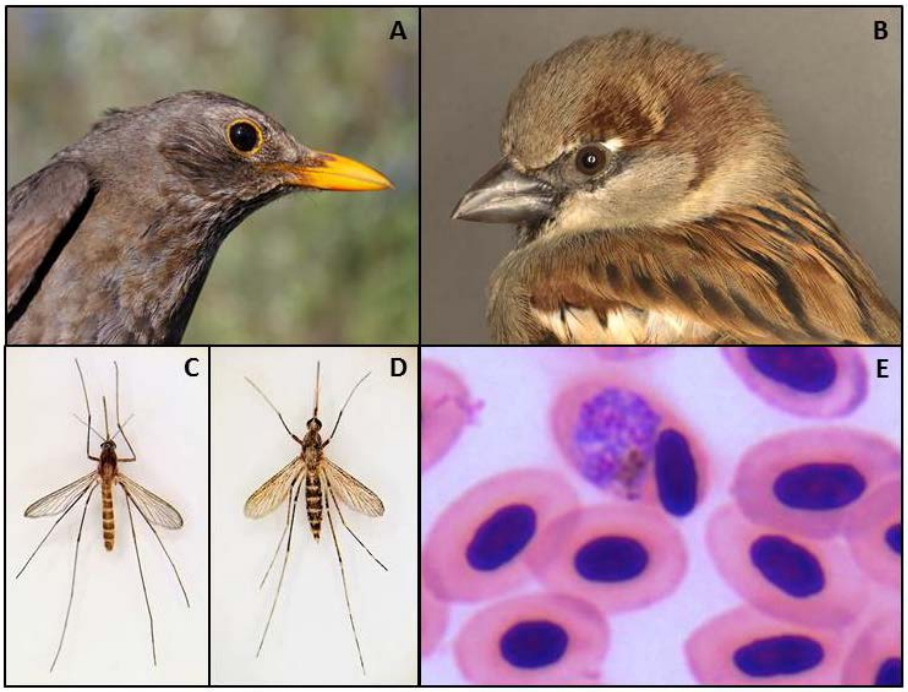
Haemosporidian parasites

I used birds infected by parasites of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* (Fig. 2 E). The life cycle of these parasites includes different phases in the vertebrate (intermediate) host and the insect vector. In brief, female mosquitoes feed on an infected bird and acquire parasite gametocytes present in the blood. The sexual phase of the parasite begins and these gametocytes develop into gametes and fuse as zygotes to form ookinetes that penetrate into the midgut wall. There, ookinetes develop into oocystes and when they invade the salivary glands located in the thorax, they develop into sporozoites, which is the parasite stage/form ready to be transmitted. When the mosquito bites a new host, the sporozoites pass onto birds and the asexual phase starts. The sporozoites travel to liver and develop into merozoites, which invade red blood cells and produce macro- and microgametocytes that remain in the bird blood stream. The infective forms of parasite are then ready to be transmitted to a new mosquito while feeding. The study of these parasites has notably increased during the last

decades (Marzal 2012), mainly thanks to the development of molecular tools that have facilitated the identification of the parasites (Hellgren et al. 2004).

Fig. 2. The main study models. Upper panel: the avian hosts (A) Blackbird *Turdus merula* and (B) House sparrow *Passer domesticus*. Lower panel: the vectors (C) *Culex pipiens* and (D) *Aedes* (*Ochlerotatus) caspius*, and (E) the parasite *Plasmodium sp.* in avian red blood cells.

Photo credits: A and E) Alazne Díez Fernández; B) Daniel Alonso; C and D) Juana Moreno.



OBJECTIVES

The main aim of this thesis is to test the role of the secretion of the uropygial gland of birds and the bird body odour in the attraction of mosquito vectors and the potential effects of avian malaria parasites on the attraction of mosquitoes towards birds. Consequently, this thesis focuses on the host-manipulation hypothesis, which argues that malaria parasites are able to alter some host traits to increase their transmission success. According to this hypothesis, infected individuals would be more attractive to mosquitoes than uninfected ones, finally increasing the contact rates between blood parasites and competent insect vectors (Section I, **Chapter 1**). To address this issue, I combined tools from different disciplines, including ornithology, molecular ecology, and entomology (see **Annex I**) and used experimental and multidisciplinary approaches to determine i) factors potentially affecting the composition of the uropygial gland secretion, including parasite infections (Section II, **Chapters 2-3**) and ii) the role of these secretions and the bird body odours in the attraction of mosquitoes, considering the potential effect of infections by mosquito-borne parasites on host-vector interactions (Section III, **Chapters 4-5**).

The uropygial gland is an important source of bird body odour. This gland produces the preen oil, which contains two fractions: the volatile (on which this thesis focuses) and the non-volatile fraction. Changes in the composition of the secretions of the uropygial gland could modulate the differential attraction of birds to mosquitoes. Thus, in **Chapter 2**, I assessed the potential effects of both intrinsic, i.e., host age, sex and infection status by haemosporidian parasites, and extrinsic factors, i.e., habitat type, on the composition of the secretions of the uropygial gland of wild birds, using common blackbirds (*Turdus merula*) as study models (**Objective 2**). As a result, the presence of the pesticide dichlorodiphenyldichloroethylene (DDE), a metabolite of the pesticide dichlorodiphenyltrichloroethane (DDT) was detected in bird secretions. Therefore, in **Chapter 3,** I studied those factors potentially determining the

presence of this pesticide in bird secretions and their consequences for bird health (**Objective 3**). To do that, I evaluated the impact of the presence of DDE in bird's secretions on the prevalence of infection by blood parasites and the bird's body condition.

Subsequently, after identifying the factors potentially affecting the composition of the secretions of the uropygial gland of birds, in **Chapter 4** I studied the role of these secretions as attractants of mosquitoes in dual choice olfactometers using wild house sparrows (*Passer domesticus*) as host models (**Objective 4**). Because, mosquitoes show a wide range of feeding patterns with clear interspecific differences, I used two mosquito species with differential feeding preferences, the mammophilic *Aedes caspius* and the ornithophilic *Culex pipiens*, to test the hypothesis that the attraction of host-seeking mosquitoes towards uropygial gland secretion may depend on mosquitoes innate feeding preferences. Finally, in **Chapter 5** I tested the host-manipulation hypothesis by testing the prediction that mosquitoes are more attracted towards malaria infected birds. To do that, I extracted the secretions of the uropygial gland and the body odour of birds naturally infected by *Plasmodium* and uninfected ones and determined the attraction of mosquitoes towards these stimuli in a dual-choice olfactometer (**Objective 5**).

GENERAL MATERIAL AND METHODS

Study area and sampling

The fieldwork was performed in the provinces of Huelva, Seville and Cadiz (Andalusia, Southern Spain). Wild birds were sampled in the field (**Chapters 2-4**) or transferred to the animal experimentation facilities of Doñana Biological Station (EBD-CSIC, Seville) (**Chapter 5**). Mosquito larvae were collected in the same provinces (Huelva and Seville) and transferred to the facilities of EBD-CSIC, where they were maintained in climatic chambers under controlled conditions (28º ± 1ºC, 60-65% relative humidity (RH) and 12:12h light:dark cycle). Adult mosquitoes were sexed and identified to species level by morphology (Schaffner et al. 2001) and confirmed by molecular tools when necessary (see **Annex I**). Subsequently, mosquito females were maintained in insect rearing cages supplemented *ad libitum* with 1% sugar solution, using the same conditions detailed above. The sugar solution was removed 24h before the experiments and they had only access to water without added sugar, which was removed from cages 1h before the start of the trials.

Composition of uropygial gland secretions and bird body odour

Uropygial gland secretions extracted from blackbirds and house sparrows were used in **Chapters 2-3 and 4-5**, respectively. Bird secretions were extracted at the field sites, conserved at -80ºC, and subsequently analysed by gas chromatography coupled with mass spectrometry (GC-MS). To identify the lipophilic components of the uropygial gland secretions, we compared the mass spectra of compounds in the sample with those available in the NIST/EPA/NIH 2002 mass spectra library. To conduct the mosquito behavioural assays in the dual-choice olfactometers, additional samples of the uropygial gland secretions from house sparrows were extracted while birds were at the animal experimentation facilities at EBD-CSIC. Two samples of the whole-body odours

of birds kept in captivity were extracted at least 15 days after the acclimation period (Fig. 3), and were preserved in 600µl of hexane at -80ºC until its use in the behavioural assays in **Chapter 5.**

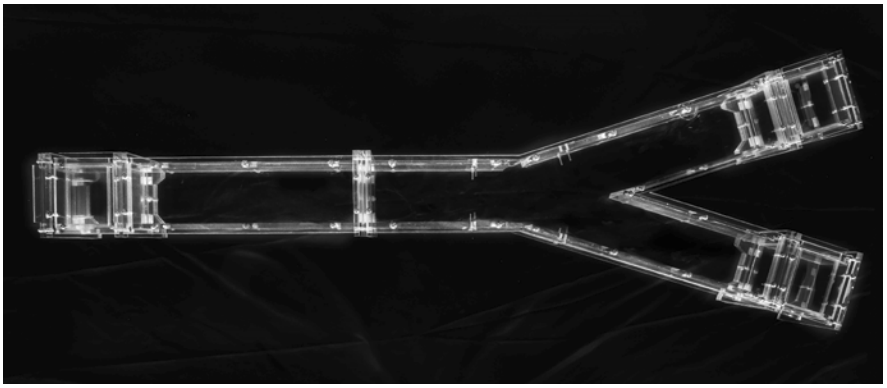
Fig. 3. Extraction of the whole body odours of birds. A house sparrow individual was introduced in a glass container. With an air pump and a flow meter a constant flow of air was generated and circulated through a charcoal-filter, which was connected to a glass desiccator. After passing during 15min. through the glass recipient containing the live bird the body volatiles in the air were retained in ORBO-402 Tenax® TA (60/80) SPT 100/5mg cartridge situated on the top of the glass recipient.

Mosquito behavioural assays in the olfactometer

To test the behavioural response of mosquitoes to bird stimuli in **Chapters 4-5**, I used two dual-choice olfactometers. The Y-shape olfactometer consists of three parts: the acclimation section, the flight section, and two terminal ports with vertical doors to separate the different compartments (Fig. 4). The behavioural responses of mosquitoes towards bird stimuli were measured as the number of mosquitoes moving to one of the two ports in relation to the total number of mosquitoes that responded, i.e., that flew to any one of the two ports. Mosquitoes that did not move or remained in the middle of the olfactometer were considered unresponsive. In **Chapter 4,** I used *Cx. pipiens* and *Ae. caspius* to test their

response towards uropygial gland secretions of uninfected house sparrows + CO_2 *vs* a control without any bird odour (only CO_2). In **Chapter 5** I used *Cx. pipiens* and two types of stimuli: uropygial gland secretion and whole body odour from individuals birds infected by *Plasmodium* and uninfected birds.

Fig. 4. One of the two dual-port olfactometers used for the experiments in this thesis. The Y-shape olfactometer consists of three parts: acclimation section, flight section and two ports with vertical doors to separate the two compartments.



SECTION I

Chapter 1

Are malaria infected birds more attractive to mosquito vectors?

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Abstract

According to the host manipulation hypothesis, parasites modify the hosts' phenotype to maximize their transmission success. Avian malaria parasites and related haemosporidians are vector-borne parasites infecting birds worldwide. Recent studies have reported a higher attraction of mosquitoes to infected birds supporting the host manipulation hypothesis. Changes in the composition of the uropygial gland secretion of birds associated to infections have been proposed as the potential mechanisms explaining this pattern. Here, we critically review the published information on the host manipulation hypothesis in the context of avian malaria infections. We focus this article on the suggested role of the secretions of the uropygial gland and bird odours as mosquito attractants. The role of uropygial gland secretions as attractants of mosquitoes was poorly supported by available literature. By contrast, changes in the odour profile (headspace) of infected birds or a reduction of the anti-mosquito behaviour of infected individuals may explain the parasite-mediated effects on mosquito attraction and biting rates. Finally, we propose future research prospects to identify the role of parasite infections on the interaction between birds and insect vectors.

Keywords: Culicoides, *Haemoproteus*, *Leucocytozoon*, mosquitoes, parasites, *Plasmodium*.

Introduction

The host manipulation hypothesis argues that parasites are able to modify host' phenotype to enhance parasite transmission success. This hypothesis has been largely tested using different study models composed by a diversity of parasites infecting invertebrate and vertebrate hosts including birds (reviewed in Heil 2016).

Avian malaria parasites belonging to the genus *Plasmodium* are widespread parasites of the phylum Apicomplexa, commonly found infecting birds in every continent except Antarctica (Valkiūnas 2005). Avian *Plasmodium* is phylogenetically related to other common parasites also infecting birds such as the malaria-like parasites of the genera *Haemoproteus* and *Leucocytozoon*. These three parasite genera are transmitted from an infected bird to a new susceptible host by different dipteran insect vectors. Species of *Plasmodium* are transmitted by mosquitoes (Culicidae) (Santiago-Alarcón et al. 2012). Hippoboscids (Family: Hippoboscidae) and biting midges (Family: Ceratopogonidae) are considered the main vectors of *Haemoproteus* species while parasites of the genus *Leucocytozoon* are mainly transmitted by blackflies (Family: Simuliidae) (Valkiūnas 2005; Santiago-Alarcón et al., 2012). However, some exceptions have been reported to this general pattern, with species such as *Leucocytozoon caulleryi* that is transmitted by biting midges (*Culicoides* spp.) instead that by blackflies (Valkiūnas 2005).

Avian malaria and malaria-like parasites are important selective forces affecting the ecology, evolution and conservation of their bird hosts. These parasites are known to induce deleterious effects on bird health (Marzal et al. 2008), breeding success (Merino et al. 2000) and survival (Marzal et al. 2008; Martínez-de la Puente et al. 2010; Asghar et al. 2015), acting as drivers of some avian population declines (Van Riper III et al. 1986; Niebuhr et al. 2016; Dadam et al. 2019). Thus, factors increasing contact rates between infected and susceptible birds and insect vectors could have important consequences for individual fitness and population dynamics and should be studied. According to

the host manipulation hypothesis, vector-borne avian malaria parasites may improve their transmission success by increasing the feeding rate of competent vectors on infected birds. These effects could be driven by an increase in the attractiveness of infected birds for vectors. Birds protect themselves from mosquito bites by moving their legs, wings and head to drive away mosquitoes (Darbro & Harrington 2007). An increase in the biting rate of mosquitoes on vertebrate hosts could be also favoured by a reduction in the anti-mosquito behaviour of infected individuals. These possibilities have been broadly studied using multidisciplinary approaches during the last years providing contrasting results. Thus, the ability of these parasites to modify bird phenotype, and thereby the mechanisms used by parasites to increase their transmission remain unclear. In this article, we critically review the published information on the bird manipulation hypothesis by avian malaria infections and focus on the suggested role of the secretions of the uropygial gland and body odour of birds as attractants of mosquitoes. In addition, we propose future research prospects to test the host manipulation hypothesis considering novel approaches based on the study of the role of bird odours in determining mosquito-bird-parasite interactions.

Vector attraction toward malaria infected birds

According to the host manipulation hypothesis, parasites manipulate the phenotype of the infected individuals to increase parasite transmission (Heil 2016). In the case of avian malaria, host manipulation hypothesis proposes that parasites may manipulate infected birds to attract more mosquitoes than uninfected ones. Researchers have used different approaches to compare the attraction of mosquitoes towards infected and uninfected birds. For instance, Lalubin et al. (2012) used a dual-choice olfactometer to compare the attraction of *Culex pipiens* towards wild Great tits (*Parus major*) uninfected or naturally infected by *Plasmodium* parasites. Contrary to the predictions of the host manipulation hypothesis, these authors found that mosquitoes were more

attracted to uninfected birds than by infected ones. However, the use of naturally infected birds does not allow to discard the possibility that vectors were attracted to uninfected individuals because of any other physiological difference which could decrease their initial probability of infection. In a field experiment, a lower number of biting midges were captured in nest-boxes of Blue tit (*Cyanistes caeruleus*) pairs medicated with the antimalarial drug primaquine than in nests of control pairs (Tomás et al. 2008). These results support the host avoidance hypothesis which argues that insect vectors may develop different mechanisms to reduce the contact rates with infected vertebrates, because the parasites may produce also deleterious effects on the vectors, especially after biting birds with high intensities of infection (Anderson et al. 2000; Valkiūnas et al. 2014; Bukauskaitė et al. 2016; Gutiérrez-López et al. 2019a).

Dipteran insect vectors use different cues to locate their hosts such as chemical and visual stimuli (Lehane 2005). It has been proposed that insect vectors are attracted by the secretion of the uropygial or preen gland. This secretion contains different compounds including alcohols, aldehydes and waxes that could be used by mosquitoes to locate their hosts. If parasites modify the composition of the secretion of the uropygial gland, this could be a potential mechanism to increase the attractiveness of infected birds to mosquitoes. In this case, it could be expected that i) parasites modify the composition of the uropygial gland secretion of birds and ii) mosquitoes are more attracted to the secretions of parasite infected birds than uninfected ones.

On one hand, Grieves et al. (2018) found that malaria infection (*Plasmodium* sp. lineage 99% similar to P-SOSP 2) modified the wax ester composition of the secretions of the uropygial gland of Song sparrows (*Melospiza melodia*). However, it is unclear if the volatile and semivolatile compounds of the uropygial gland secretions of birds derived from these wax esters. By contrast, Díez-Fernández et al. (2020b) did not find differences in the composition of the volatile fraction of uropygial gland secretions of House sparrows (*Passer domesticus*) uninfected and those infected by *Plasmodium*

parasites. Birds in this study were naturally infected by *Plasmodium* parasites corresponding to four different genetic lineages (SGS1, GRW11, COLL1 and PADOM01). These contrasting results suggest that differences in the effect of parasites on the composition of the secretions of the uropygial gland may vary between bird species, parasite lineages and the fractions of the secretion analysed (volatile fraction vs wax esters).

On the other hand, different authors have analysed the attraction of mosquitoes towards the secretion of the uropygial gland. Russell & Hunter (2005) found that CDC traps located at 5m above the ground level and supplemented with uropygial gland secretions captured more *Cx. pipiens* and *Cx. restuans* mosquitoes than un-baited traps. However, this attractive effect of the bird´s secretion was not found when traps were located at 1.5m above ground level. No attraction toward uropygial gland secretions have been observed in *Cx. pipien*s nor *Aedes caspius* mosquitoes using dual choice olfactometers (Díez-Fernández et al. 2020a). In addition, while live adult House sparrows attracted more *Cx. pipiens* mosquitoes than nestlings, similar attractions were observed when mosquitoes were exposed to uropygial gland secretions of these bird age classes (Garvin et al. 2018). No differences were also reported by Martínez-de la Puente et al. (2011) when comparing the attraction of biting midges towards miniature UV-CDC traps with and without uropygial gland secretions of pigeons. Moreover, biting midges were absent in unoccupied nest-boxes baited with secretions of the uropygial gland of Blue tit (*Cyanistes caeruleus*) (Martínez-de la Puente et al. 2011). Thus, in spite of the results by Russell & Hunter (2005), the role of secretions of the uropygial gland as attractants of mosquitoes is poorly supported.

