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Sex and age, but not blood parasite infection nor habitat, affect the composition of the uropygial gland secretions in European blackbirds

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The uropygial gland of birds produces an oily secretion with different functions, mainly related to plumage protection. In addition, the volatile compounds of this secretion may act as chemical signals that provide information to conspecifics, but it is also possible that those compounds may further 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 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 European 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 the chemical composition of the uropygial gland secretion of birds 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, odour, preen oil, sexual dimorphism, *Turdus merula*, volatile compounds

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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 and Ziswiler 1982). This secretion has multiple functions (Moreno-Rueda 2017), 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. 2004b). Although different authors have investigated the role of different life-history traits, such as habitat (e.g. aquatic versus terrestrial), migratory behaviour (resident versus migrant), social behaviour (social versus 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 (Moreno-Rueda 2017), and differences in its composition may therefore play an important role in bird intraspecific and interspecific communication (Caro et al. 2015). The avian malaria parasites *Plasmodium* and the malaria-like parasites *Haemoproteus* and *Leucocytozoon* 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). Parasites can manipulate their hosts to increase their transmission success (Heil 2016). One of the potential mechanisms of parasite manipulation is the modification of host odour profiles that could affect, for example, the composition of the secretions from the uropygial gland (Martínez-de la Puente et al. 2020). In particular, the volatile compounds of the uropygial secretion might serve as olfactory cue for host-seeking mosquitoes (Russell and Hunter 2005) with parasites increasing the attraction of mosquitoes towards infected individuals. Enhanced attraction of mosquitoes towards infected birds would in turn increase parasite transmission. 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, only two studies have investigated the relationship between uropygial gland secretion composition and the infection by avian malaria parasites, suggesting that parasites alter the wax ester profiles in song sparrows *Melospiza melodia* (Grieves et al. 2018) but not the volatile fraction of secretions of house sparrows (Díez-Fernández et al. 2020a). However, it is unclear if the wax fraction of these secretions affects its volatile compounds in other bird species, and therefore, their importance on bird odour profiles and bird–vector interactions.

Although the uropygial gland secretions have a rather constant and specific qualitative composition within taxa (Montalti et al. 2005) the composition of the secretion may be affected by the environmental conditions experienced by birds, including habitat type and diet (Apandi and Edwards 1964, Thomas et al. 2010), which may in turn influence the bacterial community residing in the uropygial gland (Rodríguez-Ruano et al. 2015). In an experimental study, Thomas et al. (2010) found differences in the compounds of the secretions of white-throated sparrows *Zonotrichia albicollis* between captive and wild birds and also between different diets. However, Whittaker et al. (2010) found that the volatile profiles of dark-eyed juncos differed between individuals from different geographic areas, despite being held in captivity in a common environment and fed with an identical diet. Nonetheless, the studied populations also differed in other traits due to recent divergence, which may affect within species differences. Although differences between habitat types, such as those associated with food resources could potentially affect the composition of the secretions, evidence on this possibility is still very limited. Therefore, it remains unclear whether meso-scale habitat differences may lead to within species variations in the chemical composition of uropygial secretions.

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 European 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 the habitat type occupied by European 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

European blackbirds were captured using mist nests during the breeding season from the end of 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). These localities are 25 km far from each other. Birds were ringed with numbered metal rings. The age (juveniles: fledglings born in 2015 and consequently < 1 year old versus adults: > 1 year old) and sex of adult birds were determined according to plumage characteristics (Svensson 1998). The sex of juveniles was molecularly determined (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 2-ml 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 −80°C 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 Maxwell16 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 (30 m length × 0.25 mm inner diameter × 0.25 µm film thickness), coupled to an Agilent 5975C Triple Axis Detector mass spectrometer (MS). We injected in splitless mode 2 µl of each sample previously dissolved in 50 µl of hexane with helium as the carrier gas. The oven temperature program started at 80°C and was maintained during 3 min, then increased to 300°C at rate of 5°C min^{-1} and finally maintained at 300°C during 35 min.

