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Effects of sex and sampling site on the relative proportion of pesticides in uropygial gland secretions of European Blackbirds (*Turdus merula*)

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Dichlorodiphenyltrichloroethane (DDT) is a pesticide that was commonly used for decades worldwide. The use of DDT was banned in the 1970s and 1980s in Europe because of 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, providing valuable information about the exposure to pesticides and potential differences between species and over time. Here, we analysed the relative proportion of DDE in the uropygial gland secretions of European Blackbirds *Turdus merula* from two localities in southern Spain. Given the negative effects of this pollutant on animal immunity, we also tested for associations between the prevalence of haemosporidians and the relative proportion of DDE in their secretions. Relative proportions of DDE varied between sampling sites and were higher in females than in males, regardless of their age. In spite of the potential immunosuppressive effect of DDE, haemosporidian infection was not associated with DDE presence.

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

Organisms are directly or indirectly exposed to environmental pollutants worldwide, including in remote areas (Welch *et al.* 1991, Blais *et al.* 1998). Persistent organic pollutants are considered one of the most important environmental contaminants, including compounds such as organochlorine pesticides, among which the widely used dichlorodiphenyltrichloroethane (DDT) is of particular concern (Jones & De Voogt 1999). This insecticide was commonly used against mosquitoes and other invertebrate pests after the Second World War and was applied in large doses to agricultural crops (Turusov *et al.* 2002). Due to its adverse effects on both humans and wildlife, its use was banned in Europe during the 1970s and 1980s (Turusov *et al.* 2002). DDT degrades into metabolites including dichlorodiphenyldichloroethylene (DDE), an extremely persistent and highly toxic compound that bioaccumulates in

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living organisms, and is thereby magnified at higher 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 (e.g. modifications in endocrine system functioning; Guillette & Gunderson 2001), and reduced haemoglobin levels and anaemia (Rivera-Rodríguez & Rodríguez-Estrella 2011). In general, persistent organic pollutants are responsible for alterations to the immune system, mainly through a decrease in the number of white blood cells and the concentration of antibodies against antigens, as well as a reduction in weight of immunological organs such as the spleen and thymus (Tryphonas 2005). Further, an increase in levels of organochlorines has been linked with immunosuppression and a higher susceptibility to parasite infections in the Glaucous Gull *Larus hyperboreus* (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 including migratory behaviour (Herzke *et al.* 2002), diet and habitat use (Clatterbuck *et al.* 2018) greatly affect the extent of exposure to pesticides and the degree of accumulation in tissues. Levels of organic pollutants have been monitored intensively in wild bird 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). 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 non-invasive studies have focused on the measurement of organochlorine pesticides in non-hatched eggs (Wang *et al.* 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 (e.g. females during the laying season). In addition, there are concerns of external contamination where moult strategy and preening behaviour as well as the particular type of feather sampled could be impacted by DDT and its metabolites due to its settling directly on the feathers (Jaspers *et al.* 2011). Uropygial gland secretions represent a promising source to measure organochlorines 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 of birds is a holocrine gland that produces a 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 (Giraudeau *et al.* 2010, Moreno-Rueda 2017). Here, we quantified the levels of DDE in the secretions of the uropygial gland of a ubiquitous and omnivorous wild bird, the European Blackbird *Turdus merula*. We sampled individuals of different age and sex from two populations located in southern Spain: a natural forest area in the Corredor Verde del Guadiamar and the María Luisa urban park. We expected higher relative proportions of DDE in adults than in juvenile birds, probably reflecting the bioaccumulation of this pollutant over time, as has been shown 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 relative to body size) were associated with the relative proportion of DDE in bird secretions.

METHODS

Study species and area

The European Blackbird is a medium-sized passerine widely distributed throughout Europe. Although this species was originally a forest-dweller, it is also very common in urban areas (Ibáñez-Álamo & Soler 2010). The home-range, the entire area exploited by individuals, including their breeding territory (around 3300–4800 m^2) and larger foraging areas are of special importance for exposure to contaminants (Wysocki

et al. 2004). Studies using biologging show that sedentary individuals typically move within 500 m of their breeding grounds and occasionally up to 1500 m during winter (Fudickar *et al.* 2013). 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 European Blackbirds are abundant and mostly sedentary (Carrascal & Salvador 2016). In contrast to other species such as birds of prey that are often used as biomarkers of environmental pollutants, European Blackbirds may be good indicators of the local presence of DDT because of their comparatively smaller home-ranges.