Díez-Fernández et al. (2020b) found differences in the attraction of *Cx. pipiens* to stimuli from birds uninfected and infected by *Plasmodium* parasites with a higher attraction of mosquitoes to the body odours, but not to uropygial gland secretions of infected House sparrows. In this study, the authors used birds naturally infected by avian *Plasmodium* parasites. Therefore, it was not possible

to distinguish if observed differences are the cause or the consequence of parasite infections. In spite of this limitation, these results support the absence of a direct impact of the infection by avian *Plasmodium* parasites on mosquito attraction to birds though the uropygial gland secretions. While the odour of infected birds attracts more mosquitoes, these results open new questions on the potential causes of the changes in bird odour profiles and the substances involved in the higher attraction for mosquitoes associated to parasite infections. Previous studies on other malaria models support a link between host odours, parasite infections, and vector attraction (De Moraes et al. 2014, Schaber et al. 2018). For example, *Plasmodium falciparum* infections are associated with the presence of terpenes (alpha-pinene and 3-carene) in the breath of children that may increase the attraction of mosquitoes toward infected individuals (Schaber et al. 2018). Some components of bird odours, such as nonanal, have been identified as attractants of mosquitoes (Syed & Leal 2009). The odour profile of birds may be determined, at least in part, by the surface microbiota on the skin and feathers (Krause et al. 2018). Bird microbiota may be altered by the insecticide, antimicrobial and nematicide properties of the uropygial gland secretions (Table 1) potentially affecting bird's odours and their interaction with vectors (see Magallanes et al. 2016). In addition, secretions of the uropygial gland may protect plumage by forming a physical barrier to microbes (Reneerkens et al. 2008). Blood parasite infections may reduce the antimicrobial activity of secretions of the uropygial gland (Magallanes et al. 2016), which could explain, at least in part, the differential mosquito attraction to the odours of *Plasmodium* spp. infected birds and uninfected ones. New studies are necessary to test these possibilities.

Table 1. Different compounds of bird uropygial gland secretions potentially affecting the bird-mosquito interactions. Some of these components may act as attractants or repellents of insect vectors as well as may have deleterious effects on arthropods and other organisms potentially affecting the mosquito-host interactions.

Compound	Potential function	References	Examples of bird species with the compound	References
Ketones				
2-Tridecanone	Insecticide	Williams et al. (1980)	*Dumetella carolinensis*	Shaw et al. (2011)
			Junco hyemalis	Whittaker et al. (2018) Soini et al. (2007)
			Sturnus unicolor	Amo et al. (2012)
			Zonotrichia albicollis	Tuttle et al. (2014)
Aldehydes				
Nonanal	Mosquito attractant	Syed & Leal (2009)	*Junco hyemalis*	Soini et al. (2007)
Carboxilic acids				
Dodecanoic acid (=lauric acid)	Antimicrobial	Huang et al. (2011)	*Bombycilla garrulous* *Bombycilla japonica*	Zhang et al. (2013)
			Dumetella carolinensis	Shaw et al. (2011)
			Junco hyemalis	Whittaker et al. (2018) Soini et al. (2007)
			Zonotrichia albicollis	Tuttle et al. (2014)

Tetradecanoic acid	Mosquito repellent and larvicidal	Sivakumar et al. (2011)	*Bombycilla garrulous* *Bombycilla japonica*	Zhang et al. (2013)
			Dumetella carolinensis	Shaw et al. (2011)
			Junco hyemalis	Whittaker et al. (2018)
Hexadecanoic acid*	Mosquito repellent and adulticide	Anuradha & Yogananth (2015)	*Bombycilla garrulous* *Bombycilla japonica*	Zhang et al. (2013)
			Junco hyemalis	Whittaker et al. (2018)
			Melopsittacus undulatus	Zhang et al. (2010)
Aromatic compound				
Phenol	Nematicide	Gu et al. (2007)	*Phoeniculus purpureus*	Burger et al. (2004)
			Upupa epops	Martín-Vivaldi et al. (2009)

*contained in *Halophila ovalis* extracts in combination with other compounds.

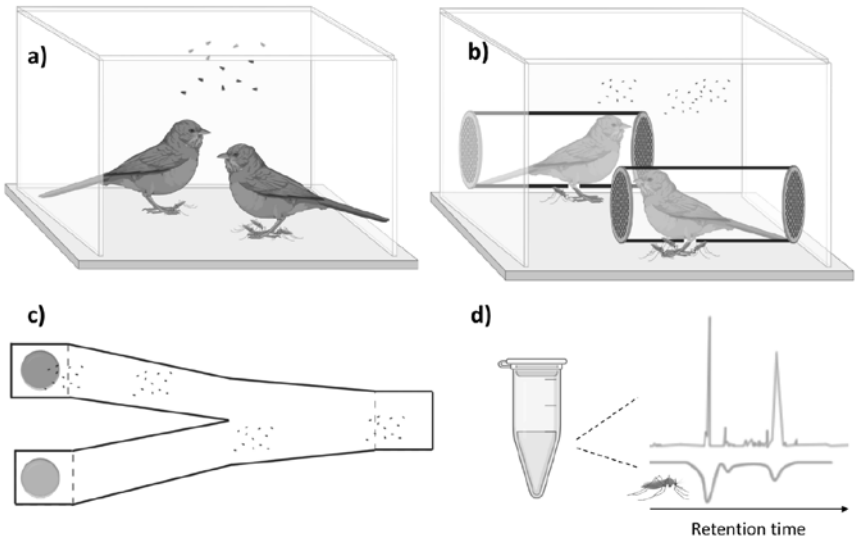
Mosquito biting rates and avian malaria infections

After reaching the bird host, mosquitoes and other dipteran insect vectors feed on blood by biting in un-feathered body parts of birds such as tarsus and eye-rings. Different methods have been used to identify the susceptibility of avian malaria infected and uninfected birds to mosquito attacks by exposing birds individually (Gutiérrez-López et al. 2019b) or in pairs (Cornet et al. 2013a, 2013b; Yan et al. 2018) to the bites of insects under controlled conditions in the laboratory (Fig. 1). In this last case, molecular techniques could be used to assess the individual origin of the blood meals present in the abdomen of vectors. Cornet et al. (2013a) found that a higher number of *Cx. pipiens* mosquitoes bit Canaries (*Serinus canaria*) experimentally infected with *P. relictum* lineage SGS1 than uninfected birds. However, such differences were only significant during the chronic phase of infection. Similarly, Yan et al. (2018) performed two experiments to test whether the infection status or the intensity of infection affect the number of bites that each bird received by mosquitoes. Although the biting rate of infected and uninfected individuals did not differ in this study, they found a higher rate of mosquito feeding on birds infected by *Plasmodium* parasites (controls) than on those infected individuals with an experimentally reduced parasite load. However, contrary to the case of Cornet et al. (2013a), birds in Yan et al. (2018) were free to respond to mosquitoes attempting to bite on them and consequently the results were affected both by the attraction of mosquitoes and by the susceptibility of birds to mosquito bites. This is especially relevant because a lower proportion of mosquitoes may be able to complete a blood meal on birds displaying a more active anti-mosquito behavior, such as feather preening with bill or scratching with legs. Overall, these studies support that avian *Plasmodium* parasites affect the contacts/choice of *Cx. pipiens* mosquitoes and birds, with the phase of infection and/or parasite load in the bird host likely eliciting a larger response on mosquito behaviour. Thus, infection characteristics in the bird needs to be considered in epidemiological studies on avian malaria parasites because these variables can explain part of the heterogeneity in vector

attacks (Cornet et al. 2013a, Yan et al. 2018), the success of parasite development in the mosquito (Pigeault et al. 2015) and the impact of parasite infections in the insect vectors (Bukauskaitė et al. 2016; Gutiérrez-López et al. 2019a).

Interestingly, the infection by *Plasmodium* parasites may also affect the behaviour of vectors (Rossignol et al. 1986; Choumet et al. 2012). Parasites may increase their transmission through different factors including the increase in the frequency or duration of the contacts between insect vectors and susceptible hosts (Heil 2016). *Aedes aegypti* mosquitoes carrying *P. gallinaceum* sporozoites ingested a lower blood volume during a blood meal and were more likely to probe for a second meal than uninfected mosquitoes (Koella 2002). Further experiments revealed that mosquitoes carrying the infective forms of avian malaria parasites need more time to complete a blood meal than uninfected mosquitoes and are more likely to take reduced blood meals (Rossignol et al. 1986). Thus, the mosquitoes may bite several times to obtain a complete blood meal. This change in mosquito behaviour could be explained by changes in the regulation/activity of the enzyme apyrase that is involved in the anticoagulation activity of mosquito saliva (Rossignol et al. 1984; Thiévent et al. 2019).

Figure 1. Different approaches used to test the host manipulation hypothesis using birds and avian malaria parasites, including the use of free-moving (a) and immobilized birds (b) exposed in pairs (e.g. infected *vs* uninfected birds; high vs low intensity infected birds). c) Dual choice olfactometers have been used to test the attraction of mosquitoes to bird stimuli (e.g. secretions of the uropygial gland or body headspace). d) Gas chromatography coupled with electroantennographic detection can be used to identify the compounds in bird odours (e.g. uropygial gland secretions or body headspace) and the response of mosquitoes to these compounds according to the bird infection status. The figure was created with BioRender.com.



Concluding remarks and future prospects

The host manipulation hypothesis has been mainly tested considering a handful of bird-parasite-vector assemblages and virtually no study has analysed the *Haemoproteus*/biting midge - hippoboscid nor *Leucocytozoon*/black fly systems. This is especially relevant considering the high number of vector species potentially involved in the transmission of these parasites, which may affect the observed patterns of host-parasite-vector associations. In addition, more than 4000 lineages of avian malaria and malaria-like parasites have been recorded until now (according to Malavi; Bensch et al. 2009). These lineages may present different virulences in their bird hosts (Ilgūnas et al. 2019a; 2019b) that may determine different degrees of vector attraction to infected individuals. However, the relationship between parasite virulence and vector attractivity has never been tested. Interspecific differences in the intensity of anti-mosquito behaviours (Darbro & Harrington 2007) and bird body size and coloration (Yan et al. 2017) may determine the differential susceptibility of each bird species to vector attacks, with some species being preferred by mosquitoes, while others are bitten less often than expected from their densities in the wild (Simpson 2009; Rizzoli et al. 2015). The different species of vectors involved in the transmission of avian malaria and malaria-like parasites may use different cues to locate their bird hosts, potentially explaining discrepancies between studies. It is also important to standardize the methods used in the studies considering aspect such as the use of immobilized/free moving exposed birds and the number of insect vectors and/or hosts included in each experimental trial. For example, studies using immobilized birds may provide information on the importance of cues such as odour or temperature in host selection by mosquitoes, while using free-moving birds allow to consider the impact of anti-mosquito behaviour on mosquito feeding success. In addition, anti-mosquito behaviours may be more frequent/intense in trials using a higher number of mosquitoes (Darbro & Harrington 2007). The number of birds exposed to mosquitoes may also affect the relative attractiveness of a specific host with respect to those closely

available, finally affecting the feeding patterns of insect vectors. For example, in cavity nesting species, nestlings are exposed to insect attacks in close proximity and consequently the bites received by an individual will be the outcome of the relative attractiveness to mosquitoes of this individual in relation to its nest mates (Christe et al. 1998). However, most studies conducted until now have been performed by exposing birds individually or in pairs to vector bites. Additionally, different host related factors including the previous experience to mosquito attacks, haematocrit, body temperature or sex classes could be also important determinants of the susceptibility of individuals to vector attacks. Again, for the case of bird sex, experiments analysing its impact on mosquito feeding preferences have given mixed results, because differences have only been reported in some bird-mosquito species combinations (Gutiérrez-López et al. 2019b; Cozzarolo et al. 2019; Burkett-Cadena et al. 2014). For example, Gutiérrez-López et al. (2019b) exposed House sparrows and Jackdaws (*Corvus monedula*) to the bites of two species of mosquitoes, *Cx. pipiens* and *Ae. caspius*. In this study, *Ae. caspius* showed a higher biting rate on female Jackdaws than on males. However, authors did not find any significant difference in the biting rates of mosquitoes on House sparrows nor for the case of Jackdaws exposed to *Cx. pipiens*. On the other hand, mixed infections by different parasite genera of avian malaria and malaria-like parasites are frequently found in wild birds (Marzal et al. 2008; Martínez et al. 2009; Ciloglu et al. 2019). In addition, infections by other vector-borne blood parasite taxa such as microfilariae, *Trypanosoma* spp., *Hepatozoon* spp. and *Lankesterella*-like parasites are common in birds (Merino et al. 1997; 2007). These mixed infections could affect the attractiveness of avian hosts to insect vectors in different ways as these parasites are transmitted by different vector groups. In addition, parasites could produce strong deleterious effects on non-competent insects potentially favouring an avoidance rather than an attractive effect. For instance, *Haemoproteus* parasites, which are transmitted by biting midges (Culicoides spp.), increase mortality in mosquitoes (Valkiūnas et al. 2014). Thus, bird-mosquito interactions may be driven by the arm-races between

mosquitoes and parasites with the last potentially increasing the attraction of mosquitoes for infected individuals, and selection acting on mosquitoes to reduce the contact rates with the more virulent parasites infecting birds.

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SECTION II

Chapter 2

Sex and age, but not avian malaria infection, affect the composition of the uropygial gland secretions of forest- and urban-dwelling blackbirds

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Abstract

The uropygial gland of birds produces an oily secretion with different functions, mainly related to plumage protection. The volatile compounds of this secretion may also act as chemical signals that provide information to conspecifics, but it is also possible that those compounds also attract hematophagous insect vectors such as those responsible for avian malaria transmission. Individual characteristics such as sex and age are usually associated with variation in the composition of the uropygial secretion. Different studies have shown that mosquitoes are more attracted towards individual birds infected by avian malaria parasites. However, whether the individual infection status by these parasites may lead to differences in the composition of this secretion remains poorly known. We used Gas Chromatography-Mass Spectrometry (GC-MS) to characterise the chemical composition of the volatile lipophilic fraction of the uropygial gland secretions of wild common blackbirds and compare its composition in an urban and a forest locality according to their age, sex and infection status by blood parasites. We found differences in the composition of the secretion between age classes and also between sexes within adult birds. However, no differences were found in chemical composition of the uropygial gland secretion of blackbirds according to their infection status by blood-parasites and habitat type. These results suggest that haemosporidian infection does not alter the composition of the volatile fraction of uropygial gland secretions in infected birds.

Keywords: haemosporidians, preen oil, sexual dimorphism, *Turdus merula*, odour, volatile compounds.

Introduction

The uropygial gland, also called preen gland, is a holocrine gland present in almost all bird species. It produces a secretion that birds spread over their plumage while preening (Jacob & Ziswiler 1982). This secretion has multiple functions, most notably feather waterproofing (Moyer et al. 2003) and protection against ectoparasites and microbes (Burger et al. 2004, Reneerkens et al. 2008, Ruiz-Rodríguez et al. 2013). The gland size is related to the volume of the secretion produced (Møller et al. 2009), with higher wax production occurring in larger uropygial glands (Sandilands et al. 2004). Although different authors have investigated the role of different life-history traits, such as habitat (e.g., aquatic *vs*. terrestrial), migratory behaviour (resident *vs*. migrant), social behaviour (social *vs*. non-social) and feather degrading bacteria abundance in explaining differences in the uropygial gland size (Vincze et al. 2013; Fülöp et al. 2016), the factors potentially affecting the chemical composition of secretions have been less studied.

The uropygial gland secretion is composed by both volatile and non-volatile fractions (Leclaire et al. 2011). According to Campagna et al. (2012), the volatile fraction includes a complex mixture of alkanes, alcohols, ketones, aldehydes and carboxylic acids with, at least, some of them producing odours, while waxes dominate the non-volatile fraction. The composition of this secretion varies among species (Haribal et al. 2009) and age classes (Shaw et al. 2011) and may differ between sexes (Whittaker et al. 2010). Sexual differences in the composition have been detected in some species such as bengalese finches (*Lonchura striata*) (Zhang et al. 2009) and dark-eyed juncos (*Junco hyemalis*) (Soini et al. 2007), but not in others, such as crested auklets (*Aethia cristatella*) (Hagelin et al. 2003), cory's shearwaters (*Calonectris borealis*) and scopoli's shearwaters (*C. diomedea*) (Gabirot et al. 2016). The uropygial secretion contributes to a large extent to bird odour (Soini et al. 2013; Moreno-Rueda 2017), and differences in its composition may therefore play an important role in bird intraspecific and interspecific communication (Rajchard 2007; Hagelin &

Jones 2007; Caro et al. 2015). The avian malaria parasites *Plasmodium* and the malaria-like parasites *Haemoproteus* and *Leucocytozoo*n share similar life cycles, but are transmitted by different insect vectors. While *Plasmodium* is transmitted by mosquitoes, *Haemoproteus* are transmitted by biting midges (Ceratopogonidae, *Culicoides* spp.) and louse flies (Hippoboscidae) and *Leucocytozoon* by black flies (Simuliidae). The volatile compounds of the uropygial secretion might serve as olfactory cue for host-seeking mosquitoes (Russell & Hunter 2005; Garvin et al., 2018) with parasites increasing the attraction of mosquitoes towards infected individuals. Cornet et al. (2013a) showed that mosquitoes were more attracted by canaries (*Serinus canaria*) chronically infected with *Plasmodium relictum* than by uninfected or acutely infected individuals, with no apparent effects of the infection status of mosquitoes on their attraction towards birds (Cornet et al. 2013b). Yan et al. (2018) provided further evidence on the effect of parasite infections on mosquito attraction, showing a higher attraction towards house sparrows (*Passer domesticus*) with higher intensities of infection. This differential attraction of mosquitoes according to parasite infections could be mediated by changes in the composition of the uropygial gland secretion. To our knowledge a single study has investigated the relationship between uropygial gland secretion composition and the infection by avian malaria parasites, reporting that parasites alter the wax ester profiles (Grieves et al. 2018). However, it is unclear if the wax fraction of these secretions affects its volatile compounds, and therefore, their importance on bird odour profiles and bird-vector interactions.

Here, we assessed the potential effects of individual traits, i.e. bird age, sex and infection status by haemosporidian parasites on the chemical composition of the volatile fraction of the uropygial gland secretions of wild common blackbirds (*Turdus merula*). Because differences in the volatile production have usually been described between age classes and sexes (Amo et al. 2012; Tuttle et al. 2014), we expect that the composition of the uropygial secretion vary between juvenile and adult blackbirds as well as between sexes. In

addition, we expect to find differences in the composition of the secretion between infected and uninfected birds because parasites could alter the composition of these secretions to increase their transmission success (i.e. host manipulation hypothesis, Heil 2016). Finally, we also assessed the effect of habitat type occupied by common blackbirds, i.e. forest and urban areas, on the composition of these secretions. We expect to find differences between birds living in these habitat types likely associated with differences in their diet (Thomas et al. 2010) or the degree of exposure to pollutants (Gómez-Ramírez et al. 2012).

Material and methods

Study area

Common blackbirds were captured using mist nests during the breeding season from March to June 2015 in two localities from southern Spain: the forest "Corredor verde del Guadiamar" (37°18'23" N, 6°15'44" W, Seville province) and the urban "María Luisa Park" (37°22'29" N, 5°59'19" W, in the city centre of Seville). Birds were ringed with numbered metal rings. The age (juveniles: <1 year old vs adults: >1 year old) and sex of adult birds was determined according to plumage characteristics (Svensson 1998), while the sex of juveniles was molecularly determined (see below). We collected uropygial gland secretions from each individual by pressuring and gently massaging the papilla with non-heparinized capillary tubes. Secretions were directly collected in 2ml gas chromatography vials. Subsequently, birds were blood sampled from the brachial vein with heparinized capillary tubes and samples transferred to Eppendorf tubes. During the fieldwork, blood samples and secretions of the uropygial gland were maintained in cold boxes (4°C). In the laboratory, we separated the plasma and cell fractions of blood samples and subsequently stored at -80ºC together with uropygial gland secretions. All birds were immediately released after handling in the same place without any apparent damage.

Molecular analyses

Genomic DNA was extracted from the cell fraction of blood samples using the Maxwell®16 LEV system Research kit (Promega, Madison, WI). Detection of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* parasites was performed using the protocol by Hellgren et al. (2004). The presence of amplicons was verified in 1.8% agarose gels and positive samples were sequenced (Macrogen Inc. Madrid, Spain). Sequences were edited with Sequencher™ v 4.9 (Genes Codes Corp., Ann Arbor, MI, USA) and compared with those deposited in public databases (i.e., GenBank, National Center for Biotechnology Information) to assess parasite identity. Juvenile individuals were molecularly sexed following Griffiths et al. (1996, 1998).