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, ver. 2.0, Faircom Corporation, USA). After examining the chromatograms, we used a limit of 45 min. of retention time (RT) to consider volatile compounds (Supporting information), 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 (TIC) until the 45 min. 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

We assessed whether the total number of compounds per individual differed between sexes, age classes and habitat types using generalized linear models (GLMs) with Poisson error distribution and log-link function. The overall prevalence of infection by avian haemosporidians in the study population was high (88.46%, 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 permutational multivariate analysis of variance test (PERMANOVA, McArdle and Anderson 2001). Because sex and age showed significant differences (Results), we also tested their interaction in a two-factor PERMANOVA. By including only infected individuals, we ruled out possible effects associated with the infection status on the relationships tested. To do that, we first calculated Euclidean distances between every pair of individuals to produce a resemblance matrix that was the basis of the following analyses. We then used the PERMANOVA test with 999 permutations to analyse possible differences in the composition of the uropygial gland secretions. In addition, we tested for potential differences between these factors (i.e. age, sex and habitat type) restricting the analyses to those compounds that appeared in at least 70% of individuals using a three-factor PERMANOVA. We also assessed the effect of sex within age classes using one-factor PERMANOVA for both datasets including all the identified compounds and those that appeared in at least 70% of the individuals. Additionally, we used one-factor PERMANOVA to assess possible age differences in the composition, with different models run for each sex and for both datasets. In a preliminary analysis we found that the date of sampling did not significantly affect the composition of the uropygial secretions ($p = 0.49$). Therefore, this variable was not included in our models. Statistical analyses were conducted with PRIMER V6.1.13 (Clarke and Gorley 2006) with the PERMANOVA V1.0.3 add-in package (Anderson et al. 2008) and STATISTICA (v. 8.0 Stat Soft. Inc. 1984–2007).

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 (Results), we assessed differences in these major classes of compounds using GLMs with quasibinomial error distribution, where age, sex and their interaction were included as explanatory variables.

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. Additionally, we assessed the effect of infection status on the proportion of each compound present in at least 30% of infected juveniles using GLMs with quasibinomial distribution and logit link function, and subsequently controlled for multiple comparisons with post-hoc Benjamini–Hochberg adjustment of significance. Differences between factor levels were assessed with post-hoc Tukey tests. These analyses were performed in R software (<www.r-project.org>) 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 European 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 found a total of 213 different compounds in the volatile fraction of the uropygial secretions of European blackbirds (Supporting information), including 153 and 210 compounds in juveniles and adults, respectively, and 191 and 156 in males and females, respectively (Table 2). The total number of compounds per individual differed significantly between age classes, with adults having more compounds than juveniles (GLM, Estimate ± standard error (SE) $= -0.16 \pm 0.04$, $p < 0.001$) and also between sexes, with males having more compounds than females (Estimate $= 0.19 \pm 0.04$ SE, $p < 0.001$). In addition, birds from the forest habitat tended to have a higher number of compounds than those from the urban habitat (Estimate $= -0.08 \pm 0.04$ SE, $p = 0.051$).

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. The interaction between sex and age was not significant ($PseudoF_{1,42} = 1.35$, $p = 0.18$). In addition, analyses considering only compounds present in at least 70% of the individuals showed significant differences between age classes ($PseudoF_{1,42} = 6.32$, $p = 0.001$), but not between sexes ($PseudoF_{1,42} = 1.84$, $p = 0.09$) or habitat types ($PseudoF_{1,42} = 1.23$, $p = 0.26$). When analysing age classes separately and considering all the compounds identified in the secretions, significant differences between sexes were found in adult birds ($PseudoF_{1,28} = 5.30$, $p = 0.006$) but not in juveniles ($PseudoF_{1,14} = 0.89$, $p = 0.54$). However, when considering only those compounds present in at least 70% of individuals, no differences were found neither in adults ($PseudoF_{1,28} = 5.88$, $p = 0.50$) or juveniles ($PseudoF_{1,14} = 1.34$, $p = 0.25$). When analysing sexes separately and considering all compounds, we found significant differences in the composition between age classes in males ($PseudoF_{1,22} = 11.76$, $p = 0.001$) but not in females ($PseudoF_{1,20} = 1.68$, $p = 0.08$). However, when considering those compounds present in at

Table 2. Mean relative proportion (± SD) of the major classes of volatile compounds found in the uropygial gland secretions of infected and uninfected European blackbirds of different sex and age 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)

least 70% of individuals, significant differences between age classes were found in both sexes (males $PseudoF_{1,22}=3.47$, $p=0.02$, females $PseudoF_{1,20}=3.28$, $p=0.01$).

When analysing the compounds of the nine major classes as a whole within infected individuals, we found significant differences between age classes ($F_{1,42}=18.39$, $p < 0.001$), but not between sexes ($F_{1,42}=1.34$, $p=0.25$) or their interaction ($F_{1,42}=0.53$, $p=0.47$) (Fig. 1). However, when assessing these groups of compounds individually, significantly higher relative proportions of alcohols were found in adults than in juveniles, while the opposite pattern was found for ketones, steroids and pyzarines (Table 3, Fig. 2A–D). In addition, a significant effect of both sex and the interaction between sex and age was found for the proportions of alkanes. Females showed a higher proportion of alkanes than males (Table 3, Fig. 2E). 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: $p=0.99$). In addition, no significant differences were found in the proportions of alkanes between age classes of each sex (all $p > 0.44$).