Bird sampling was conducted from the end of March to June 2015, overlapping with the species' breeding season. Study sites included the Corredor Verde del Guadiamar (37°18′23″N, 6°15′44″W, Seville Province, Spain) where poplar and oak groves surrounded by agricultural fields dominate. In addition, birds were sampled at the urban park of María Luisa that is located in the city centre of Seville (37°22′29″N, 5°59′19″W). The two sites are separated by approximately 25 km.

Bird capture and sampling

Birds were captured using mist-nests from sunrise to midday. A total of 52 European Blackbirds were sampled: 33 individuals at the Corredor Verde del Guadiamar (15 juveniles and 18 adults; 17 males and 16 females), and 19 individuals at the María Luisa park (six juveniles and 13 adults; nine males and 10 females). All birds were ringed with numbered metal rings, weighed and their wing length was measured to the nearest millimetre. Two individuals escaped before measurement of their body mass. Age (juveniles: < 1 year old versus adults: > 1 year old) and sex of adult birds were determined according to plumage characteristics (Svensson 1998), but the sex of juveniles was molecularly determined (see below). Blood samples were taken by brachial venepuncture, transferred to Eppendorf tubes and maintained in cold boxes (4°C) during the fieldwork. In the laboratory, blood samples were separated into plasma and cell fractions and stored at −80 °C until further analyses. In addition, during the fieldwork, only for the case of adult birds, a drop of blood was smeared on a slide (n = 30, we could not obtain a blood smear for one individual). A few hours later, the blood smears were fixed with absolute methanol in the laboratory. Smears were stained with Giemsa during July 2015. We quantified the presence and abundance of blood parasites following Merino and Potti (1995) with minor modifications. Parasitaemia of *Plasmodium*, *Haemoproteus* and *Lankesterella* was calculated as the number of parasites per 10 000 erythrocytes.

The uropygial gland was measured using a digital calliper (± 0.01 mm) and its volume was estimated following Magallanes *et al.* (2016), as the product of length, height and width of the gland in millimetres. We collected uropygial gland secretions from all individuals by gently pressuring and massaging the upper part of the papilla with a non-heparinized capillary. In this way, secretions did not pass through the capillary but were directly collected in 2-mL gas chromatography glass vials (EULABOR®, Seville, Spain). We used a glass capillary to press the papilla to avoid any external contamination such as from gloves or cotton swabs. We followed the same procedure but without collecting any secretion to obtain blank control vials for 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 fieldwork and subsequently stored at −80 °C.

Molecular analyses

Genomic DNA was extracted from the cell fractions of blood using a semi-automatic Maxwell kit method (Maxwell®16 LEV system Research; Promega, Madison, WI, USA). Juvenile birds were molecularly sexed using primers P2 (5′-TCTGCATCGCTAAATCCTTT-3′) and P8 (5′-CTCCCAAGGATGAGRAAYTG-3′) following Griffiths *et al.* (1996, 1998). In the detection of parasites of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* a fragment of 478 base pairs (excluding primers) of the cytochrome b gene (cyt b) was amplified following Hellgren *et al.* (2004). The presence of amplicons was verified in 1.8% agarose gels. We included a negative control for both polymerase chain reactions (PCRs) (at least one per plate). In addition, samples providing negative results were rescreened with the complete PCR protocol to avoid the occurrence of false negatives. Positive samples were sequenced using the facilities at Macrogen (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 (National Center for Biotechnology Information) using BLAST to identify the parasites infecting the birds. Parasite lineages were named according to Malavi (Bensch *et al.* 2009).

Detection of DDE in uropygial gland secretions

Uropygial gland secretions were analysed using an Agilent 7890A gas chromatograph fitted with a poly (5% diphenyl, 95% dimethylpolysiloxane) column HP5-MS (30 m length × 0.25 mm inner diameter × 0.25 µm film thickness) and an Agilent 5975C Triple Axis Detector mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA). 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 programme started at 80 °C and was maintained for 3 min, then increased to 300 °C at a rate of 5 °C/min and finally was maintained at 300 °C for 35 min. 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, Columbia, MO, USA), and later confirmed DDE presence with 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; ThermoFisher Scientific Inc., Waltham, MA, USA). To correct for the non-independence of proportions, we performed compositional analysis by logit transformation of the proportion data by taking the natural logarithm of proportion/(1–proportion; Aebischer *et al.* 1993).