Composition of the uropygial gland secretions

Analyses of the composition of uropygial gland secretions were performed using an Agilent 7890A gas chromatograph (GC), fitted with a poly (5% diphenyl, 95% dimethylpolysiloxane) column HP5-MS (30m length x 0.25mm inner diameter x 0.25µm film thickness), coupled to an Agilent 5975C Triple Axis Detector mass spectrometer (MS) as detector. We injected in splitless mode 2µl of each sample previously dissolved in 50µl of hexane with helium as the carrier gas. The oven temperature program started at 80ºC and was maintained during 3 minutes, then increased to 300ºC at rate of 5ºC/minute and finally maintained at 300ºC during 35 minutes.

We tentatively identified the lipophilic compounds of the secretions by comparing their mass spectra with the list of potential compounds available in the NIST/EPA/NIH 2002 (NIST Mass Spectral Library, Version 2.0©, Faircom Corporation, USA). After examining the chromatograms, we used a limit of 45min. of retention time (RT) to consider volatile compounds (see Table 1 in Supplementary Material), as only complex waxes of high molecular weight were found above this RT. Nevertheless, low proportions of some diester waxes

appeared below this RT limit and were also used for calculations. The percentage of each volatile compound in relation to the total amount of compounds detected in the sample was determined from the relative proportion of each compound in relation to the total ion current until the 45min. RT limit (García-Roa et al. 2018), using the peak area integration capability of the Xcalibur 2.2 software (Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA). For unidentified compounds, we used the RT and the mass spectra characteristics to ensure that the same compound appeared in different individuals. To correct for the non-independence of proportions, we performed compositional analysis consisting in logit transforming the proportion data by taking the natural logarithm of proportion/(1-proportion) (Aebischer et al. 1993).

Statistical analysis

To analyse whether the chemical profiles of the uropygial gland secretions varied between sexes and age classes, haemosporidian infections and habitat types, we calculated Euclidean distances between every pair of individuals to produce a resemblance matrix that was the basis of following analyses. We used the permutational multivariate analysis of variance test (PERMANOVA, McArdle & Anderson, 2001) with 999 permutations to analyse possible differences in the composition of the uropygial gland secretions. The overall prevalence of infection by avian haemosporidians in the study population was high (88.46%, see Results). Because nearly all adult blackbirds (30 out of 31) were infected by avian haemosporidians, we used infected juveniles and infected adults (n=46) to assess the effect of bird sex (male/female), age (juvenile/adult) and habitat type (urban/forest) on the composition of the uropygial secretions using a three-factors PERMANOVA. We also assessed the effect of sex within age classes using one-factor PERMANOVA. In addition, we used the subset of juvenile blackbirds (n=21) to assess the effect of infection status (uninfected/infected) on the composition of uropygial secretions using a one-factor PERMANOVA. These analyses were conducted with PRIMER V6.1.13 (Clarke & Gorley 2006)

with the PERMANOVA V1.0.3 add-in package (Anderson et al. 2008). Additionally, we assessed the effect of infection status on the proportion of each compound present in at least 30% of infected juveniles using independent Generalized Linear Models (GLMs) with quasibinomial distribution and logit link function, and subsequently controlled for multiple comparisons with post-hoc Benjamini-Hochberg adjustment of significance. To test for differences within infected individuals in the major classes of chemical compounds, we grouped the relative proportion of the different volatile compounds present in the uropygial secretions of each individual into nine major classes: alcohols, alkanes, amides, carboxilic acids, esters of carboxylic acids, ketones, pyrazines, steroids, and waxy esters. Aldehydes were excluded from this analysis because they were only present in three juvenile males and were not found in juvenile females. Furanones and tocopherol were also excluded because the former was detected in a single juvenile female and absent in juveniles males, and the later was only detected in one juvenile male and one adult male. Because sex and age were the only factors explaining differences in the composition of uropygial secretions (see results), we assessed differences in these major classes of compounds using independent GLMs with quasibinomial error distribution, where age, sex, and their interaction were included as explanatory variables. Differences between factor levels were assessed with post-hoc Tukey tests. Analyses were performed in R software (R Core Team 2017) with the package lme4 (Bates et al. 2015).

Results

Overall, 52 birds (21 juveniles and 31 adults; 26 males and 26 females) were captured in the forest (n=33) and urban (n=19) areas. Forty-six out of 52 birds (88.46%) were infected by at least one parasite genus. In particular, 43 birds (82.69%) were infected by *Plasmodium* spp. and 23 individuals (44.23%) were infected by *Leucocytozoon* parasites (Table 1). One individual showed evidence of co-infection (i.e. presence of double peaks in the chromatogram) by two lineages. A single individual was infected by *Haemoproteus* parasites, although

the sequence presented double peaks. Twenty-two birds (42.31%) showed mixed infections by both *Plasmodium* and *Leucocytozoon* parasites.

Table 1. Number of infected birds and prevalence (in parenthesis) for each parasite genus and lineage found in blackbirds from southern Spain.

Parasite identity	Prevalence of infection
Plasmodium	43 (82.69%)
SYAT05	41 (78.85%)
pSPHUjJ	2 (3.85%)
Leucocytozoon	23 (44.23%)
TUMER01	20 (38.46%)
TUMER02	2 (3.85%)
NEVE1	1 (1.92%)
Haemoproteus (Unknown lineage)	1 (1.92%)

We identified a total of 213 different compounds in the volatile fraction of the uropygial secretions of common blackbirds (Table 1 in Supplementary Material). Qualitative analyses showed that secretions of juvenile birds contained less compounds than those from adults (153 *vs* 210 compounds, respectively), while the number of compounds found in males were higher than in females (191 *vs* 156, respectively) (Table 2).

The overall chemical composition of secretions differed between sexes and age classes (three-factors PERMANOVA, age: $PseudoF_{1,42}$=8.03, *P*=0.001; sex: $PseudoF_{1,42}$=5.12, *P*=0.004) but not between habitat types ($PseudoF_{1,42}$=1.23, *P*=0.23). Qualitatively similar results were also obtained when testing each of these factors alone, instead of including the three factors in the same analyses. When analysing age classes separately, significant differences between sexes were found in the case of adult birds ($PseudoF_{1,28}$=5.30, *P*=0.006) but not in juveniles ($PseudoF_{1,14}$=0.89, *P*=0.54). We did not find a significant effect of the infection status by avian haemosporidians on the composition of

secretions in juvenile blackbirds ($PseudoF_{1,19}$=1.13, P=0.31). No significant differences were found in the relative proportion of each compound associated with infection status (P > 0.19 in all cases).

When analysing the nine major classes of compounds within infected individuals, significantly higher relative proportions of alcohols were found in adults than in juveniles, while the opposite pattern was found for ketones, pyzarines and steroids (Table 3, Fig. 1A-D). In addition, a significant effect of both sex and the interaction between sex and age for the proportions of alkanes. Females showed a higher proportion of alkanes than males (Fig. 1E). However, post-hoc analyses for alkanes revealed that sexual differences were observed in adults (post hoc Tukey test: P=0.012) but not in juveniles (post hoc Tukey test: P=0.99) (Fig. 1F). In addition, no significant differences were found in the proportions of alkanes between age classes of each sex (all P > 0.44).

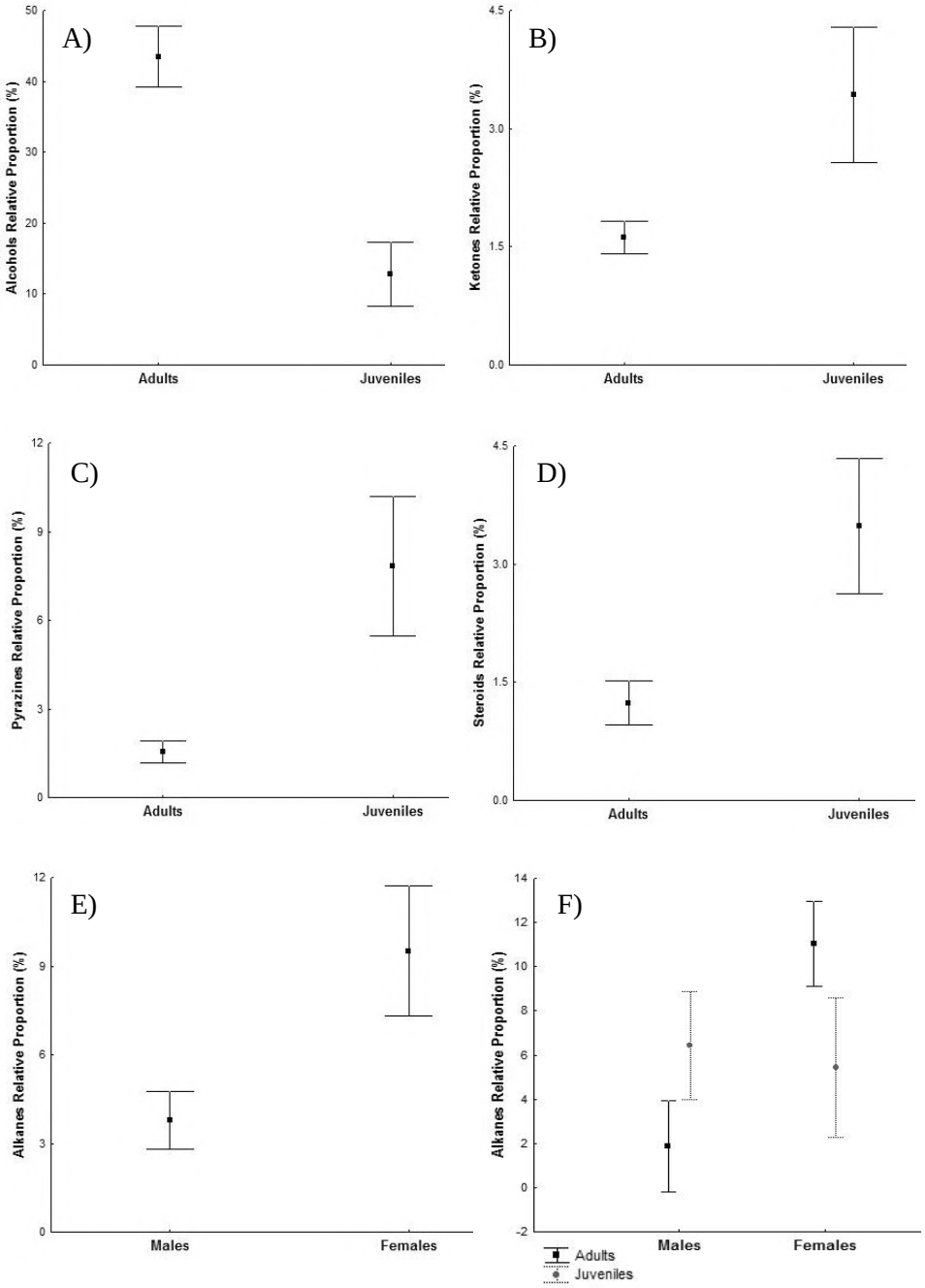
Table 2. Mean relative proportion (± S.D.) of the major classes of volatile compounds found in the uropygial gland secretions of infected and uninfected blackbirds (*Turdus merula*) of different age and sex classes.

	Juveniles		**Adults**	
Compounds	**Males (*n*=12)**	**Females (*n*=9)**	**Males (*n*=14)**	**Females (*n*=17)**
Alcohols	18.45 (19.44)	8.02 (13.52)	49.31 (14.59)	39.35 (28.13)
Aldehydes	0.08 (0.14)	0.02 (0.07)	0.16 (0.18)	0.53 (0.61)
Alkanes	6.26 (5.08)	9.84 (11.47)	1.89 (3.29)	11.57 (11.52)
Amides	0.66 (1.34)	0.74 (0.79)	0.18 (0.16)	0.36 (0.84)
Carboxilic acids	15.83 (17.15)	6.15 (8.18)	21.7 (7.71)	17.46 (17.93)
Esters of carboxilic acids	11.49 (10.44)	21.17 (22.69)	4.45 (5.21)	6.8 (15.27)
Furanones	ND	0.02 (0.05)	0.03 (0.04)	0.14 (0.31)
Ketones	2.88 (3.20)	2.62 (3.44)	1.69 (1.10)	1.49 (1.19)
Others (Diacetin)	ND	ND	0.12 (0.12)	ND
Pollutants (DDE)	0.22 (0.25)	0.52 (0.97)	0.44 (0.35)	0.78 (0.76)
Pyrazines	6.75 (9.49)	0.12 (0.06)	2.17 (2.55)	1.03 (1.13)
Steroids	2.35 (3.21)	3.69 (3.27)	0.73 (0.83)	1.64 (1.80)
Tocopherol	0.007 (0.03)	ND	0.003 (0.01)	ND
Waxes	34.81 (23.14)	41.63 (19.01)	16.91 (13.04)	18.7 (11.10)
Waxy esters	0.23 (0.42)	0.18 (0.29)	0.22 (0.40)	0.15 (0.36)

Table 3. Summary statistics of GLMs (estimate, standard error (S.E.), and *P*-value) assessing the effects of bird sex (male/female), age (juvenile/adult) and their interaction on the relative proportion of the major classes of compounds of the uropygial gland secretions of common blackbirds (*Turdus merula*) infected by avian haemosporidians. Significant differences are highlighted in bold.

Compounds	**Sex**			**Age**			**Sex*Age**		
	Est.	**S.E.**	***P***	**Est.**	**S.E.**	***P***	**Est.**	**S.E.**	***P***
Alcohols	0.45	0.36	0.22	-2.24	0.87	**0.01**	0.67	0.99	0.51
Alkanes	-1.86	0.60	**0.003**	-0.77	0.56	0.18	2.04	0.87	**0.02**
Amides	-0.72	0.74	0.34	0.84	0.60	0.17	-0.43	1.05	0.69
Carboxilic acids	0.20	0.36	0.59	-0.91	0.63	0.16	0.52	0.76	0.49
Esters of carboxilic acids	-0.49	0.75	0.52	0.88	0.69	0.21	0.01	1.04	0.99
Ketones	0.09	0.39	0.82	0.91	0.40	**0.03**	-0.24	0.54	0.66
Pyrazines	0.81	0.76	0.29	2.19	0.71	**0.004**	-0.87	0.89	0.33
Steroids	-0.84	0.56	0.14	1.08	0.41	**0.01**	0.25	0.69	0.72
Waxy esters	0.29	0.74	0.70	0.24	0.96	0.80	-0.01	1.21	0.99

Fig. 1. Relative proportion (± S.E.) of the major classes of compounds present in the uropygial gland secretions of common blackbirds (*Turdus merula*) that differed between age classes: A) Alcohols, B) Ketones, C) Pyrazines, and D) Steroids, and between sexes: E) Alkanes, and their interaction (F).



Discussion

Here, we compared the chemical composition of the volatile fraction of the uropygial gland secretion of wild common blackbirds in relation to their age, sex, infection status by haemosporidians, and habitat type. We found clear differences between age classes and also between male and female adults when considering only infected birds. However, we did not find any significant effect of parasite infections on the composition of juvenile birds´ secretions.

Our results add further support to the existence of age-related differences in the composition of uropygial gland secretions, as reported in species such as hens (Sandilands et al. 2004), starlings (*Sturnus unicolor*) (Amo et al. 2012) and gray catbirds (*Dumetella carolinensis*) (Shaw et al. 2011), but not in others such as black kites (*Milvus migrans*) (Potier et al. 2018). We found that secretions of adults had more compounds than juveniles, with significantly higher relative proportions of alcohols, but lower proportions of pyrazines, steroids, and ketones. Similarly, Amo et al. (2012) found significant differences in the composition of the secretions of adult and nestling starlings, with a higher proportion of ketones in nestlings. These differences between age classes may be related to the sexual maturity of birds, and among other factors, to differences in the levels of different hormones (Whelan et al. 2010). Hormones affect the behaviour and physiology of birds and may be related to the production of compounds present in the uropygial gland secretions (Whittaker et al. 2011).

As expected, we found that the volatile compounds of the uropygial gland secretion of common blackbirds varied between sexes, but only within adult birds. In particular, adult females had significantly higher proportions of alkanes than adult males. This result suggests a direct or indirect effect of hormones on the composition of the uropygial secretion. Sexual differences have been reported in different species, as in the case of budgerigars (*Melopsittacus undulatus*), with males showing higher relative abundance of hexadecanoic acid and alkanols than females (Zhang et al. 2010). Similar results were found by Leclaire et al. (2011) in black-legged kittiwakes (*Rissa tridactyla*) during the bird

breeding season. However, the composition of the uropygial gland secretion did not differ between sexes in other species, such as crested auklet (*Aethia cristatella*) (Hagelin et al. 2003) and Cory's and Scopoli's shearwaters (*Calonectris borealis* and *C. diomedea*, respectively) (Gabirot et al. 2016). In addition, non-significant differences between sexes were found in the composition of the volatile fraction of uropygial gland secretions of juvenile house sparrows (Díez-Fernández et al. 2020a), further suggesting that sexual differences may be only apparent in adult/sexually mature birds. Differences in the composition of the secretion, especially during the breeding season, may be related to sexual hormones and with the importance to maintain high quality plumage, which is often involved in sexual selection (Piersma et al. 1999; Soini et al. 2007). Soini et al. (2007) found that several volatiles of the uropygial secretion of dark-eyed juncos (*Junco hyemalis*) varied according to the breeding and non-breeding seasons, together with changes in plasma testosterone levels in both males and females (Ketterson et al. 2005). The composition of these secretions may vary coinciding with an active testicular activity as shown in the sub-tropical passerine (*Pycnonotus cafer*) (Bhattacharyya & Chowdhury 1995), which may explain the fact that sexual differences in this study were found in adults but not in juvenile birds.

In contrast to our predictions in the light of the host manipulation hypothesis (Heil 2016), we did not find any significant effect of the infection by haemosporidians on the composition of the uropygial gland secretion of juvenile blackbirds. We expected to find differences in the composition based on the potential role of these secretions in bird-vector interactions (Takken & Knols 2010) as attractants of black flies (Fallis & Smith 1964) and mosquitoes (Russell & Hunter 2005). However, other studies failed to find support for these associations in, for instance, the absence of attraction to the uropygial gland secretions in biting midges (Martínez-de la Puente et al. 2011) and black flies (King & Adler 2012). In addition, two species of mosquitoes, including an important vector of avian haemosporidian parasites, had similar responses when

exposed to uropygial gland secretions of house sparrows and controls (only CO_2) in a dual choice experiment (Díez-Fernández et al. 2020b). Altogether, these results suggest that stimuli other than these secretions may play a role in vector attraction, thus differences may not be found between birds according to their infection status in the context of the host manipulation hypothesis. Díez-Fernández et al. (2020a) found that mosquitoes were more attracted by the headspace (the body odour) of *Plasmodium*-infected house sparrows, but no differences in mosquito attraction were found when using only the secretion of the uropygial gland of infected and uninfected birds. Current evidence in humans support that *Plasmodium* modify the odour profile of their hosts to increase mosquito biting rates on infected individuals (De Moraes et al. 2018; Robinson et al. 2018). These results suggest that parasites could manipulate bird odours in different ways other than changes in uropygial gland secretions. A previous study using experimental *Plasmodium*-infected and uninfected birds reported changes in the wax composition of birds associated to the infection treatment (Grieves et al. 2018), although the role of this fraction on bird odours remains unclear. Discrepancies between studies could be due to different factors, including the different compounds of the uropygial gland secretion analysed between studies. In addition, in our study most birds were infected, many of them (42.31%) showing mixed infections by different parasite genera, while only six birds were uninfected. Moreover, this high prevalence of infection precluded us to assess whether differences might occur within infected and uninfected adult birds. For example, nonanal may play a key role as an attractant of bird-biting mosquitoes (Syed & Leal 2009), although this compound was only found in seven individuals (all of them adults) in our study. Alternatively, another compound such as diacetin, which is an insect attractant found in plants (Schäffler et al. 2015), was previously found in the extracellular vesicles of in-vitro *Plasmodium falciparum* infected red blood cells (Correa et al. 2017) and we detected diacetin in ten *Plasmodium*-infected adult males in this study.