We did not find any significant effect of the infection status by avian haemosporidians on the composition of secretions in juvenile birds ($PseudoF_{1,19}=1.13$, $p=0.31$). No significant differences were found in the relative proportion of each compound associated with infection status ($p > 0.19$ in all cases).

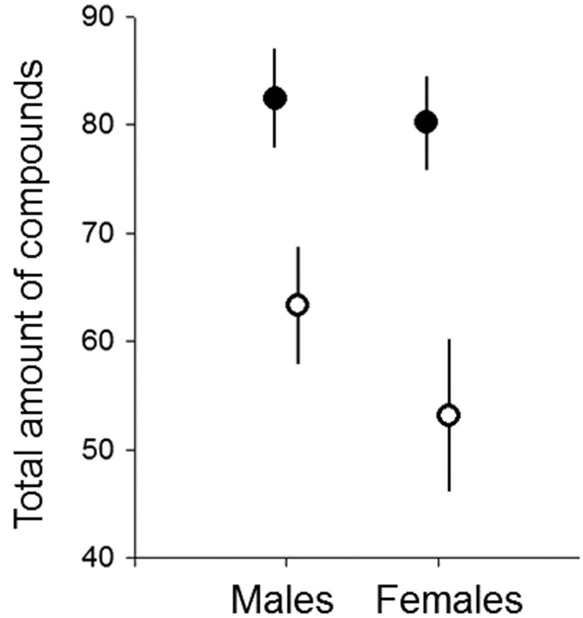

Figure 1. Total amount (mean ± SE) of the nine major classes of compounds (alcohols, alkanes, amides, carboxilic acids, esters of carboxilic acids, ketones, pyrazines, steroids, waxy esters) found in the uropygial gland secretions of European blackbirds for different sex and age classes.

Discussion

Here, we compared the chemical composition of the volatile fraction of the uropygial gland secretion of wild European 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 domestic chickens (Sandilands et al. 2004a), 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. Grieves et al. (2019) also detected differences between age classes in the composition of wax esters of the non-volatile fraction of secretions of song sparrows. 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

Table 3. Summary statistics of GLMs (estimate, standard error (SE) and p-values) 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 European blackbirds infected by avian haemosporidians. Significant differences are shown in bold.

Compounds	Sex			Age			Sex × Age		
	Estimate	SE	p	Estimate	SE	p	Estimate	SE	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

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 European blackbirds varied between sexes based on the total number of compounds per individual. In particular, adults showed higher number of compounds than juveniles, suggesting 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 (Hagelin et al. 2003) and Cory's and Scopoli's shearwaters (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. The difference in the proportion of alkanes between sexes was higher in adults than in juveniles (Fig. 2) suggesting that this compound may be also associated with the maturity of 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 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 and Chowdhury 1995), which may explain the fact that sexual differences in this study were found in adults but not in juvenile birds in the analysis considering the relative proportion of all compounds identified in the secretions.

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 birds. We expected to find differences in the composition based on the potential role of these secretions in bird–vector interactions (Takken and Knols 2010) as attractants of black flies (Fallis and Smith 1964) and mosquitoes (Russell and 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 and 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 (i.e. the whole body odour of birds) 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