Statistical analyses

Initially, we assessed whether body mass differed between sexes, age classes and sampling sites. To this end, we calculated the scaled mass index of body condition of all individuals to standardize their body mass relative to body size (estimated as wing length) (Peig & Green 2009). We used wing length as a proxy for body size because it was measured in all individuals. Body mass was unrelated to wing length ($r = 0.24$, $F_{1,48} = 1.70$, $P = 0.10$). Then, we assessed the factors associated with variation in the body condition index using a generalized linear model (GLM) with Gaussian error distribution and identity link function, where age class (juveniles and adults), sex (males and females) and sampling site (Corredor Verde del Guadiamar and María Luisa park) were included as explanatory variables. Subsequently, we used a GLM with quasibinomial error distribution and logit-link function to assess whether the relative proportion of DDE differed between age classes, sexes and sampling sites. Because the body condition index differed between birds and sampling sites (see below), we also included this index as a covariate in the model and the two-way interactions between sampling site and all the explanatory variables. We used a backward stepwise procedure to include only significant terms ($P < 0.05$) in the final model.

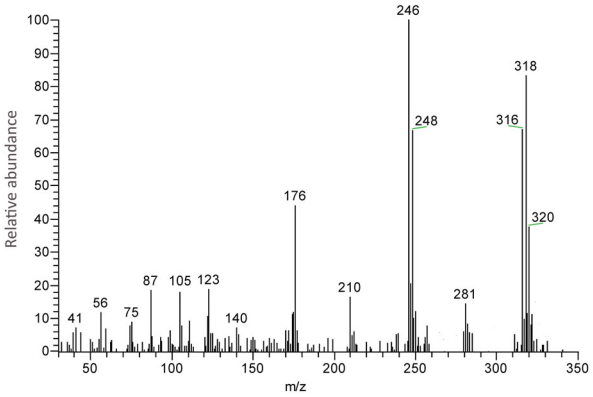

Figure 1. Representative mass spectrum of the compound identified as dichlorodiphenyldichloroethylene (DDE) found in samples of uropygial gland secretions of European Blackbirds.

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 determined by PCR (infected versus uninfected birds) and the relative proportion of DDE, sex and sampling site by using a GLM with quasibinomial error distribution and logit-link function. For the subset of adults, we used independent GLMs with Poisson error distribution and log-link function to assess whether the intensity of

infection by *Plasmodium*, *Leucocytozoon* and filarial worms (the most common parasites identified in blood smears) was associated with the relative proportion of DDE, sex, sampling site and body condition index.

The relationship between the relative proportion of DDE and the volume of the uropygial gland was tested by a Spearman correlation. The volume of the uropygial gland of two individuals was not measured, which explains the differences in the sample size included in the different analyses. All statistical analyses were performed using the R software environment (R Core Team 2017) with the package *lme4* (Bates *et al.* 2015).

RESULTS

DDE was found in the secretions of 36 (69.23%) individuals. Control vials did not show any trace of this compound. The body condition index estimated for sampled European Blackbirds differed significantly between age classes (GLM, Estimate ± standard error (se) −4.68 ± 1.48, $P = 0.003$), sexes (Estimate ± se −4.36 ± 1.43, $P = 0.004$) and sampling sites (Estimate ± se −4.04 ± 1.47, $P = 0.008$). Following the backward stepwise procedure for the analyses of the relative proportion of DDE in bird secretions, significant differences were only found between sexes (Estimate ± se −0.74 ± 0.30, $P = 0.016$) and sampling sites (Estimate ± se −1.20 ± 0.37, $P = 0.002$). Females had higher relative proportions of DDE than males (Fig. 2) and birds from the Corredor Verde del Guadiamar had higher levels than those from the María Luisa park (Fig. 3).

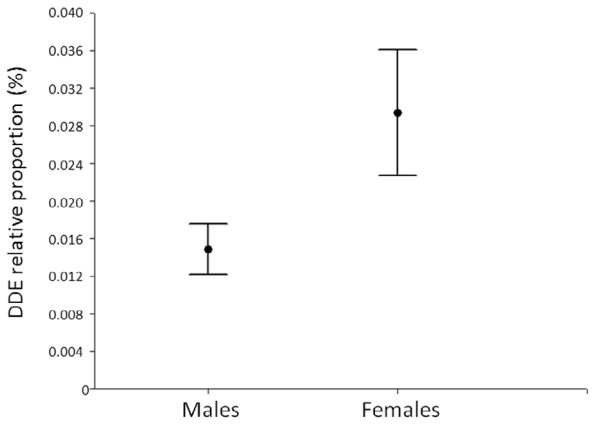

Figure 2. Relative proportion (± standard error) of dichlorodiphenyldichloroethylene (DDE) in the uropygial gland secretions of European Blackbirds in relation to sex: males and females.