Common blackbirds have widespread distributions that include urban and rural habitats (Aparicio 2011). In spite of living in habitats with dissimilar availability of resources, we did not find differences in the composition of the uropygial gland secretion. Previous studies suggested that these compounds are acquired passively through food, thus individual variation may simply result from variation in diet (Sandilands et al. 2004). It is possible that common blackbirds, despite using different habitats share a similar diet, resulting in non-significant differences in the composition of their secretions. Nevertheless, Whittaker et al. (2010) analysed the volatiles compounds in dark-eyed juncos from two populations that were maintained in the same environment under controlled conditions, and found significant differences between them. These authors suggested that genetic instead of environmental (including diet) factors might be responsible for differences in the composition of the secretion. However, environmental factors associated with habitat use such as exposure to pollutants, may indeed lead to differences in the composition of the uropygial secretion. For instance, common blackbirds from forest areas showed higher relative proportion of the pesticide dichlorodiphenyldichloroethylene (DDE) in their secretions than those from urban areas (Díez-Fernández et al. unpublished).

In sum, we provide further evidence on the role of two intrinsic factors largely affecting the composition of secretions of the uropygial gland, i.e. age and sex. By contrast, in spite that secretions of the uropygial gland have been proposed to play a role in host-vector-parasite interactions, we did not find any significant association between the infection by two common vector-borne parasites and the composition of the volatile fraction of uropygial gland secretions in juvenile blackbirds. Further studies considering differences between infected and uninfected adult birds and in the volatile and non-volatile fractions of the secretions should be conducted, including experimental approaches manipulating factors such as the infectious status and parasitaemia to compare potential changes affecting the composition of uropygial secretions.

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Supplementary Material

Table 1. Mean relative proportion (± S.D.) of compounds found in the secretions of the uropygial gland of common blackbirds. The number of individuals presenting each compound is shown in brackets.

		Juveniles (n=21)				Adults (n=31)			
RT	Compounds	Males (n=12)		Females (n=9)		Males (n=14)		Females (n=17)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Alcohols									
20.12	Unknown alcohol	0.04 (5)	0.06	0.04 (2)	0.08	0.07 (11)	0.09	0.11 (10)	0.13
22.35	Unknown alcohol	ND		ND		0.004 (3)	0.01	0.01 (4)	0.03
23.23	Tetradecanol	0.24 (2)	0.82	0.35 (2)	0.70	0.01 (4)	0.01	0.03 (6)	0.06
24.43	Unknown alcohol	ND		ND		0.06 (8)	0.08	0.13 (10)	0.15
25.35	Pentadecanol	0.02 (2)	0.06	0.19 (4)	0.30	0.05 (11)	0.05	0.14 (10)	0.22
27.36	Hexadecanol	0.16 (5)	0.25	0.76 (4)	1.45	0.02 (6)	0.03	0.12 (12)	0.12
29.22	Octadecanol	ND		0.06 (2)	0.11	0.004 (1)	0.01	ND	
33.11	Unknown alcohol	ND		0.06 (2)	0.15	ND		0.24 (1)	1.00
33.54	Eicosanol	0.73 (3)	2.44	0.06 (2)	0.13	ND		3.39 (4)	13.67
34.59	Unknown alcohol	ND		3.70 (1)	11.11	0.29 (4)	0.73	ND	
34.69	9-Octadecen-1-ol	ND		ND		0.01 (2)	0.02	ND	
36.21	Henicosanol	1.74 (1)	6.02	2.78 (1)	8.35	0.05 (3)	0.10	0.23 (4)	0.75
36.77	Unknown alcohol	ND		ND		0.57 (2)	1.45	0.03 (3)	0.07
37.60	Docosanol	11.52 (5)	18.38	ND		48.01 (14)	14.92	17.01 (5)	28.14
39.20	Unknown alcohol	ND		ND		0.01 (2)	0.02	ND	
40.59	Tetracosanol	ND		ND		ND		2.59 (2)	10.47
41.76	Hexacosanol	ND		ND		0.08 (1)	0.32	ND	
42.20	Unknown alcohol	ND		ND		ND		4.38 (2)	18.01
42.76	Unknown alcohol	3.98 (1)	13.79	0.02 (1)	0.06	0.03 (1)	0.13	2.93 (1)	12.08
43.36	Unknown alcohol	ND		ND		ND		8.02 (3)	19.48
45.01	Unknown alcohol	ND		ND		0.04 (2)	0.11	ND	

Aldehydes									
7.56	Nonanal	ND		ND		0.01 (5)	0.02	0.01 (2)	0.03
22.85	Dodecanal	0.01 (2)	0.02	ND		0.01 (3)	0.01	0.04 (10)	0.05
25.00	Tetradecanal	0.07 (4)	0.13	0.02 (1)	0.07	0.13 (9)	0.18	0.42 (13)	0.58
28.98	Octadecanal	ND		ND		0.01 (2)	0.02	0.05 (3)	0.16
Alkanes									
7.55	Undecane	0.001 (1)	0.00	0.43 (5)	0.78	ND		0.02 (2)	0.07
10.10	Dodecane	0.21 (5)	0.44	0.50 (5)	0.99	ND		0.05 (5)	0.11
12.27	Unknown branched alkane	ND		0.02 (1)	0.07	ND		0.03 (3)	0.08
12.83	Unknown branched alkane	0.31 (6)	0.38	0.77 (7)	1.26	0.01 (2)	0.03	0.10 (8)	0.24
13.49	Tridecane	0.003 (1)	0.01	ND		ND		0.04 (4)	0.08
15.43	Tetradecane	0.41 (8)	0.56	0.70 (8)	0.85	0.12 (11)	0.29	0.51 (16)	0.92
17.79	Unknown branched alkane	ND		ND		ND		0.01 (3)	0.03
17.93	Pentadecane	0.45 (6)	0.82	0.62 (5)	1.30	0.09 (11)	0.10	0.33 (14)	0.37
18.88	Unknown branched alkane	0.005 (2)	0.01	ND		0.03 (3)	0.12	ND	
19.11	Unknown branched alkane	0.005 (1)	0.02	ND		ND		0.02 (4)	0.04
19.32	Unknown branched alkane	ND		ND		ND		0.01 (4)	0.03
19.56	Unknown branched alkane	0.001 (1)	0.00	ND		ND		0.005 (1)	0.02
20.27	Hexadecane	0.28 (7)	0.31	0.57 (8)	0.53	0.14 (2)	0.51	0.98 (16)	1.66
20.34	Unknown branched alkane	ND		ND		ND		0.04 (2)	0.12
21.84	Heptadecane	ND		ND		ND		0.001 (1)	0.00
22.52	Octadecane	ND		ND		ND		0.08 (5)	0.19
22.72	Unknown branched alkane	ND		ND		ND		0.01 (4)	0.04
23.64	2,6,11,15-Tetramethyl-hexadecane	ND		ND		ND		0.02 (3)	0.06
23.65	Unknown branched alkane	0.01 (3)	0.02	ND		0.04 (3)	0.15	0.15 (8)	0.24
23.86	Unknown branched alkane	0.003 (1)	0.01	ND		ND		0.01 (3)	0.02
24.05	Unknown branched alkane	ND		ND		ND		0.01 (2)	0.04
24.18	Unknown branched alkane	ND		ND		ND		0.01 (1)	0.02
24.21	1-Chloro-tetradecane	ND		ND		ND		0.04 (2)	0.11
24.61	Nonadecane	0.18 (4)	0.35	0.12 (3)	0.20	0.20 (7)	0.60	1.02 (12)	2.41

25.49	Unknown branched alkane	ND		ND		0.002 (1)	0.01	ND	
25.59	Unknown branched alkane	0.002 (1)	0.01	0.03 (2)	0.08	0.003 (2)	0.01	ND	
26.29	1-Chloro-hexadecane	1.49 (12)	1.83	1.81 (8)	2.40	0.31 (14)	0.13	0.87 (16)	0.92
27.94	2,6,10,14-Tetramethyl-hexadecane	0.46 (3)	1.23	ND		ND		0.14 (6)	0.24
28.26	2,6,10-Trimethyl-dodecane	ND		ND		ND		0.02 (2)	0.05
28.59	Eicosane	0.22 (7)	0.34	0.18 (5)	0.23	0.19 (2)	0.68	0.59 (9)	0.88
29.33	Unknown branched alkane	0.12 (5)	0.17	0.38 (5)	0.53	0.01 (1)	0.04	0.06 (7)	0.09
30.20	Unknown branched alkane	0.13 (8)	0.14	0.06 (3)	0.09	0.02 (5)	0.03	0.12 (9)	0.34
30.42	Unknown branched alkane	ND		ND		0.01 (2)	0.02	0.01 (4)	0.03
31.12	Unknown branched alkane	0.09 (6)	0.10	0.18 (5)	0.30	0.02 (5)	0.02	0.11 (9)	0.18
31.85	Heneicosane	ND		ND		ND		0.07 (3)	0.20
32.19	Unknown branched alkane	0.19 (3)	0.40	0.18 (4)	0.27	0.29 (6)	0.69	0.64 (11)	0.80
32.74	Unknown branched alkane	ND		ND		ND		0.11 (3)	0.43
32.82	Unknown branched alkane	ND		ND		ND		0.06 (6)	0.12
32.85	Docosane	0.07 (2)	0.18	0.06 (3)	0.14	0.11 (10)	0.16	0.15 (7)	0.26
33.51	Tricosane	ND		ND		0.02 (3)	0.05	ND	
33.58	Unknown branched alkane	0.05 (2)	0.11	ND		ND		0.03 (3)	0.06
33.90	Tetracosane	0.25 (9)	0.32	0.52 (7)	0.64	0.03 (8)	0.04	0.14 (11)	0.23
34.04	Unknown branched alkane	0.02 (1)	0.08	ND		ND		0.005 (1)	0.02
36.02	Hexacosane	ND		ND		ND		0.23 (4)	0.79
37.10	Unknown branched alkane	0.25 (8)	0.27	1.05 (4)	2.00	0.05 (5)	0.08	0.35 (11)	0.81
37.44	Unknown branched alkane	0.05 (2)	0.17	ND		ND		0.02 (2)	0.07
38.62	Heptacosane	0.34 (4)	0.63	1.14 (3)	2.61	0.01 (1)	0.04	1.07 (7)	2.07
40.09	Octacosane	0.54 (8)	1.09	0.21 (2)	0.45	0.05 (5)	0.08	2.83 (8)	8.17
41.60	Unknown branched alkane	0.11 (2)	0.26	0.02 (1)	0.07	0.11 (5)	0.24	0.39 (6)	0.71
44.15	Nonacosane	0.03 (1)	0.11	0.27 (1)	0.80	ND		0.06 (1)	0.26
Amides									
21.24	Butanamide, N-methyl-4-(methylthio)-2-(2,2-dimethylpropylidene)	ND		ND		0.02 (8)	0.02	ND	
35.00	9-Octadecenamide	0.23 (5)	0.43	0.12 (2)	0.32	0.05 (5)	0.09	0.25 (7)	0.76
41.31	13-Docosenamide	0.42 (4)	0.98	0.62 (6)	0.83	0.12 (7)	0.17	0.10 (2)	0.40

Carboxilic acids

8.43	Pentanoic acid	ND		ND		0.01 (2)	0.03	ND	
10.26	Octanoic acid	ND		ND		0.11 (7)	0.15	0.04 (4)	0.08
12.81	Nonanoic acid	ND		ND		0.17 (10)	0.23	0.13 (6)	0.30
15.05	Decanoic acid	ND		ND		0.03 (6)	0.05	ND	
17.18	Undecanoic acid	ND		ND		0.01 (2)	0.03	ND	
19.87	Dodecanoic acid	0.06 (3)	0.14	0.08 (2)	0.21	0.19 (12)	0.14	0.14 (6)	0.24
24.00	Tetradecanoic acid	0.11 (4)	0.26	0.29 (3)	0.64	0.66 (14)	0.54	0.35 (11)	0.49
26.03	Pentadecanoic acid	0.24 (4)	0.54	0.35 (3)	0.95	0.47 (11)	0.56	0.45 (7)	0.87
27.98	Hexadecanoic acid	3.28 (9)	3.00	2.00 (4)	3.09	3.79 (14)	1.63	3.47 (14)	3.66
29.12	Heptadecanoic acid	0.20 (2)	0.48	ND		0.11 (5)	0.25	ND	
31.20	9,12-Octadecadienoic acid	0.37 (4)	0.60	ND		0.81 (14)	0.92	0.74 (8)	1.47
31.27	9-Octadecenoic acid	0.21 (2)	0.55	ND		0.29 (9)	0.33	0.28 (5)	0.77
31.70	Octadecanoic acid	2.29 (7)	3.49	0.95 (5)	1.16	4.04 (14)	3.56	3.65 (11)	5.83
33.46	Nonadecanoic acid	0.08 (2)	0.22	ND		0.20 (6)	0.34	ND	
34.23	5,8,11,14-Eicosatetraenoic acid	0.22 (2)	0.56	ND		0.29 (9)	0.83	ND	
35.19	Eicosanoic acid	4.80 (8)	4.89	1.64 (4)	2.71	6.56 (14)	2.45	5.36 (11)	7.20
36.76	Heneicosanoic acid	0.24 (3)	0.46	ND		0.92 (10)	1.51	0.05 (4)	0.09
38.34	Docosanoic acid	3.72 (4)	6.29	0.83 (2)	2.23	3.04 (10)	2.42	2.80 (10)	3.74

Esters of carboxilic acids

18.49	Dodecanoic acid, methyl ester	ND		ND		0.01 (4)	0.01	0.002 (1)	0.01
21.59	Cyclopentaneacetic acid, 3-oxo-2-pentyl-, methyl ester	ND		ND		0.02 (5)	0.03	ND	
23.10	Tetradecanoic acid, methyl ester	ND		ND		0.01 (2)	0.02	ND	
24.13	Tetradecanoic acid, 9-methyl-, methyl ester	0.01 (2)	0.01	0.15 (3)	0.38	0.01 (2)	0.05	ND	
24.98	Pentadecanoic acid, 14-oxo-, methyl ester	ND		ND		0.06 (3)	0.15	ND	
25.18	Pentadecanoic acid, methyl ester	ND		ND		0.02 (4)	0.05	0.08 (7)	0.16
25.87	9-Hexadecenoic acid, ethyl ester	ND		ND		0.003 (1)	0.01	ND	
27.17	Hexadecanoic acid, methyl ester	0.28 (7)	0.34	1.12 (5)	1.61	0.06 (6)	0.11	0.03 (2)	0.08

28.19	Hexadecanoic acid, 15-methyl-, methyl ester	0.38 (5)	0.56	0.61 (5)	0.79	0.10 (2)	0.32	0.23 (10)	0.25
29.10	Heptadecanoic acid, methyl ester	0.11 (4)	0.23	0.74 (4)	1.11	0.33 (8)	0.56	0.05 (2)	0.14
29.43	Octadecanoic acid, 2-oxo-, methyl ester	0.23 (5)	0.34	0.31 (2)	0.76	0.09 (10)	0.10	0.11 (9)	0.18
29.87	3-Octadecenoic acid, methyl ester	0.02 (2)	0.08	ND		0.03 (2)	0.09	ND	
29.95	Heptadecanoic acid, 16-methyl-, methyl ester	ND		ND		0.15 (5)	0.32	ND	
30.37	9,12-Octadecadienoic acid, methyl ester	0.44 (10)	0.47	0.47 (4)	0.82	0.36 (11)	0.80	0.64 (11)	1.52
30.48	9-Octadecenoic acid, methyl ester	0.65 (10)	0.73	0.59 (5)	0.92	0.17 (10)	0.28	0.21 (7)	0.54
30.59	10-Octadecenoic acid, methyl ester	ND		ND		0.005 (1)	0.02	ND	
30.84	Hexadecanoic acid, 14-methyl-, methyl ester	0.03 (3)	0.07	ND		0.01 (1)	0.04	0.02 (3)	0.04
30.95	Octadecanoic acid, methyl ester	0.72 (11)	1.05	2.20 (8)	2.96	0.18 (12)	0.26	0.17 (11)	0.24
31.64	Unknown methyl ester of an octadecenoic acid	0.11 (4)	0.21	0.98 (3)	2.17	ND		0.07 (4)	0.20
31.98	Hexadecanoic acid, butyl ester	0.43 (6)	0.70	0.84 (6)	1.20	ND		0.12 (3)	0.28
32.12	Nonadecanoic acid, ethyl ester	ND		0.002 (1)	0.01	0.002 (1)	0.01	0.04 (1)	0.16
32.22	Octadecanoic acid, 17-methyl-, methyl ester	0.14 (2)	0.44	0.17 (1)	0.50	0.03 (1)	0.10	ND	
32.70	Nonadecanoic acid, methyl ester	0.16 (6)	0.25	0.48 (5)	0.65	0.17 (7)	0.39	ND	
33.27	5,8,11,14-Eicosatetraenoic acid, ethyl ester,	0.39 (8)	0.77	0.85 (6)	0.98	0.22 (8)	0.45	0.13 (6)	0.24
33.39	5,8,11,14,17-Eicosapentaenoic acid, methyl ester	0.77 (2)	2.30	0.07 (1)	0.21	0.07 (3)	0.16	0.06 (3)	0.15
33.57	7,10,13-Eicosatrienoic acid, methyl ester	ND		ND		0.04 (5)	0.07	ND	
33.90	10,13-Eicosadienoic acid, methyl ester	ND		ND		0.01 (1)	0.04	ND	
34.40	Eicosanoic acid, methyl ester	1.07 (9)	1.19	2.05 (8)	2.19	0.29 (13)	0.27	1.14 (10)	3.49
35.00	Heneicosanoic acid, methyl ester	0.17 (2)	0.45	0.76 (4)	1.03	0.06 (5)	0.13	ND	
35.48	Eicosanoic acid, ethyl ester	0.26 (3)	0.60	0.23 (2)	0.49	0.14 (4)	0.27	0.09 (2)	0.29
36.22	13-Docosenoic acid	0.18 (2)	0.50	0.16 (1)	0.47	0.02 (2)	0.05	0.05 (1)	0.20
36.54	6,9,12,15-Docosatetraenoic acid, methyl ester	0.07 (3)	0.12	ND		0.01 (2)	0.03	0.03 (3)	0.08
36.60	Heneicosanoic acid, methyl ester	0.26 (3)	0.48	0.23 (2)	0.47	0.07 (7)	0.08	ND	
37.04	Nonadecanoic acid, ethyl ester	0.11 (4)	0.26	0.06 (1)	0.18	0.04 (4)	0.07	ND	
37.58	Docosanoic acid, methyl ester	1.75 (10)	1.86	2.85 (7)	3.27	0.83 (10)	1.25	1.66 (10)	4.95
38.12	Tricosanoic acid, methyl ester	0.82 (9)	0.99	2.30 (5)	3.13	0.38 (6)	0.84	0.91 (9)	2.88