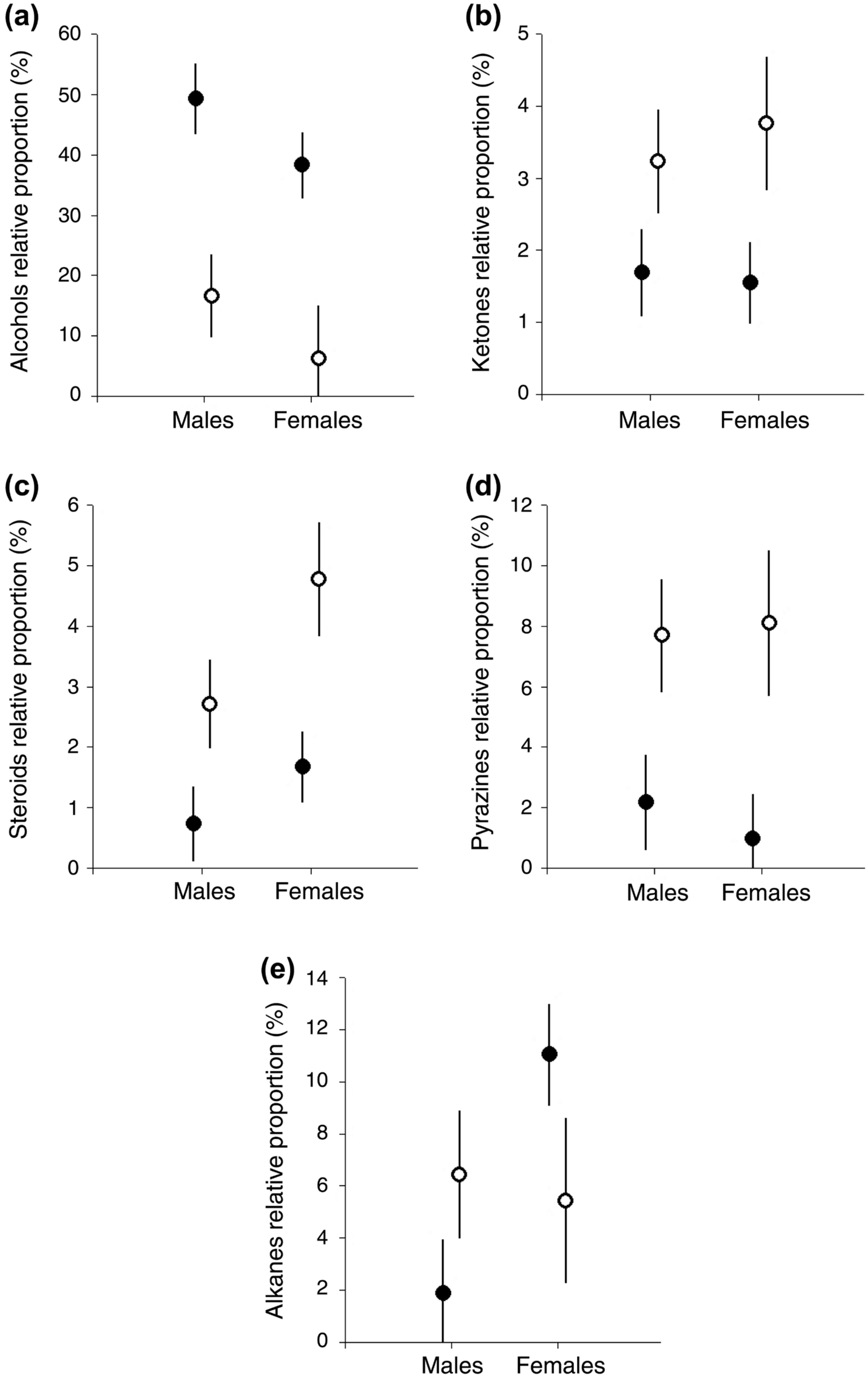

Figure 2. Relative proportion (mean ± SE) of the major classes of compounds present in the uropygial gland secretions of European blackbirds that differed between the interaction of sex and age classes: (A) alcohols, (B) ketones, (C) pyrazines, (D) steroids and (E) alkanes.

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 and Leal 2009), although this compound was only found in seven individuals (all of them adults) in our study. Alternatively, another component 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.

European 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, although the number of different compounds tended to be higher in birds from the forest that from the urban habitat. Previous studies suggested that these compounds are acquired passively through food, thus individual variation may simply result from variation in diet (Sandilands et al. 2004a). It is possible that European 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 (originally separated ca 95 km) 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, European 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. unpubl.).

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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Alazne Díez-Fernández: Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (equal); Validation (equal); Writing – original draft (lead); Writing – review and editing (lead). **Josué Martínez-de la Puente**: Conceptualization (lead); Formal analysis (supporting); Investigation (equal); Methodology (equal); Supervision (equal); Validation (equal); Writing – review and editing (equal). **José Martín**: Data curation (lead); Formal analysis (lead); Supervision (equal); Writing – review and editing (equal). **Laura Gangoso**: Conceptualization (lead); Formal analysis (equal); Investigation (equal); Methodology (equal); Supervision (equal); Validation (equal); Writing – review and editing (equal). **Pilar López**: Data curation (equal); Formal analysis (equal); Supervision (supporting); Writing – review and editing (supporting). **Ramón Soriguer**: Conceptualization (lead); Funding acquisition (lead); Investigation (equal); Methodology (equal); Project administration (lead); Resources (lead); Supervision (supporting); Validation (equal); Writing – review and editing (equal). **Jordi Figuerola**: Conceptualization (lead); Data curation (equal); Formal analysis (equal); Funding acquisition (lead); Investigation (lead); Methodology (equal); Project administration (lead); Resources (lead); Supervision (lead); Validation (lead); Visualization (lead); Writing – review and editing (lead).

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Data availability statement

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.20350/digitalCSIC/13835> (Díez-Fernández et al. 2021).

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