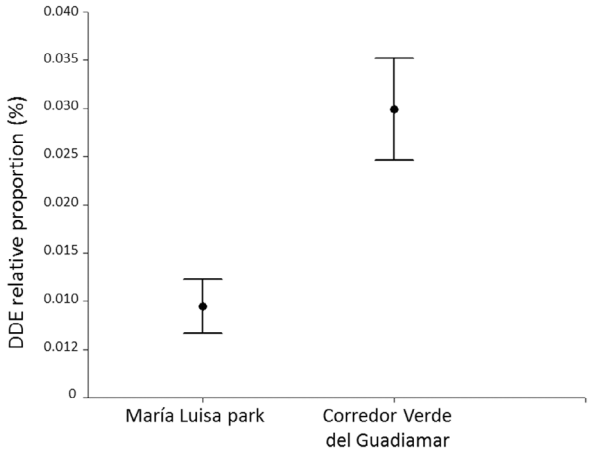

Figure 3. Relative proportion (± standard error) of dichlorodiphenyldichloroethylene (DDE) in the uropygial gland secretions of European Blackbirds in relation to sampling site: Maria Luisa park ($n = 19$) and the Corredor Verde del Guadiamar ($n = 33$).

Molecular analyses showed that 46 (88.46%) birds were infected by blood parasites, including 30 out of 31 adults and 16 out of 21 juveniles. Negative controls remained negative after running the PCR. *Plasmodium* was the most common blood parasite found (prevalence 82.69%), with parasites corresponding to two different lineages: SYAT05 (*Plasmodium vaughani*; $n = 41$) and LINN1 (= pSPHUjJ; $n = 2$). *Leucocytozoon* infection showed a prevalence of 44.23%, corresponding to three different lineages: TUMER01 ($n = 19$), TUMER02 ($n = 2$) and NEVE01 ($n = 1$); one individual showed evidence of co-infection (e.g. the presence of double peaks in the chromatogram) by two lineages. Finally, an unidentified lineage of *Haemoproteus* sp. was present only in one adult bird (prevalence 1.92%). Thirty of the 33 individuals from the Corredor Verde del Guadiamar were infected by blood parasites, with 29 birds (87.88%) infected by *Plasmodium* and 21 (63.66%) by *Leucocytozoon* parasites; i.e. multiple birds were infected by both parasite lineages. In the María Luisa park, 16 of 19 individuals were infected, with 14 birds (63.68%) infected by *Plasmodium*, three birds (15.79%) by *Leucocytozoon* and a single individual (5.26%) by *Haemoproteus* (Supporting Information Table S1).

Parasite screening of smears from adult birds (n = 30) revealed the presence of five parasite genera. *Plasmodium* was the most prevalent parasite genus (prevalence 56.67%), followed by microfilaria (30.00%) and *Leucocytozoon* (20.00%). In addition, *Lankesterella* (6.67%), *Haemoproteus* (3.33%) and *Trypanosoma* (3.33%) were also found.

Parasite detection varied between the two methods used, with a higher prevalence of infection found for both *Plasmodium* and *Leucocytozoon* when using molecular tools (Table 1). The mean (range) parasitaemia by each parasite genus was: 2.23 (0–21) for *Plasmodium*, 1.43 (0–15) for microfilaria, 0.30 (0–9) for *Haemoproteus*, 0.27 (0–3) for *Leucocytozoon*, 0.20 (0–6) for *Trypanosoma* and 0.07 (0–1) for *Lankesterella*. No significant differences were found in the intensity of infection by the three most common parasites (*Plasmodium*, *Leucocytozoon* and microfilaria) with respect to the relative proportion of DDE, location, body condition index or sex ($P > 0.08$ in all cases). The infection status for the subset of juveniles was not associated with the relative proportion of DDE for either with sex or sampling site ($P > 0.14$ in all cases).

The volume of the uropygial gland was not significantly related to the relative proportion of DDE (Spearman $\rho = 0.05$, $P = 0.73$).

DISCUSSION

The use of DDT has been banned in Spain since 1977 (BOE 1975). Despite this ban, our results add to the widespread evidence that birds are still exposed to its metabolite DDE in both natural and urban areas (Naso *et al.* 2003, Martínez-Lopez *et al.* 2007). By using a non-invasive method, we found that European Blackbirds tested positive for DDE in two sampling sites, the María Luisa park and in the Corredor Verde del Guadiamar, with detected DDE levels higher at the latter. Moreover, the relative proportion of DDE differed between sexes, with females showing a higher relative proportion than males. In contrast, the relative proportion of DDE was not associated with parasite infections.