38.55	Docosanoic acid, ethyl ester	0.39 (5)	0.83	0.11 (1)	0.33	0.20 (6)	0.31	0.68 (4)	2.08
39.28	Tricosanoic acid, ethyl ester	0.21 (6)	0.34	ND		0.01 (1)	0.03	ND	
39.59	Tetracosanoic acid, methyl ester	0.06 (3)	0.12	0.47 (3)	0.75	0.05 (4)	0.09	0.14 (3)	0.43
40.03	Tetracosanoic acid, ethyl ester	ND		0.99 (2)	2.14	0.01 (1)	0.06	ND	
40.53	Unknown derivative of tetracosanoic acid, methyl ester	0.34 (8)	0.44	0.49 (5)	1.10	0.01 (2)	0.02	ND	
41.01	Pentacosanoic acid, methyl ester	0.23 (6)	0.38	0.26 (2)	0.63	0.11 (6)	0.14	0.06 (3)	0.15
41.42	Hexacosanoic acid, methyl ester	0.11 (2)	0.34	0.34 (1)	1.02	0.04 (4)	0.07	0.10 (3)	0.26
42.10	Hexacosanoic acid, ethyl ester	0.47 (3)	1.02	0.27 (1)	0.81	0.02 (1)	0.09	ND	
42.31	Heptacosanoic acid, methyl ester	0.11 (1)	0.37	0.03 (1)	0.08	ND		ND	
Furanones									
22.15	5-Hexyldihydro-2(3H)-furanone	ND		0.01 (1)	0.03	0.01 (6)	0.01	0.04 (8)	0.05
30.56	5-Dodecyldihydro-2(3H)-furanone	ND		0.01 (1)	0.02	0.02 (5)	0.03	0.10 (7)	0.31
Ketones									
7.24	2-Nonanone	0.11 (1)	0.38	0.02 (3)	0.04	ND		ND	
12.67	2-Undecanone	0.03 (2)	0.08	0.04 (3)	0.06	0.01 (3)	0.02	0.004 (2)	0.01
14.02	2,4,4-Trimethyl-3-(3-methylbutyl) cyclohex-2-enone	ND		ND		0.03 (7)	0.05	0.22 (12)	0.28
15.31	2-Dodecanone	0.02 (2)	0.05	0.03 (2)	0.06	0.01 (5)	0.01	0.01 (3)	0.02
17.49	3-Tridecanone	0.01 (2)	0.02	0.09 (3)	0.19	0.02 (6)	0.04	0.07 (7)	0.12
17.82	2-Tridecanone	0.15 (5)	0.19	0.21 (3)	0.45	0.06 (7)	0.07	0.07 (6)	0.16
19.02	2-Hexanone, methyl derivative	ND		0.08 (2)	0.19	0.01 (5)	0.02	0.07 (8)	0.10
20.24	2-Tetradecanone	0.03 (2)	0.08	0.03 (1)	0.10	0.02 (2)	0.07	ND	
22.52	2-Pentadecanone	0.05 (5)	0.10	0.06 (3)	0.14	0.08 (14)	0.09	0.07 (9)	0.09
23.61	Unknown ketone	ND		ND		0.01 (1)	0.03	0.02 (3)	0.04
24.67	2-Hexadecanone	0.05 (2)	0.12	0.05 (2)	0.11	0.05 (6)	0.12	0.06 (5)	0.16
26.71	2-Heptadecanone	0.23 (8)	0.39	0.09 (4)	0.13	0.09 (12)	0.12	0.19 (16)	0.16
27.61	Unknown ketone	ND		ND		0.04 (5)	0.07	0.11 (5)	0.22
28.44	1,13-Tetradecadien-3-one	0.05 (4)	0.08	0.11 (3)	0.19	ND		ND	
28.47	4-Dodecylcyclohexanone	ND		ND		0.14 (11)	0.13	ND	
28.57	3-Octadecanone	ND		ND		0.14 (12)	0.10	0.05 (3)	0.15
29.32	2-Octadecanone	0.19 (7)	0.21	0.03 (1)	0.08	0.12 (12)	0.10	ND	
30.07	3-Eicosanone	0.14 (7)	0.18	0.13 (3)	0.25	0.07 (10)	0.07	0.01 (1)	0.04

30.54	2-Nonadecanone	0.005 (1)	0.02	0.02 (1)	0.06	0.01 (4)	0.02	ND	
32.25	2-Eicosanone	0.11 (4)	0.16	0.14 (2)	0.34	0.08 (7)	0.13	0.09 (3)	0.22
32.90	Unknown ketone	0.64 (8)	0.73	0.62 (5)	0.71	0.17 (11)	0.17	0.12 (8)	0.21
33.03	Unknown ketone	0.39 (9)	0.42	0.31 (4)	0.42	0.15 (12)	0.14	0.08 (2)	0.28
34.59	Unknown ketone	0.10 (6)	0.15	0.11 (3)	0.19	0.03 (5)	0.05	ND	
36.19	Unknown ketone	0.46 (4)	1.09	0.42 (3)	0.63	0.23 (7)	0.29	0.19 (6)	0.31
36.87	3,6-Nonadecadione	0.04 (3)	0.09	ND		0.05 (3)	0.12	ND	
39.26	Unknown ketone	0.08 (2)	0.22	0.04 (1)	0.12	0.05 (3)	0.12	0.07 (2)	0.25
Pyrazines									
21.51	Unknown pyrazine	0.12 (2)	0.29	0.18 (2)	0.48	0.04 (3)	0.08	ND	
23.58	Unknown pyrazine	ND		ND		0.13 (4)	0.26	ND	
25.08	Unknown pyrazine	0.66 (6)	0.95	0.36 (3)	0.57	0.08 (5)	0.17	0.05 (2)	0.16
27.06	Unknown pyrazine	3.10 (7)	4.44	2.58 (4)	3.99	0.79 (8)	1.02	ND	
27.13	Unknown pyrazine	ND		ND		0.10 (8)	0.11	0.86 (15)	1.09
27.34	Unknown pyrazine	0.77 (4)	1.44	0.27 (1)	0.81	0.41 (7)	0.61	ND	
27.49	Unknown pyrazine	2.07 (7)	2.97	1.86 (4)	2.81	0.55 (8)	0.73	0.07 (5)	0.15
39.73	Unknown pyrazine	0.04 (2)	0.11	0.15 (1)	0.45	0.07 (7)	0.10	0.04 (2)	0.14
Steroids									
41.13	Cholest-5-en-3-ol, tetradecanoate	1.47 (9)	3.19	0.96 (5)	1.47	0.16 (11)	0.16	0.59 (13)	0.58
42.23	Cholest-2-ene	0.21 (4)	0.37	0.31 (5)	0.45	0.06 (2)	0.22	ND	
42.48	Unknown steroid (187,213,255,353,368)	0.33 (2)	1.13	0.18 (3)	0.31	ND		0.08 (3)	0.21
42.96	Cholesterol margarate	0.34 (3)	0.68	2.23 (6)	2.59	0.51 (6)	0.81	0.97 (8)	1.30
Waxy ester									
28.78	3-Pentadecyl octanoate	0.02 (2)	0.05	0.05 (1)	0.15	0.09 (6)	0.26	0.002 (1)	0.01
30.58	Unknown ester of octanoic acis	ND		ND		0.01 (2)	0.03	ND	
38.27	Hexadecyl octanoate	ND		ND		ND		0.09 (4)	0.20
41.95	Hexadecanoic acid, 2-(octadecyloxy) ethyl ester	0.10 (2)	0.32	0.09 (1)	0.26	0.11 (3)	0.34	ND	
43.79	Tetradecyl dodecanoate	0.11 (3)	0.28	0.05 (1)	0.15	ND		0.05 (2)	0.19
Others									
14.20	Diacetin (=1,2,3-Propanetriol, diacetate)	ND		ND		0.12 (10)	0.12	ND	

Pesticide									
32.02	DDE	0.22 (8)	0.25	0.52 (4)	0.97	0.44 (12)	0.35	0.78 (12)	0.76
Tocopherol									
44.85	γ-Tocopherol	0.01 (1)	0.03	ND		0.003 (1)	0.01	ND	
Waxes									
23.92 to 44.34	Different types of complex waxes	34.80	23.14	41.63	19.01	16.90	13.03	18.70	11.10

RT=retention time. ND=Not detected.

Chapter 3

Habitat type and age affect the relative proportion of pesticides in uropygial gland secretions of common blackbirds

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(*Submitted Manuscript*)

Abstract

Dichlorodiphenyltrichloroethane (DDT) is a pesticide that was commonly used worldwide during decades. The use of DDT was banned decades ago in Europe due to its high toxicity and persistence in the environment, bioaccumulation in living organisms and biomagnification through food webs. However, monitoring using both invasive and non-invasive methods has routinely reported the occurrence of DDT metabolites such as dichlorodiphenyldichloroethylene (DDE) in wild birds, which evidence the differential exposure of organochlorine pesticides between and within species in areas along years. Here, we compared the exposure to DDE in urban and forest areas in southern Spain by analysing relative proportion of DDE in the uropygial gland secretions of common blackbirds (*Turdus merula*). Given the negative effects of this pollutant on animal immunity, we also tested for associations between the prevalence of haemosporidians in common blackbirds and the relative proportion of DDE in their secretions. Individuals from the forest area presented higher relative proportion of DDE than those from the urban area and proportions were also higher in adults than in juvenile birds, regardless of their sex. In spite of the potential immunosuppressive effect of DDE, prevalence of infection was not associated with DDE. Overall, our results support the role of habitat use in explaining the exposure to DDE and its excretion through the uropygial gland secretions in a wild passerine.

Keywords: bioaccumulation, blood parasites**,** organochlorine pesticides **(**OCPs), preen gland secretion, *Turdus merula*, urban ecology.

Introduction

Living beings are directly or indirectly exposed to environmental pollutants worldwide, including remote areas (Welch et al. 1991; Blais et al. 1998). Persistent organic pollutants (POPs) are considered one of the most important environmental contaminants, including key compounds such as organochlorine pesticides (OCPs), among which the widely used dichlorodiphenyltrichloroethane (DDT) stands out (Jones & Voogt 1999). This insecticide was commonly used against mosquitoes and other invertebrate pests since the Second World War and was massively applied to agricultural crops (Turusov et al. 2002). Due to its deleterious effects on health of human and other vertebrates, its use was banned between 1970-1980 in Europe (Turusov et al. 2002). DDT degrades into metabolites such as dichlorodiphenyldichloroethylene (DDE), which is an extremely persistent and highly toxic compound that bioaccumulates in living organisms, escalating through trophic levels of the food chain (Bouwman et al. 2013). The negative effects of DDT include reduced eggshell quality in birds (Blus 2011), reproductive disorders (i.e. modifications in endocrine system functioning) (Guillette & Gunderson 2001) and reduced haemoglobin levels and anaemia (Rivera-Rodríguez & Rodríguez-Estrella 2011). Furthermore, an increase in levels of OCPs has been linked with immunosuppression and a higher susceptibility to parasite infections (Sagerup et al. 2000).

Accumulation of pesticides is not uniform among vertebrate species and their concentration can vary depending on the species ability for biotransformation and elimination (Borgå et al. 2007). Species-specific life-history traits greatly affect the exposure to pesticides and its accumulation in animals, including their migratory behaviour (Herzke et al. 2002), diet and habitat use (Clatterbuck et al. 2018). Levels of organic pollutants have been monitored intensively in wild birds populations, especially in raptors and seabirds, which are considered good sentinel species mainly due to their long lifespan, extended home ranges and apex position in food webs (Guruge et al.

2001; García-Fernández et al. 2008; Gómez-Ramírez et al. 2012). Due to the high capacity of these pollutants to accumulate in adipose tissues, organs, and muscles (van Drooge et al. 2008; Clatterbuck et al. 2018), invasive methods have been broadly used to monitor their presence and concentration in dead animals (Hop et al. 2002). Nevertheless, non-invasive methods may facilitate biomonitoring in wildlife. In birds, most studies focused on the measurement of OCPs in non-hatched eggs (Wang 2011), droppings (Sun et al. 2006), feathers (Abbasi et al. 2016) or blood samples (Espín et al. 2018). Although these studies have provided valuable results, the use of these samples could potentially have important limitations affecting the conclusions obtained. For instance, sampling eggs may provide information on the pollutants present in adults during a specific period (i.e. females during laying season). In addition, according to Jasper et al. (2011), the external contamination, moult strategy and preening behaviour as well as the particular type of feather sampled could settle directly in the feathers altering the final levels of DDT and its metabolites. Uropygial gland secretions represent a promising source to measure OCPs in birds (van den Brink et al. 1998) that may allow researchers to estimate the burden of pollutants in adipose tissues (Yamashita et al., 2007).

The uropygial gland is a bird holocrine gland that synthetize an oil secretion with a mixture of different compounds such as alkanes, ketones, aldehydes, alcohols and waxes (Campagna et al. 2012). This secretion has several functions including waterproofing of feathers and protection against solar radiation, among others (Giraudeau et al. 2010; Moreno-Rueda 2017). Here, we tested the hypothesis that habitat determines the exposure to DDE of a ubiquitous and omnivorous wild bird, the common blackbird (*Turdus merula*). We compared the relative proportions of DDE in the secretions of the uropygial gland of birds from two populations located in an urban and a forest area, respectively. Due to the widespread use of DDT-based insecticides in agricultural crops, we predict a higher DDE relative proportion in individuals from the forest than the urban area. We also predicted higher DDE relative

proportion in adults than in juvenile birds, likely reflecting the bioaccumulation of this pollutant over time, as has been showed for other contaminants such as heavy metals in different tissues of this species (Kim et al. 1996). In addition, owing to the negative consequences of DDE on bird health, for example, due to its immunosuppressive effects (Bustnes et al. 2004), we assessed whether the prevalence of infection by blood parasites and bird body condition (body mass corrected by body size) were associated with the relative proportion of DDE in bird secretions.

Material and methods

Study species and area

The common blackbird is a medium-sized passerine species widely distributed throughout Europe. The species is commonly found in both urban and natural areas with trees, shrubs and open areas (Ibáñez-Álamo & Soler 2010). The home range, i.e. the whole area exploited by individuals, including foraging areas (which are of special importance for exposure to contaminants) covers about 12.700m^2 (Karakaya & Arikan, 2015). It is an omnivorous species that feeds mainly on the ground, with insects and earthworms constituting an important part of its diet (Chamberlain et al. 1999). Spanish populations of common blackbirds are sedentary (Carrascal & Salvador 2016).

Bird sampling was conducted in two localities in southern Spain from March to June 2015, overlapping with the species' breeding season. The forest area was located at the "Corredor Verde del Guadiamar" (37°18'23"N, 6°15'44"W, Seville province). Vegetation in this area is dominated by poplar and oak groves surrounded by agricultural fields. The urban area was the park of "Maria Luisa" (37°22'29"N, 5°59'19"W), which is located in the centre of the city of Seville. Both sampling sites are separated by approximately 25km. These study areas were selected because they were maintained their landscape characteristics during years. Bird trapping was carried out with all the necessary

permits issued by the Regional Department of the Environment (Consejería de Medio Ambiente, Junta de Andalucía) and CSIC bio-ethics committee.

Bird capture and sampling

We captured a total of 52 common blackbirds in forest (n=33) and urban (n=19) areas (21 juveniles and 31 adults; 26 males and 26 females), by using mist nests from sunrise to midday. Playbacks were not used to attract birds to avoid any potential bias in bird sampling (Figuerola & Gustamante 1995). Birds were ringed with numbered metal rings, weighed and wing length was measured. The age (juveniles: <1 year old *vs* adults: >1 year old) and sex of adult birds was determined according to plumage characteristics (Svensson 1998). The sex of juveniles was molecularly determined (see below). Birds were blood sampled by brachial venipuncture in heparinized capillary tubes and samples transferred to Eppendorf tubes. Blood samples were maintained in cold boxes (4°C) during the fieldwork. In the laboratory, samples were separated in plasma and cell fractions and stored at -80ºC until further analyses.

The uropygial gland was measured using a digital calliper (± 0.01mm) and its volume was estimated following Magallanes et al. (2016), as the product of length, height and width of the gland in mm. We collected uropygial gland secretions from all individuals by gently pressuring and massaging the papilla with a non-heparinized capillary and secretions were stored in 2ml gas chromatography vials. We followed the same procedure but without collecting secretion to obtain blank control vials to exclude contaminants from the handling procedure or the environment, and to examine potential impurities in the solvent or analytical procedure. The vials were maintained in a cold box during the fieldwork and, subsequently, stored at -80ºC.

Molecular analyses

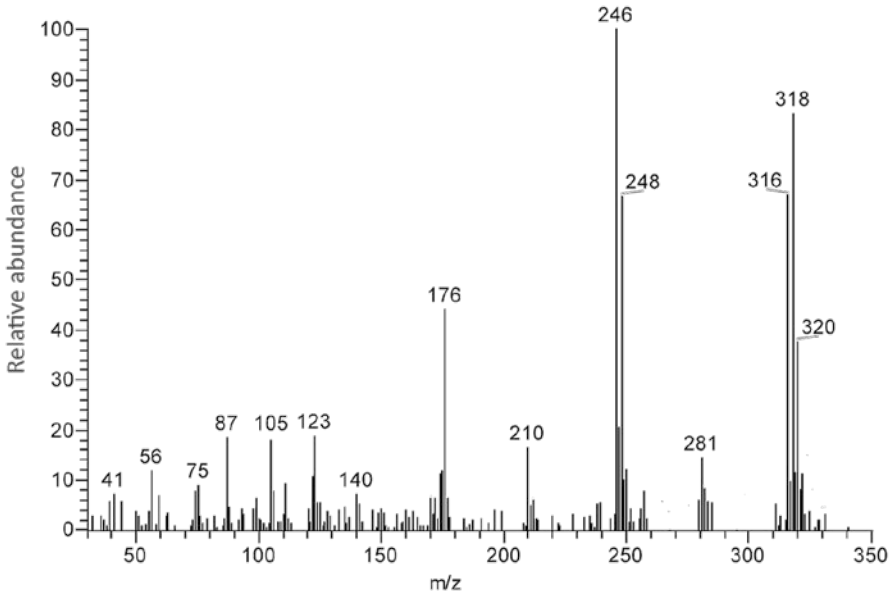
Genomic DNA was extracted from the cell fractions using a semi-automatic Maxwell kit method (Maxwell®16 LEV system Research, Promega, Madison, WI). Juvenile birds were sexed following Griffiths et al. (1996, 1998). Detection of parasites of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* was performed following Hellgren et al. (2004). The presence of amplicons was verified in 1.8% agarose gels. Positive samples were sequenced using the facilities of Macrogen Company (Macrogen Inc. Madrid, Spain). Sequences were edited with the software Sequencher™ v 4.9 (Genes Codes Corp., Ann Arbor, MI, USA) and compared with those deposited in GenBank Database (National Center for Biotechnology Information) using BLAST to identify the parasites to the genus level.

Detection of DEE in uropygial gland secretions

Uropygial gland secretions were analysed using an Agilent 7890A gas chromatograph (GC) fitted with a poly (5% diphenyl, 95% dimethylpolysiloxane) column HP5-MS (30m length x 0.25mm inner diameter x 0.25µm film thickness) and an Agilent 5975C Triple Axis Detector mass spectrometer (MS) as detector. We injected in splitless mode 2µl of each sample previously dissolved in 50µl of hexane with helium as the carrier gas. The oven temperature program started at 80ºC and was maintained during 3 minutes, then increased to 300ºC at a rate of 5ºC/minute and finally was maintained at 300ºC for 35 minutes. To identify the uropygial gland secretion lipophilic components, we compared the mass spectra of compounds in the sample with the standards available in the NIST/EPA/NIH 2002 (NIST Mass Spectral Library, Version 2.0©, Faircom Corporation, USA), and later confirmed with authentic standards (from Sigma-Aldrich Chemical Co., St. Louis, MO, USA). DDE was identified based on its characteristic mass spectrum (with typical m/z: 105, 176, 210, 246, 318) (Fig. 1), but we did not discriminate between the isomers *p,p*'-DDE and *o,p*'-DDE. The relative proportion of DDE was determined as the percentage of

the total ion current (Xcalibur 2.2 software, Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA). To correct for the non-independence of proportions, we performed compositional analysis consisting in logit transforming the proportion data by taking the natural logarithm of proportion/(1-proportion) (Aebischer et al. 1993).

Figure 1. Representative mass spectrum of the compound identified as DDE found in samples of uropygial gland secretions of common blackbirds.



Statistical analysis

Relative proportion of DDE was non-normally distributed. Thus, non-parametric Mann-Whitney U tests were used to compare the DDE relative proportion in secretions of birds of different ages (juveniles and adults), sexes (males and females) and habitats (urban and forest areas). The relationship between DDE relative proportion and the volume of uropygial gland was tested by a Spearman correlation. Because nearly all adult blackbirds (30 of 31) were infected by avian haemosporidians, we used the subset of juveniles to assess the association

between the infection status by any haemosporidian parasite (infected *vs* uninfected birds) and the relative proportion of DDE by using a Generalized Linear Model (GLZ). Finally, potential differences in bird body mass according to DDE proportions were tested using a Linear Model (LM) by including the body mass as the dependent variable and the relative proportion of DDE and wing length (a correlate of body size) as independent variables. The body mass of two individuals and the volume of uropygial gland of other two birds were not measured, which explain differences in the sample size included in the different analyses. Statistical analyses were performed in STATISTICA 8.0 (StatSoft. Inc. 1984-2007).

Results

DDE was found in the secretions of 36 (69.23%) common blackbirds. Control vials did not show any trace of this compound in any case. The relative proportion of DDE was significantly higher in birds from forest than from urban areas (Z adjusted= -2.34, *P*=0.019; Fig. 2). The relative proportion of DDE was higher in adults than in juveniles (Z adjusted= -2.25, *P* =0.024, Fig. 3), while not differ between sexes (Z adjusted= -0.65, *P*=0.52). Parasite infections were recorded in 46 (88.46%) birds, including 30 out of 31 adults and 16 out of 21 juveniles. *Plasmodium* spp. was the most common parasite infecting birds (prevalence=82.69%), followed by *Leucocytozoon* spp. (prevalence=44.23%) and *Haemoproteus* spp., the later being present only in one adult bird (prevalence=1.92%). The infection status of birds was not associated with the relative proportion of DDE for the subset of juveniles (Wald=0.99, d.f.=1, *P*=0.32). Bird body mass was significantly related to relative proportion of DDE (F=5.69, d.f.=1,47, *P*=0.021) after controlling for the effect of wing length (F=3.39, d.f.=1,47, *P*=0.07). The volume of uropygial gland was not significantly related to the relative proportion of DDE (N=50, Spearman R=0.12, *P*=0.42).

Figure 2. Relative proportion (± S.E.) of DDE in the uropygial gland secretions of common blackbirds in relation to habitat characteristics, urban (n=19) *vs* forest (n=33) areas.

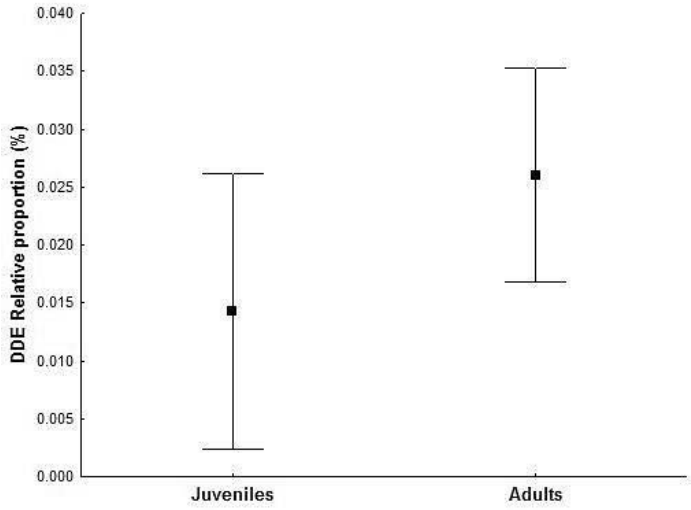
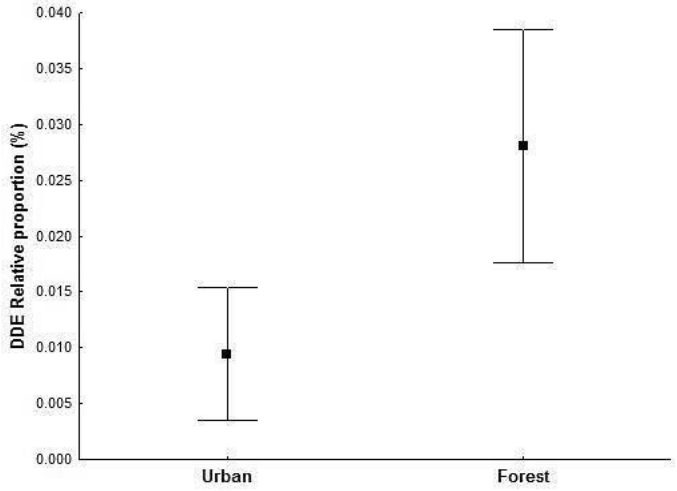


Figure 3. Relative proportion (± S.E.) of DDE in the uropygial gland secretions of common blackbirds in relation to age classes, juveniles: <1 year old (n=21) *vs* adults: >1 year old (n=31).



Discussion

In spite that the use of DDT, similarly to other European countries, was banned in Spain since 1977 (BOE 1975), our results add to the widespread evidence that birds are still exposed to its metabolite DDE in these areas (Martinez-Lopez et al. 2007; Naso et al. 2003). By using a non-invasive method, we found that common blackbirds from forest areas had a higher relative proportion of DDE than those from urban areas. Furthermore, this pollutant differed between age classes, with adults showing a higher relative proportion than juvenile birds, which suggests that this pollutant bioaccumulates in bird tissues over time. Finally, the relative proportion of DDE was not associated with parasite infections but a positive association was found with bird´s body mass

Although exposure to DDE seems to be widespread throughout Europe, it may, however, differ according to the species' life history traits (e.g. food habits, migratory behaviour) and across the different habitats used during their annual cycle (Yu et al. 2014). For instance, Kunisue et al., (2002) reported the accumulation of organochlorines in migratory birds in their Asian wintering grounds, suggesting the existence of a high exposure in certain areas. Differences in exposure to DDE also occur at finer spatial scales, as was reported in Eurasian Eagle owl eggs from different locations in Spain (Gómez-Ramírez et al. 2012). Indeed, and in agreement with this possibility, our results support important differences associated to habitat characteristics. DDT was commonly used in large amounts in crops for the control of insect pests in past decades, whereas its use was likely less intense in urban areas. Even so, up to 84.21% of birds from the urban area had detectable concentrations of DDE in their uropygial secretions, which suggests that DDT was used in the city or was spread from surrounding areas due to the semivolatility of this pesticide (Meijer et al. 2003). However, we can not exclude that the difference found between habitats were due to a differential feeding behaviour of birds, with birds from urban areas feeding more on fruits and those from forest areas feeding more on invertebrates. Earthworms, important feeding sources for this bird species, accumulate high

levels of DDT and DDE (Harris et al. 2000). The positive relationship between DDE and bird´s body mass could be due to a link between these variables and the levels of lipid resources, where DDE may be accumulated prior to its excretion.

DDT and its metabolites are highly persistent pollutants in the environment, being progressively bioaccumulated in animal tissues (Turusov et al. 2002). Growing evidence support the accumulation capacity of these chemicals in animals and biomagnification through food webs of terrestrial, freshwater and estuarine habitats (Kidd et al. 2001). Bioaccumulation may be evidenced by comparing age classes of the same species, as we did here. For instance, Espín et al. (2018) reported higher blood concentrations of DDE in adults than in nestlings of Montagu's and pallid harriers (*Circus pygargus* and *C. macrourus*) from Spain and Kazakhstan, respectively. Similarly, Garcia-Heras et al. (2018) detected higher DDE levels in adults than in nestling black harriers (*C. maurus*) from South Africa. In the same way, we found that adult blackbirds had a higher DDE relative proportion than juveniles. On the other hand, we did not find significant differences in the relative proportion of DDE between sexes. Although, several studies have suggested that female birds accumulate less contaminant than males, due to maternal transfer of contaminants to eggshell and yolk (Drouillar & Nostrom 2001; Kubota et al. 2013), we found no significant differences between the sexes. However, our results confirm that both sexes eliminate DDE from their bodies in similar proportions through the secretions of the uropygial gland.

Although accumulation of OCs has been associated with weakened health, we did not find any significant relationship between parasite infections and the relative proportion of DDE in common blackbirds. Nonetheless, the effects of pollutants on the prevalence of infectious diseases are poorly known. The high exposure to OCs of pre-fledgling chicks of caspian terns (*Hydroprogne caspia*) and herring gulls (*Larus argentatus*) has been associated with immunosuppression, in particular, a decrease in T-cells, which facilitates parasitic infections (Grasman et al. 1996). However, these effects may differ

between pollutants (or be only apparent at high pollutant concentrations), bird and parasite species studied. In a study on other pollutants, Bichet et al. (2013) found a positive relation between lead concentration and *Plasmodium* prevalence in house sparrows (*Passer domesticus*), but not between zinc and cadmium, suggesting immunotoxic effects of these contaminants. Because haemoporidians usually produce chronic infections, our results could be explained in the base that birds were infected prior to DDE exposure. Further studies are necessary to also analyse the relationship between DDE and parasite load instead of parasite infection status as they may reflect two different faces of parasite infections in birds (Westerdahl et al. 2011).

In conclusion, DDE could be easily detected using non-invasive and non-destructive methods in birds allowing the biomonitoring of contaminants as well as the identification of factors (i.e. habitat use and age, in our study) potentially affecting the exposure of birds to this pollutant. Our results indicate that exposure to DDE is higher in forest areas and that DDE accumulates with age, increasing in relation with body mass of blackbirds.

Acknowledgments

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SECTION III

Chapter 4

House sparrow uropygial gland secretions do not attract ornithophilic nor mammophilic mosquitoes

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Abstract

Mosquito feeding preferences determine host-vector contact rates and represent a key factor in the transmission of vector-borne pathogens. The semiochemical compounds of which vertebrate odours are composed probably play a role in mosquito host choice. Birds spread secretions from uropygial gland over their feathers to protect their plumage, compromising behaviour that may in turn affect odour profiles. Although uropygial secretions are expected to modify the attractiveness of birds to mosquitoes, contradictory findings have been reported. Mosquito species differ in their feeding preferences, with some species feeding mainly on birds (ornithophilic species) and others on mammals (mammophilic species). Consequently, it is possible that ornithophilic and mammophilic species differ in their response to uropygial gland secretions. Using a dual-choice olfactometer, the present study test this hypothesis by comparing the behavioural response to uropygial gland secretions from juvenile male and female house sparrows (*Passer domesticus*) in the ornithophilic *Culex pipiens* and the mammophilic *Aedes* (*Ochlerotatus) caspius* mosquitoes. No differences were found in the response of either mosquito species to the uropygial gland secretions. Therefore, the preference of ornithophilic mosquitoes for avian hosts is apparently not explained by a greater attraction of mosquitoes to the uropygial gland secretion odour when presented in combination with CO_2-enriched airflow.

Keywords: *Aedes (Ochlerotatus) caspius*, *Culex pipiens*, host attraction, mosquito behaviour, olfactometer, wild birds

Contact rates between insect vectors and hosts have important consequences for the transmission dynamics of vector-borne pathogens. Blood-seeking female mosquitoes detect their vertebrate hosts via a combination of visual, thermal and chemical stimuli (Lehane 2005). The attraction that mosquitoes feel to different vertebrate groups differs widely between mosquito species: some species feed preferentially on mammals (mammophilic species), others mainly feed on birds (ornithophilic species) and yet others mainly feed on other vertebrate groups (Martínez-de la Puente et al. 2015). Different compounds, including octenol, ammonia and lactic acid, have been reported as attractants to mammophilic mosquitoes such as *Anopheles gambiae* (Takken & Knols 1999). Birds produce, in their uropygial gland, a mixture of organic compounds that varies between species, ages and sexes (Tuttle et al. 2014; Moreno-Rueda 2017), which they spread over their plumage when preening and which mainly functions as waterproofing and conditioning for plumage. This secretion has been reported to attract insect vectors such as mosquitoes (Russell & Hunter 2005), whereas other studies have found the opposite (i.e. that this secretion in fact acts as a repellent) (Douglas III et al. 2005). Thus, how secretion from the uropygial gland of the bird affects host attractiveness to mosquitoes still remains unclear.

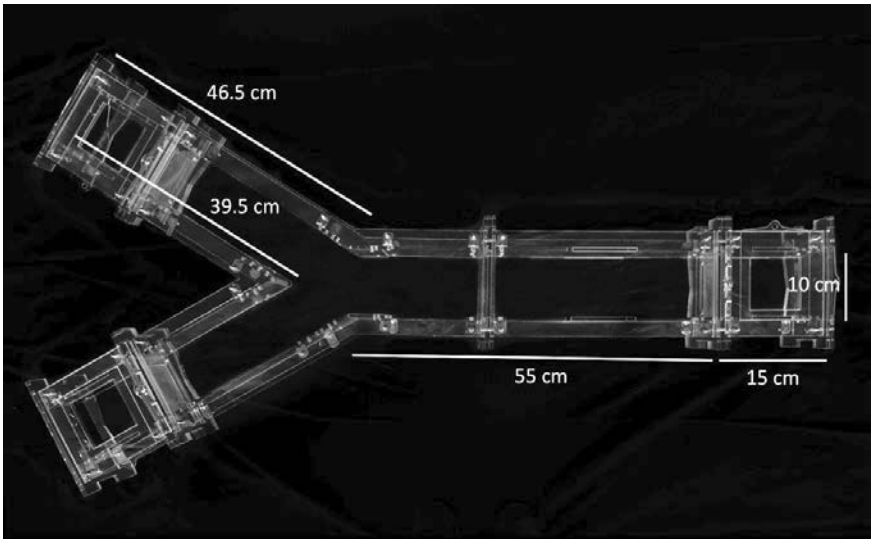
The present study aimed to investigate the effects of uropygial gland secretion on the host-seeking behaviour of mosquitoes and to explore whether these effects differ between mosquito species with different feeding preferences. Using a dual-choice olfactometer, the present study assessed the effect of house sparrow (*Passer domesticus*) uropygial gland secretion on the attraction of the ornithophilic common house mosquito *Culex pipiens* and the mammophilic marshland mosquito *Aedes* (*Ochlerotatus*) *caspius* (Muñoz et al. 2012; Martínez-de la Puente et al. 2015). It would be expected that *Cx. pipiens* would be more responsive to avian stimuli. House sparrows were selected as the study avian species because they are common mosquito hosts and are naturally infected by different mosquito-borne pathogens (Ferraguti et al. 2018).

Juvenile house sparrows were captured using mist nests in the provinces of Cadiz and Huelva (southern Spain) from mid-September to mid-October 2013 in the context of different studies (Ferraguti et al. 2018; Jiménez-Peñuela et al. 2019). Some additional individuals were also captured in the province of Seville in May 2015. A blood sample (of up to 100µL) was obtained from the jugular vein using sterile syringes, never exceeding 1% of the body mass of birds, aiming to determine bird sex and infection status by haemosporidian parasites. Genomic DNA from the blood samples was extracted using a Maxwell® 16 LEV system Research (Promega, Madison, WI, U.S.A.). Birds were molecularly sexed using primers P2 (5´-TCTGCATCGCTAAATCCTTT-3´) and P8 (5´-CTCCCAAGGATGAGRAAYTG-3´) and the infection status by the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* was determined molecularly as described in Gutiérrez-López et al. (2019). The secretion of the uropygial gland was extracted by gently massaging the papilla of the gland and immediately placed in 2-mL gas chromatography vials. Immediately after sampling, birds were released at the place of capture. Blood samples and uropygial gland secretions were maintained in cold boxes in the field and stored in the laboratory at -20ºC and -80ºC, respectively, until the behavioural assays were performed. Only secretions from birds not infected by haemosporidians (overall infection prevalence of 59%) (Jiménez-Peñuela et al. 2019) were used for the experiments to avoid the potential effect of parasite infections on the response of the mosquitoes. The final sample size included in the present study was 51 juvenile birds, comprising 29 male and 22 females.

Mosquito larvae were collected in Huelva and Seville provinces during August to December 2015 and July/August 2016. They were then transported to the laboratory where they were kept in a climatic chamber under standard conditions (12:12 light/dark photocycle at 28ºC and 65-70% relative humidity) and fed *ad libitum* with Mikrozell (20mL/22g; Hobby Mikrozell; Dohse Aquaristik GmbH & Co. KG, Gelsdorf, Germany). When mosquitoes emerged, they were placed in insect cages and fed *ad libitum* with 1% sugar solution and

deprived of sucrose solution 24h before the experiment and thereafter only had access to water, which was removed from cages 1h before the trials. Six- to 15-day-old female mosquitoes were used in the experiments.

Figure 1. Dual-port olfactometer used in the present study. This Y-shape consists of three parts: acclimation section (length 15cm, width 10cm, height 10cm), flight section (length 55cm, width 10cm, height 10cm) and two ports with vertical doors to separate the two compartments (external length 46.5cm, internal length 39.5cm, width 10cm, height 10cm).



The behavioural responses of mosquitoes to the secretions from the uropygial glands of birds were assessed using a dual-choice olfactometer (Mimétrica Diseña a tu Medida S.L., Pozuelo de Alarcón, Madrid, Spain) (Fig.1). This Y-shape olfactometer is made of methacrylate and consists of three parts: acclimation section (length 15cm, width 10cm, height 10cm), flight section (length 55cm, width 10cm, height 10cm) and two ports with vertical doors to separate the two compartments (external length 46.5cm, internal length 39.5cm, width 10cm, height 10cm). The doors were closed before the start and after the trials to be able to reliably count the number of mosquitoes caught in

each section. Airflow was generated with an air pump taking air from a partially open 60-L plastic bucket enriched with a continuous 180mL/min flow of CO_2 (purity ≥ 99.7%). The mixture was passed through a charcoal-filter, before being humidified and warmed (60% relative humidity and 27 ± 1ºC) and then equally distributed into both ports of the olfactometer via a flow meter (Dual Pump System and Flow Measurement System, Sable Systems, Las Vegas, NV, U.S.A) with a flow 1800-mL/min. Airflow enriched by CO_2 was present in both ports of the olfactometer, although the vial with the secretion from one bird was only placed in one of the two ports. For every trial, the secretion of one individual bird was placed in each port alternatively to discard any positional effects. In each trial, 6-15-days-old female mosquitoes (n=20 ± 1) were allowed to fly in completely darkness and follow an upwind air current towards the secretion port or control port for 15min. Then, the doors separating the different compartments were closed and the number of mosquitoes caught in each section was counted. All trials were carried out 2-3h before the beginning of the dark cycle in the climatic chamber. Mosquitoes were only used for one trial and no individuals were re-used in subsequent trials. The olfactometer was cleaned between consecutive trials with water and allowed to dry; a minimum of 24h was allowed to elapse between consecutive trials.

Only mosquitoes that actively responded to the stimuli or the control and thus were caught in one of the two ports were considered for statistical purposes. First, the number of mosquitoes attracted to bird stimuli (secretion + CO_2) and control (CO_2) ports was compared using non-parametric Wilcoxon matched-paired tests in R, version 3.4.3 (R Core Team 2017) with the package *dplyr* (Wickham et al. 2015). Tests were two-tailed and separate analyses were conducted for each mosquito species. Second, the response of *Cx. pipiens* and *Ae*. *caspius* towards the avian stimuli was analysed using generalized linear mixed models with binomial error and logit link function with the packages *lme4* (Bates et al. 2015) and *blmeco* (Korner-Nievergelt et al. 2015). The response variable was defined as the number of mosquitoes that chose the bird secretion in

relation to the number of mosquitoes that chose the control using the *cbind* function. Mosquito species and the bird sex were included as fixed factors in the model. The variables 'port' (left or right port) and 'year' (2015 or 2016) could not be included as random terms because of convergence problems. Therefore, a preliminary analysis assessed whether the port and the year affected the variation in the response variable. Because non-significant effects were detected (in both cases $P > 0.20$), these variables were removed from further analyses. The trial ID was incorporated as a random term to account for overdispersion.

In total, 419 *Cx. pipiens* females were assayed in 21 trials and 603 *Ae. caspius* in 30 trials. Mosquitoes actively responded in 19 and 15 of these trials for *Cx. pipiens* and *Ae. caspius*, respectively ($\chi^2 = 9.11$, df=1, $P=0.003$). Of these, 165 (39.4%) *Cx. pipiens* and 27 (4.5%) *Ae. caspius* reached one of the two ports during the trials. Both *Cx. pipiens* (V=124.5, $P=0.24$) and *Ae. caspius* (V=63.5, $P=0.21$) were attracted equally to the ports containing bird´s stimuli and to the control. For the case of *Cx. pipiens*, 95 out of 165 (57.58%) mosquitoes showing an active response were attracted by the secretion, whereas 70 (42.42%) were attracted to the control port. For the case of *Ae. caspius*, 17 (62.96%) out of 27 mosquitoes showing an active response were attracted to the port containing bird´s stimuli and 10 (37.04%) were attracted to the control port. *Culex pipiens* and *Ae. caspius* responded similarly to the bird stimuli (mosquito species: $\chi^2=0.20$, d.f.=1, $P=0.66$). Bird sex did not affect significantly the response of mosquitoes (sex: $\chi^2 = 1.60$, d.f.=1, $P=0.21$).

Although the uropygial gland secretion is considered to be largely responsible for bird odour (Moreno-Rueda 2017), no support was found for a greater attraction of ornithophilic mosquitoes towards this stimulus. This result is unexpected given that previous studies have reported the ornithophilic feeding preference of *Cx. pipiens*: 69-98% of blood meals on birds (Gómez-Díaz & Figuerola 2010).

The role of the uropygial gland secretion as an attractant for blood-sucking insects is still unclear. Russel & Hunter, 2005 captured more mosquitoes in traps supplemented with uropygial gland secretion than in control traps, although these differences were only evident in traps located at a height of 5m above ground level and not at a height of 1.5m. By contrast, other studies have reported non-significant effects for uropygial gland secretions on the attraction of mosquitoes (Garvin et al. 2018a). Despite *Cx. pipiens* responding more actively than *Ae. caspius*, the results obtained in the present study agree with the latter findings because no significant differences were found in the responses of the two studied mosquito species to the presence of uropygial gland secretions. It has been suggested that uropygial gland secretions could be selected to become cryptic for ectoparasites, thus providing camouflage against hematophagous insects such as blood-seeking mosquitoes (Moreno-Rueda 2017). In addition, these secretions could play a role as repellents and insecticides rather than attractants, as suggested by the lower vector-borne parasite prevalence in house sparrows with larger uropygial glands (Magallanes et al. 2016).

It is possible that the degraded secretion deposited on the skin or feathers determines the attractiveness of mosquitoes to their hosts and not the uropygial gland secretion itself. The bacterial community on a bird's skin metabolizes these secretions, thus potentially affecting their chemical composition and, consequently, their attractiveness to mosquitoes (Bernier et al. 2008). In addition, the possibility of mosquito host-seeking behaviour being determined by cues emitted by hosts that differ from those considered in the present study (e.g. temperature, colouration or host body size) (Lehane 2005) cannot be ruled out. Although a greater attraction of *Cx. pipiens* to bird stimuli would be expected, this may not be the case for the mammophilic *Ae. caspius*. For this species, other mammal stimulus not considered here such as L-(+)-lactic acid or 1-octen-3-ol may be more relevant cues for mosquitoes when detecting their hosts (Williams et al. 2006). The absence of these compounds in the uropygial gland secretion of

wild house sparrows may explain, at least in part, the lack of significant differences found in the case of *Ae. caspius* and the low response observed in this species where only 4.5% of the individuals reached one of the two ports during the trials.

No significant differences in mosquito responses were found in terms of bird sex. In a previous study, Gutiérrez-López et al. (2019) reported that the mammophilic *Ae. caspius* bit more females than males and no preferences for any particular sex were found when *Cx. pipiens* were exposed to house sparrows (i.e. the same host species as investigated in the present study). Likewise, no significant differences were found in the attraction of mosquitoes to secretions of male or female birds. Nevertheless, the present study only used samples collected from juvenile birds and so the effect of sexual differences on the attractiveness of uropygial gland secretion in adults cannot be ruled out. Although the composition of the uropygial secretion differs between age classes in birds, including house sparrows, mosquitoes are reported to be similarly attracted to the uropygial gland secretion of both juvenile and adult house sparrows (Garvin et al. 2018b).

In summary, the results obtained in the present study suggest that two mosquito species of the genera *Culex* and *Aedes* do not show a differential attraction towards uropygial gland secretion of house sparrows.

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Ethics statement

Bird trapping was carried out with all the necessary permits issued by the regional Department of the Environment (Consejería de Medio Ambiente, Junta de Andalucía). Mosquito larvae collection and bird sampling on private land and in private residential areas were conducted with all the necessary permits and consent, and in the presence of owners. The CSIC Ethics Committee approved the experimental procedures on 9 March 2012. This study did not affect any endangered or protected species.

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Chapter 5

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

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 & 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 & 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 (CO_2), temperature, moisture, and body odour- to detect and select their hosts (Eiras & Jepson 1994; Lehane 2005). The use of synergistic cues rather than a single independent cue may greatly facilitate host choice. The combination of CO_2 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 CO_2 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 & 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.

Materials and methods

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 (20ml/22g Hobby Mikrozell; Dohse Aquaristik GmbH & Co. KG, Gelsdorf, Germany) in a climatic chamber under controlled conditions (28º ± 1ºC, 60-65% relative humidity (RH) and 12:12h 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 32.5cm) and fed a 1% sugar solution ad libitum. Mosquitoes were deprived of the sugar solution 24h before the trials began and only had access to water, which was removed from cages 1h 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.

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, 37º18´51´´N, 6º50´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 2ml 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 -20ºC and -80ºC, 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 36cm) in a vector-free room under controlled conditions with a 12:12h light:dark cycle at 22ºC (± 1ºC). 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).

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.

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 (5.8L, 20cm 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 400ml/min. Each bird remained inside the desiccator for 15min. Birds were not exposed for longer periods in order to minimize their stress levels. Overall, for each bird, 6L 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 2ml gas chromatography vials with 600µl of hexane and stored at -80ºC. 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.

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 (30m length x 0.25mm inner diameter x 0.25µm film thickness) with helium as the carrier gas and the Agilent 5975C Triple Axis Detector mass spectrometer (MS) as the detector. We added 50µl of hexane directly into the vial with the secretion and vortexed it. Then, we injected in the GC in splitless mode 2µl of the supernatant containing lipids. The oven temperature program started at 80ºC and was maintained for 3min., then increased to 300ºC at a rate of 5ºC/min., and finally was maintained at 300ºC for 35min. 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.

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 60L plastic bucket enriched with a continuous 180ml/min flow of CO_2 (purity $\geq$ 99.7%) to generate airflow passing through the

charcoal filter. The flow was then humidified (60% RH) and warmed (27 ± 1ºC), 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 1800ml/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 24h elapsed between two consecutive trials with the same olfactometer.

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).

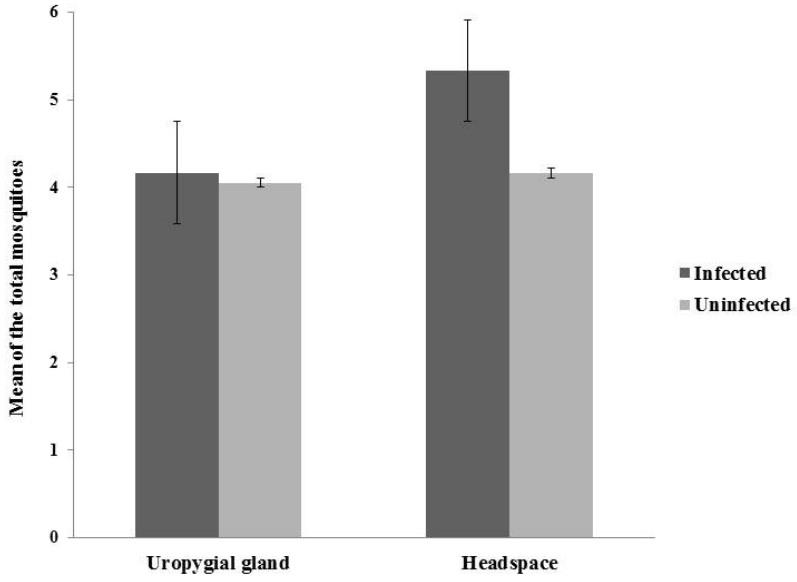
The relative proportion of each compound (e.g. the percentage relative to the total amount of volatile lipids in the sample) was determined as the percentage of the area of each peak in relation to the total ion current using the peak area integration capability of the Xcalibur 2.2 software (Thermo Fisher 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 & 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 & 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).

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 ± SE=0.98 ± 0.28, degrees of freedom (d.f.)=16, P=0.003), regardless of sex (estimate ± SE=0.00002 ± 0.26, d.f.=16, P=0.99). The stimuli did not significantly affect the mosquito attraction (estimate ± SE =0.39 ± 0.26, d.f.=16, P=0.15). However, the interaction between the infection status and the stimuli approached significance (estimate ± 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).

Figure 1. Mean (± 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*).



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: $PseudoF_{1,33}=0.79$, $P=0.73$; infection: $PseudoF_{1,33}=0.75$, $P=0.77$; sex-infection interaction: $PseudoF_{1,33}=0.62$, $P=0.92$). 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 carboxilic acids) present in the uropygial secretions (ANOVA, in all cases, $P > 0.14$) (Table 2).

Table 1. Mean relative proportion (± 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.

		Males (*n*=21)				Females (*n*=16)			
		Uninfected (*n*=10)		Infected (*n*=11)		Uninfected (*n*=8)		Infected (*n*=8)	
RT	Compounds	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)	7.96	0.97 (2)	1.97
26.9	Hexadecanol	2.48 (4)	3.86	5.11 (7)	4.87	2.69 (3)	4.82	2.36 (4)	3.61
28.9	Heptadecanol	4.08 (5)	4.57	5.69 (7)	5.51	8.25 (5)	10.43	6.24 (5)	7.22
30.8	Octadecanol	43.58 (9)	18.13	40.99 (10)	25.96	41.10 (6)	29.61	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)	0.39
23.4	Pentadecanal	0.05 (1)	0.16	ND		0.10 (2)	0.21	0.06 (1)	0.17
25.6	Hexadecanal	1.06 (4)	1.54	9.52 (3)	30.03	0.38 (3)	0.59	0.27 (1)	0.75
27.6	Heptadecanal	0.21 (2)	0.45	0.21 (2)	0.54	0.29 (2)	0.65	0.19 (1)	0.55
29.5	Octadecanal	20.28 (9)	30.30	8.18 (8)	8.53	18.64 (8)	33.59	11.64 (6)	20.35

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)	1.01
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)	0.69
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)	1.06
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)	1.08
31.5	Octadecanoic acid, ethyl ester	0.22 (2)	0.49	0.76 (4)	1.23	1.23 (3)	2.09	0.85 (3)	1.26
	Alcohol acetates								
25.4	Tetradecanol, acetate	0.05 (1)	0.15	0.32 (3)	0.69	0.60 (3)	0.92	0.69 (3)	0.99
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 (± 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

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 & 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 & 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 el 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 & 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 & 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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GENERAL DISCUSSION

Animal communication through the emission and detection of chemical cues may be important in social behaviours such as mate choice (Leclaire et al. 2019; Martín & López 2015), antipredatory behaviours (Kats & Dill 1998, Amo et al. 2008) and food acquisition (Amo et al. 2013; Borgo et al. 2006). In mosquitoes, chemical cues are of major importance for the identification of food sources, including plants (Foster & Hancock 1994; Lahondère et al. 2020) and vertebrate hosts (Takken & Verhulst 2013), the later playing a central role in pathogen epidemiology. Understanding the role of different chemical cues in host-vector relationships and the potential effects of parasites on this process is of special relevance, since the interactions between vertebrate hosts, vectors, and parasites define the transmission dynamics of vector-borne diseases.

In this thesis, I used a multidisciplinary approach to assess the role of chemical cues, namely the uropygial gland secretion and the whole body odour (headspace) of birds, in the attraction of mosquitoes and how this interaction could be mediated by the mosquito-borne avian malaria parasites. These widespread parasites play a key role in the dynamics of wild birds populations and represent excellent models in studies of ecology and evolution of vector-borne pathogens (Rivero & Gandon 2018), like those included in this thesis. In parasites with complex life cycles involving both intermediate and definitive hosts, parasites could manipulate different traits of the vertebrate, but also of the invertebrate hosts to complete their life cycle. For instance, malarial parasites are able to affect the behaviour of infected vectors (Stanczyk et al. 2017) by increasing host-seeking activity (Koella et al. 2002), frequency of biting (Wekesa et al. 1992), and blood-sucking duration (Anderson et al. 1999) when the transmissible stages of the parasites are present in mosquitoes. Likewise, competent insect vectors may increase their preference for infected vertebrate hosts (Metz & McBride 2018). Indeed, infections by malaria parasites may also induce behavioural and/or physiological changes in the vertebrate hosts that make them more attractive or susceptible to vectors. This may be the case of *Plasmodium*-infected children, who increased their attractiveness to *Anopheles*

vectors with respect to uninfected children, probably due to an increment in aldehyde levels present in feet odour (Robinson et al. 2018). In addition, De Moraes et al. (2014) exposed *Anopheles stephensi* mosquitoes to the odours of healthy and *Plasmodium chabaudii*-infected mice and found that parasite infections increased the attraction of mosquitoes to infected individuals. In birds, however, contradictory results have been found, as reviewed in **Chapter 1**, yet increasing evidence support predictions within the host manipulation hypothesis, at least for the interaction between *Plasmodium* and *Culex pipiens* mosquitoes. The host manipulation hypothesis proposes that parasites induce changes in the host that increase parasite development or transmission. However, alternative explanations suggest that these changes (e.g., increasing body temperature or fever, Hart 1988) are an adaptive response to the infection (Poulin 2010) or just a by-product of pathology (Lefèvre & Thomas 2008; Poulin 2010). Beyond the question of the generality of this phenomenon in host-parasite systems, the mechanisms underlying the increased attraction of vectors towards infected hosts and the particular traits involved in this process remain poorly known. The secretion of the uropygial gland of birds, based on their chemical characteristics (Campagna et al. 2012), has been proposed to mediate the attraction of vectors. So, it is possible that the increased attraction of mosquitoes towards *Plasmodium*-infected birds may be mediated by changes in the composition of the uropygial gland secretion (Cornet el al. 2013). However, this possibility had not been properly assessed in the bird-malaria system. In this thesis, I tested different predictions that could be expected to occur if avian malaria parasites manipulate their hosts by altering their composition of chemical cues, such as those that uropygial gland secretions may convey, to increase the attraction of mosquito vectors towards infected birds.

Throughout this thesis, I worked with two different widespread passerines, i.e. blackbirds and house sparrows, which are common hosts of mosquitoes (Hatchwell et al. 2001; Muñoz et al. 2012) and are frequently infected by avian malaria parasites (MalAvi database, Bensch et al. 2009). These

species are therefore ideal to tackle different questions related to the host manipulation hypothesis. As a first step, I identified both intrinsic and extrinsic factors, including parasite infections, potentially affecting the composition of the uropygial gland secretion of birds. Among them, bird age and sex, two traits commonly analysed for different bird species, affected the composition of the uropygial secretion, as supported by the results of **Chapters 2** and **3.** For example, Shaw et al. (2011) and Amo et al. (2012) found significant compositional differences between adults and juveniles/nestlings in gray catbirds (*Dumetella carolinensis*) and starlings (*Sturnus unicolor*). On the other hand, bird sex affected the composition of the volatile fraction of the secretion in dark-eyed junco (*Junco hyemalis*) (Whittaker et al. 2010). Variation in the composition could be related to the hormone levels associated with the sex (Piersma et al. 1999; Soini et al. 2007) and sexual maturity of birds (Whelan et al. 2010). However, the absence of significant differences between these factors has also been reported in other species, such as cory's (*Calonectris borealis*) and scopoli's shearwaters (*C. diomedea*) and black kites (*Milvus migrans*) (Gabirot et al. 2016; Potier et al. 2018). In **Chapter 5,** I did not find significant differences in the composition of the secretion of male and female house sparrows, which could be due to the fact that only juvenile -sexually immature- birds were analysed. Altogether, these results support that bird age and sex (**Chapter 2**) are related to differences in the composition of the uropygial secretion. Other factors such as habitat, which may largely affect the diet and exposure to pollutants, may also be important in determining inter-individual differences in the composition of uropygial secretions. This is especially evident for pollutants present in secretions of the uropygial gland such as DDE, a metabolite of the pesticide DDT (**Chapter 3**). I found higher levels of DDE in adult than in juvenile blackbirds, regardless of their sex, which points towards the bioacumulative potential of DDE in living organisms (Turusov et al. 2002), as found in blood samples of other species such as black harriers (*Circus maurus*) (Garcia-Heras et al. 2018). In addition, I detected DDE in the secretions of birds from both forest and urban areas, yet higher levels were found in forest habitats.

It could be expected that pesticides had a negative effect on the immune system, compromising the ability of birds to fight-off infections such as avian malaria parasites (Sagerup et al. 2000). However, I did not find any significant association between infections and DDE levels in blackbirds. Nonetheless, the effects of pollutants on the prevalence of infectious diseases are still poorly understood (Jones & De Voogt 1999). Moreover, I did not find any significant effect of malaria infections on the composition of the volatile fraction of the uropygial gland secretions, a result that contrast with my expectations and previous findings. For example, Grieves et al. (2018) found differences in wax ester profile between song sparrows (*Melospiza melodia*) experimentally infected by *Plasmodium* and uninfected ones. Based on the results of this thesis, infection by avian malaria parasites was unrelated to the composition of the volatile fraction of the uropygial gland secretion.

After testing for potential differences in the chemical composition of the uropygial gland secretions associated with parasite infections, I investigated the role of this secretion as a cue used by host-seeking mosquitoes (**Chapter 4**). Using two mosquito species with different feeding preferences, namely the mammophilic *Aedes caspius* and the ornithophilic *Culex pipiens*, I found no differences between them in the attraction to the uropygial gland secretions of house sparrows. In fact neither of the two species showed evidence of attraction to this stimulus. This suggests that the chemical composition of the secretion by itself do not underlie the attraction of mosquitoes, even for the ornithophilic *Cx. pipiens*, as was previously reported (Garvin et al. 2018). However, in my experiments, only juvenile house sparrows were used to test mosquito attraction, and differences in the composition of the uropygial secretion related to age, as shown in **Chapter 2**, could affect the observed results. To gain insight into the role of different bird stimuli in mosquito attraction in this natural host-parasite assemblage, in **Chapter 5** I compared the effect of uropygial secretion and body odour of birds (headspace) naturally infected by *Plasmodium* and uninfected ones on the attraction of *Cx. pipiens*. With this experimental approach we aimed

at disentangling the mechanisms underlying the observed enhanced attraction of mosquitoes towards infected birds. Cornet et al. (2013) reported that *Cx. pipiens* were more attracted to *Plasmodium*-infected canaries (*Serinus canaria*) than to uninfected ones or birds with acute *Plasmodium* infections. By using an olfactometer, I tested the attraction of mosquitoes to the odours of *Plasmodium*-infected individuals and found a higher attraction of mosquitoes to the headspace of infected birds, while no differences were found when the stimulus tested was the uropygial secretion. Behavioural assays combined with the chemical analyses (GC-MS) of the volatile fraction of the uropygial gland secretions could provide further information about the compounds that mediate in the attraction of mosquitoes to their hosts. However, as reported above, I did not find differences in the chemical composition of the uropygial secretions of infected and uninfected birds. Although the ultimate ways by which *Plasmodium* parasite makes infected birds more attractive to mosquitoes are still unclear, my results suggest that the odour profiles of infected individuals change with respect to uninfected ones, which seems to mediate the differential attraction of mosquitoes. Although this possibility has received support in other malaria models (e.g. rodent malaria; De Moraes et al. 2014), it remains untested for avian malaria parasites. If host odours drive the attraction of mosquitoes, the parasites could mediate this process by altering the production of specific compounds (e.g. either increasing attractants or decreasing repellents). These results link with the host manipulation hypothesis presented in **Chapter 1**, and evidence the possible modification of host traits (i.e. odours) by avian *Plasmodium* parasites to increase mosquito attraction.

Results from this thesis open new questions for future studies. For example, different factors such as the microbiota or genetic components could also affect the odours of vertebrate hosts (Krause et al. 2018). Therefore, qualitative and quantitative differences in the composition of the microbiota between infected and uninfected individuals could affect their interaction with mosquitoes. New experiments are required to identify bacteria and fungi volatile

signatures in *Plasmodium*-infected hosts, as well as potential changes in the microbiota associated with the infection (Mukherjee et al. 2019). In addition, in order to identify the specific compounds involved in mosquito stimulation, different approaches could be used, including the electroantennography technique (EAG). This procedure allows researchers to monitor the response of insects towards a single or a mixture of target compounds (Logan et al. 2008; Biessmann et al. 2010). The physiological response to the compounds only occurs when the receptor cells of the mosquito are sensitive to the stimuli. In this particular case, the mosquito antenna could be exposed to the chemical stimuli of *Plasmodium* infected and uninfected birds, or other combinations which could affect host selection by mosquitoes, such as parasite load or stages of infection, producing changes in their electric potential coming from their nerve cell. Results of EAG studies indicated that *Culex quinquefasciatus* females respond differently to a variety of carboxylic acids, alcohols, and aldehydes from human skin emanation (Puri et al. 2006). Similarly, this procedure allowed researchers to identify the key role of nonanal, a mosquito attractant, which may be involved in the bird-selection by vectors potentially playing a role in the epidemiology of zoonotic pathogens with avian reservoirs, such as the West Nile virus (Syed & Leal 2009). This knowledge may have important applications for the control and surveillance of vector-borne diseases by for instance, using these attractants to improve the efficacy of the traps for the capture of ornithophilic mosquito species. During this thesis, I only focused on uninfected mosquitoes, but further research is necessary to understand if parasites affect the response of infected mosquitoes to bird stimuli (Stanczyk et al. 2019). Finally, considering the diversity of avian malaria parasites, further studies are necessary to identify potential differences in the effects of different parasites lineages, a fact that could be linked to differences in the virulence of parasites identified among bird species (Dimitrov et al. 2015). This is extensible to other related parasites such as *Haemoproteus* and *Leucocytozoon*, which differs from *Plasmodium* in the vectors involved in their transmission.

To sum up, this thesis contributes to a better understanding of the interaction between birds, vectors and avian malaria parasites. The multidisciplinary approach used here provides key information about the importance of chemical cues such as the odour of birds in mosquito attraction, with clear implications for the epidemiology of *Plasmodium* in natural environments.

CONCLUSIONS

1. Mosquito vectors increase their attraction toward avian malaria infected birds because the parasite may modulate some host traits such as body odours. These effects support the host manipulation hypothesis that would allow parasites to maximize their transmission success.

2. The chemical composition of the volatile fraction of uropygial gland secretions of common blackbirds infected by avian Haemosporidians differs between age classes and also between sexes within adult birds. Adults had higher relative proportions of alcohols than juveniles, while the opposite pattern was found for ketones, pyzarines and steroids. In addition, adult females had higher proportions of alkanes than adult males.

3. Although the chemical composition of the uropygial gland secretions of common blackbirds was similar between habitat types, the relative proportion of the pesticide DDE present in this secretion was higher in forest- than in urban-dwelling birds, probably due to a higher historical use of DDT in forest areas. Levels of DDE were also higher in adult than in juvenile birds, regardless of their sex, supporting the bioaccumulative potential of this pollutant.

4. Both the ornithophilic *Culex pipiens* and the mammophilic *Aedes caspius* were not attracted to the uropygial secretions of a passerine bird, suggesting that the preference of ornithophilic mosquitoes for avian hosts is not mediated by the chemical cues that this secretion conveys.

5. Avian malaria infections do not alter the volatile chemical profile of the uropygial gland secretions of juveniles of two bird species, common blackbirds and house sparrows.

6. Ornithophilic mosquitoes were more attracted to the headspace of *Plasmodium*-infected than uninfected birds. However no differences in

mosquito attraction were found when vectors were exposed to the uropygial secretions of these birds. This suggests that *Plasmodium* may affect host odour profiles in a way to increase the attraction of host-seeking mosquitoes. This higher attraction is not due to changes in the composition of the uropygial secretions.

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ANNEX I

Annex I

Aedes vittatus in Spain: current distribution, barcoding characterization and potential role as a vector of human diseases

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Abstract

Background: *Aedes vittatus* is currently found in Africa, Asia and Europe, where it acts as a vector of pathogens causing animal and human diseases (e.g. chikungunya, Zika and dengue). Like other *Aedes* species, *Ae. vittatus* is able to breed in artificial containers. The ECDC has recently highlighted the need for molecular tools (i.e. barcoding characterization) that enable *Aedes* species to be identified in entomological surveys.

Results: We sampled mosquito larvae and adults in southern Spain and used a molecular approach to amplify and sequence a fragment of the cytochrome *c* oxidase subunit 1 gene (barcoding region) of the mosquitoes. The blast comparison of the mosquito sequences isolated from Spain with those deposited in public databases provided a ≥ 99% similarity with sequences for two *Aedes* mosquitoes, *Ae. vittatus* and *Ae. cogilli*, while similarities with other *Aedes* species were ≤ 94%. *Aedes cogilli* is only present in India and there are no records of this species from Europe.

Conclusions: Due to the low genetic differences between *Ae. vittatus* and *Ae. cogilli*, the barcoding region should not be used as the only method for identifying *Ae. vittatus*, especially in areas where both of these *Aedes* species are present. This type of analysis should thus be combined with morphological identification using available keys and/or the characterization of other molecular markers. In addition, further entomological surveys should be conducted in order to identify the fine-scale distribution of this mosquito species in Europe.

Keywords: DNA barcoding, *Aedes* mosquitoes, vector-borne diseases.

Background

Vector-borne pathogens are a global health concern in which mosquitoes play a central role as vectors of pathogens (Daszak et al. 2000). In Europe both native and invasive species of *Aedes* mosquitoes are involved in the transmission of pathogens including viruses (e.g. dengue and chikungunya (Tomasello et al. 2013)) and parasites (e.g. *Dirofilaria* (Cancrini et al. 2007). Of these mosquitoes, the invasive *Aedes albopictus* has received much attention in recent decades due to its role in the transmission of dengue (Succo et al. 2016) and chikungunya (Rezza et al. 2007) in Europe. Certain *Aedes* species, including *Ae. albopictus*, are able to breed in artificial containers and it is important to develop accurate identification protocols for differentiating native and invasive *Aedes* species that breed in the same area (Juliano et al. 2005; ECDC 2012; ECDC 2014). The identification of mosquito species through the characterization of a fragment of the cytochrome *c* oxidase subunit 1 (*cox*1) gene is a useful tool for monitoring the presence of species (Hebert et al. 2003; Ondrejicka et al. 2014), above all given the difficulties in identifying mosquitoes in larval stages and the current scarcity of trained taxonomists (Godfray 2002). However, this method requires a previous genetic characterization of the species (Dawnay et al. 2007). This is an important limitation in the case of *Aedes* mosquitoes as this information is not available for most of the species of this genus that breed in Europe (Schaffner et al. 2009), despite their importance in pathogen transmission (Paupy et al. 2009).

The aim of this study was to update the current distribution of *Ae. vittatus* and provide the first genetic characterization of the barcoding region of specimens of this species from Europe. Hitherto, sequences from this species were only available from China (Wang et al. 2012), India (Murugan et al. 2015) and Kenya (Ajama et al. 2016). In addition, we review here available information on the potential role of this species in the transmission of virus of public health concern.

Methods

As a part of an extensive mosquito-monitoring program, a female *Ae. vittatus* was captured in a CDC trap in Ayamonte, Huelva Province (Fig. 1; 37°13'30"N, 7°24'29"W), in June 2015. This sampling site is located in the Guadiana marshes, in the garden of a house close to the built-up area of Ayamonte. At the same time, we also trapped 19 *Ochlerotatus caspius*. In further trapping sessions during 2015 in this area we captured 1145 *Oc. caspius*, 47 *Oc. detritus*, 9 *Cx. pipiens*, 4 *Cx. theileri*, 3 *Cx. perexiguus*, 3 *Culiseta longiareolata* and 2 *Cs. annulata*. Additionally, mosquito larvae were collected from a container in July 2015 in a rural property near Castilblanco de los Arroyos, Seville Province (Fig. 1; 37°41'56"N, 5°58'44"W), in an area characterized by the presence of isolated houses surrounded by scrubland. Larvae were maintained in plastic trays with natural water and fed *ad libitum* with Mikrozell (Hobby Mikrozell 20ml/22g) in a climatic chamber at constant conditions (28°C, 65-70% relative humidity (RH) and 12:12 light:dark photocycle). Adult mosquitoes were fed *ad libitum* with 1% sugar solution. Five to seven days after emergence, adult mosquitoes were anaesthetised with diethyl ether and identified to species level using available taxonomic keys (Schaffner et al. 2001; Becker et al. 2010) under a stereo-microscope (Nikon SMZ645). The ability of laboratory-reared females to bite humans was checked by exposing the arm of one of the authors (RGL) to mosquito bites. The time elapsed between arm exposure and the beginning of blood-feeding was recorded.

Three mosquitoes (one male and two females) from Seville Province were selected for molecular characterization of the barcoding region and to confirm the morphological identification of the species. A fragment of the right hind-leg of each mosquito was cut-off using a sterile blade and placed on a Petri dish. Genomic DNA was extracted using the Maxwell 16 LEV Blood DNA Kit (Promega, Madison, WI, USA) following the manufacture's instructions. PCR reactions were performed using the primer pair LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACTT CAG GGT

GAC CAA AAA ATC A-3') (Folmer et al. 1994) following Whiteman et al. (2006) to amplify a 658 bp fragment of the *cox*1 gene (excluding primers) (see Gutiérrez-López et al. 2015). The presence of amplicons was verified on 1.8% agarose gels. Sequences were resolved in both directions by Macrogen sequencing service (Macrogen Inc., the Netherlands). Sequences were edited using the SequencherTM v4.9 software (Gene Codes Corp., Ann Arbor, MI, USA) and compared with sequences deposited in the GenBank DNA sequence database (National Center for Biotechnology Information) and the Barcode of Life Data Systems (BOLD).

Fig. 1 Distribution by provinces of *Ae. vittatus* in Spain. Light grey and dark grey indicates the provinces where the species is absent or present, respectively. The two new records of *Ae. vitattus* reported in this study are marked with stars: 1. Ayamonte (Huelva Province), 2. Castilblanco de los Arroyos (Seville Province).

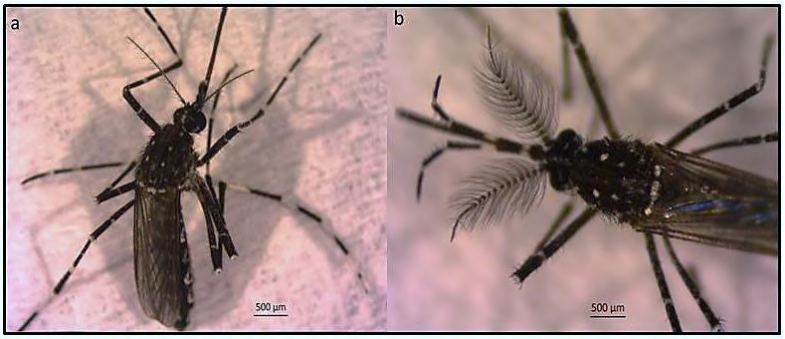
Results

Mosquitoes were morphologically identified as *Ae. vittatus* (Fig. 2). Genetic characterization of the barcoding region of the three mosquitoes provided a

unique haplotype. Using the BOLD system, the sequences obtained in our study were identified as *Ae. vittatus* (99.4%) or *Aedes* (*Phagomyia*) *cogilli* (99.0%). Likewise, a 99% overlap between *Ae. vittatus* and *Ae. cogilli* was found using a BLAST comparison with sequences in GenBank, while similarities with other *Aedes* species were ≤ 94%.

The anthropophilic feeding preference of *Ae. vittatus* females was confirmed by the fact that four mosquitoes (57.1%) fed on a human arm after < 5 min of exposure.

Fig. 2. *Aedes vittatus* female (a) and male (b) captures in Seville province



Discussion

We characterized for the first time in Europe the barcoding region of *Ae. vittatus*. A BLAST comparison of this sequence with those deposited in public databases provided a ≥ 99% similarity with sequences of two *Aedes* mosquitoes, *Ae. vittatus* and *Ae. cogilli*. However, *Ae. cogilli*, is only present in India and is not found in Europe (Young et al. 2017). The other *Aedes* sequences on GenBank differed by about 6% from the *Ae. vittatus* sequence isolated here. Although varying between taxa, interspecific differences in the barcoding region are established at 0-2% (Ashfaq et al. 2014). Based on the low interspecific differences found between *Ae. vittatus* and *Ae. cogilli*, our results do not support the use of the *cox*1 region as a method for separating these species where theycoincide; rather, this method should be combined with morphological identification using available keys or the characterization of other molecular

markers. Based on the morphological characteristics of the specimens captured here, we conclude that the mosquitoes we captured belong to the species *Ae. vittatus* (Huang 1977).

The current distribution of *Ae. vittatus* includes rural and natural areas in Africa, Asia and European countries in the Mediterranean Basin such as France, Italy, Portugal and Spain (Fig. 3). Specifically, *Ae. vitattus* has been recorded with a clear discontinuous distribution from eleven Spanish provinces (Bueno-Marí et al. 2012). Larvae of *Ae. vittatus* have been recorded in a variety of habitats including rock pools, tree holes, domestic containers and hoofprints (Service 1970; Bueno-Marí & Jiménez-Peydró 2010). In eastern Spain, this species is present in coastal mountainous areas of thermomediterranean and lower mesomediterranean thermotypes (Bernués-Bañeres & Jiménez-Peydro 2013). Here, we update the distribution of this species in the Iberian Peninsula and provide the first reports of its presence in the provinces of Huelva and Seville (Fig. 1). In Huelva, an adult female was trapped close to a built-up area, while mosquito larvae belonging to this species were sampled in a rural property in Seville. The mosquito from Huelva was captured in an area close to the town of Ayamonte, which suggests the possibility of contact between this mosquito species and human populations.

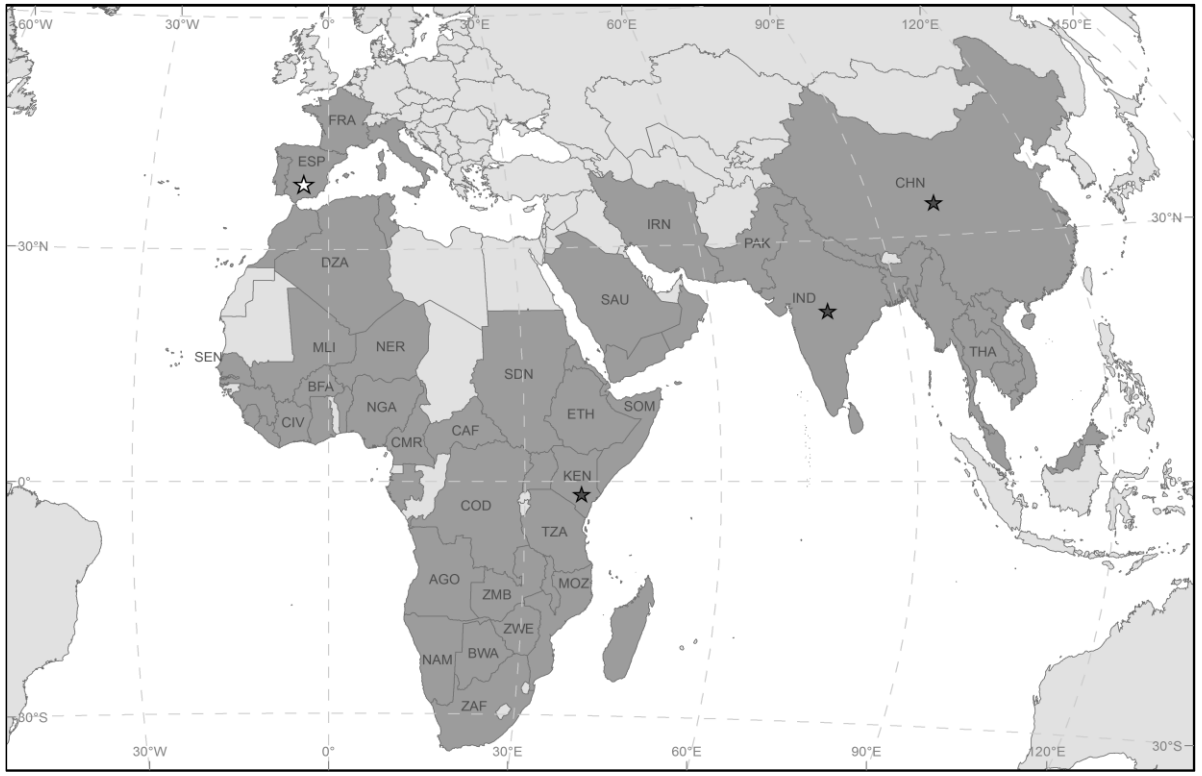
The fact that *Ae. vittatus* uses artificial containers for breeding in rural ecosystems may be particularly relevant given its ability to transmit pathogens causing human diseases. In addition to humans, *Ae. vittatus* feed on bovids, sheep/goats and porcupines (Service 1965; Wilson & Sevarkodiyone 2015), suggesting its potential role in the transmission cycle of a variety of arboviruses (Table 1). Although *Ae. vittatus* has also been reported to be involved in the transmission of viruses potentially affecting humans, including species of *Alphavirus*, *Flavivirus* and *Bunyavirus* (Table 1), this species probably only has a low risk in Spain. Diagnosis of these diseases and vector surveillance will help elucidate the potential role of *Ae. vittatus* in the transmission of viruses in Europe.

Table 1 Main viruses causing diseases transmitted by *Ae. vittatus* with information of the potential hosts and known distribution of the diseases

Family/Virus	Disease	Hosts	Distribution	Reference
Family Togaviridae (*Alphavirus*)				
Babanki virus	Babanki	Humans, birds	Africa, Europe	Ochieng et al. 2013
Chikungunya virus	Chikungunya	Humans, birds, domestic animals, monkeys, rodents	Africa, America, Asia, Europe	Vazeille et al. 2008
Middelburg virus	Middelburg	Humans, domestic animals	Africa	Attoui et al. 2007
Semliki Forest virus	Encephalitis	Humans, birds, domestic animals, non-human primates, rodents	Africa, Asia, Europe	Fazakerley 2002
Family Flaviviridae (*Flavivirus*)				
Dengue virus	Dengue	Humans, non-human primates	Africa, South America	Angel & Joshi 2008; Diallo et al. 2005

Saboya virus	Saboya	Humans, rodents	Africa	Grard et al. 2010
Wesselsbron virus	Wesselsbron	Humans, domestic animals, monkeys	Africa	Diagne et al. 2013
Yellow fever virus	Yellow fever	Humans, non-human primates	Africa, South America	Barrett & Higgs 2007; Ngoagouni et al. 2010
Zika virus	Zika	Humans, bats, birds, domestic animals, non-human primates	Africa, America, Asia	Wahid et al. 2016; Vorou 2016; Diallo et al. 2014
Family Bunyaviridae (*Bunyavirus*)				
Bunyamwera virus	Bunyamwera	Humans	Africa	Odhiambo et al. 2014

Fig. 3. Worldwide distribution of *Ae. vittatus* (dark grey colour). Stars indicate the geographical origin of the previously (black) and new (white) described genetic sequences of the barcording region.



Conclusions

When identifying *Ae. vittatus* in areas where its distribution overlaps with that of the related Asian species *Ae. cogilli*, the identification of the barcoding region should be combined with morphological identification and/or the characterization of other molecular markers. However, in Europe, molecular tools may allow for the accurate identification of this species due to the great genetic difference (6%) found between Spanish *Ae. vittatus* and other *Aedes* species. Further entomological studies should be conducted in order to identify the fine-scale distribution of *Ae. vittatus* in European countries, where it could play a role in the transmission of viruses with public health relevance.

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Availability of data and materials

Sequences generated in this study were deposited in the GenBank database under the accession number MF429950 Mosquitoes were deposited in the collection of the Museo Nacional de Ciencias Naturales (MNCN-CSIC), Madrid, Spain, under the accession numbers MNCN/ADN 86743 and 86744.

Ethics approval

All experimental procedures were approved by the CSIC Ethics Committee and Animal Health authorities, and complied with Spanish laws.

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Resulta "curioso" acabar la tesis durante una pandemia mundial. Sin embargo, esta situación consigue que dé las gracias de manera más fuerte aún si cabe a todas y cada una de las personas que forman parte de mi vida y de este gran proyecto que es la tesis. De todo corazón:

¡¡¡GRACIAS!!!


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