Although exposure to DDE seems to be widespread throughout Europe, exposure may differ according to a 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, a result consistent with high exposure in certain areas. Differences in exposure to DDE also occur at finer spatial scales, as was reported in Eurasian Eagle Owl *Bubo bubo* eggs from different locations in Spain (Gómez-Ramírez *et al.* 2012). In agreement with this possibility, our results show significant variation in DDE levels between two nearby sites. These differences could be explained by habitat characteristics, because DDT has been used in large amounts in croplands for the control of insect pests over the past few decades, whereas DDT was probably used less intensely in urban areas. Even so, up to 57.89% of birds from the María Luisa urban park had detectable concentrations of DDE in their uropygial secretions, which suggests that DDT was also used in the city or was spread from surrounding areas as a result of the semivolatility of this pesticide (Meijer *et al.* 2003). It is also possible that birds, including juveniles and adult birds, are still exposed to this pollutant in both environments because of the current use of products such as Dicofol. This pesticide contains high levels of impurity in the form of DDT-related compounds, as *o,p'*-DDT or *o,p'*-DDE, and is extensively used for controlling damage to

Table 1. Infection status of adult European Blackbirds and prevalence of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* according to the detection method: screening of blood smears and molecular (PCR) analyses.

	Blood smears (n = 30)[a]			PCR (n = 31)		
Parasite	Infected	Uninfected	Prevalence	Infected	Uninfected	Prevalence
Plasmodium	17	13	56.67	29	2	93.55
Haemoproteus	1	29	3.33	1	30	3.23
Leucocytozoon	6	24	20.00	14	17	45.16

PCR, polymerase chain reaction. [a]One individual was only analysed using PCR but not blood smears.

cotton, fruit trees, vegetables and ornamental crops by insect pests (Yang *et al.* 2008, Muñoz-Arnanz & Jiménez 2011). However, we cannot exclude the possibility that the difference found between localities was the result of differential feeding behaviour of the birds, should birds from María Luisa park feed more on fruits and those from Corredor Verde del Guadiamar feed more frequently on invertebrates. Indeed, earthworms are an important source of food for this bird species and this may facilitate the accumulation of high levels of DDT and DDE (Harris *et al.* 2000).

DDT and its metabolites are highly persistent pollutants in the environment, being progressively bioaccumulated in animal tissues (Turusov *et al.* 2002). Growing evidence supports the accumulation capacity of these chemicals in animals and biomagnification through food webs of terrestrial, freshwater and estuarine habitats (Kidd *et al.* 2001). Previous studies have reported significant differences in the presence of pollutants between bird sexes, with females usually showing lower levels of contaminants than males (Drouillard & Norstrom 2000, Kubota *et al.* 2013). This pattern may be explained on the basis of female net transfer of organochlorines to the eggshell and yolk during reproduction. However, we found a higher relative proportion of DDE in females than in males. This was also the case for Tanabe *et al.* (1998), who found lower levels of DDT in male than female White-winged Terns *Chlidonias leucopterus*, whereas other authors have reported no sex differences in residues of the DDT family (e.g. King & Krynitsky 1986, Cid *et al.* 2007, Goutner *et al.* 2011). Contrasting results between studies could be due to the sample size and the type of the sample analysed, e.g. blood, feathers, faeces, tissues (e.g. liver, muscle) or, as in our case, the secretions of uropygial gland. Samples of different origin may contain a heterogeneous distribution of lipids (Subramanian *et al.* 1986). Variability of concentrations within species could probably be attributed to different feeding habits (trophic position and specific prey species) and variable metabolic capacity, which could determine the incorporation of unfixed values of pollutants (Jaspers *et al.* 2006).

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 Harrier *Circus pygargus* and Pallid Harrier *Circus macrourus* from Spain and Kazakhstan, respectively. Similarly, García-Heras *et al.* (2018) detected higher DDE levels in adults than in nestling Black Harriers *Circus maurus* from South Africa. However, we failed to identify any difference in the relative proportion of DDE between age classes, suggesting that birds of both ages should be similarly exposed to this pollutant in the area. It is possible that during reproduction, females transfer DDE directly to the egg yolk and the chicks incorporate this pollutant into their tissues during development. Accordingly, Dauwe *et al.* (2006) detected higher concentrations of pollutants (organochlorines and PCBs (polychlorinated biphenyls)) in eggs than in 5-, 10- or 15-day-old Great Tit *Parus major* nestlings, although the proportion of DDE increased with the age of the nestlings. Again, potential differences found between studies could be due to the different tissues analysed, with differences between age classes being identified in blood samples but not in the secretions of the uropygial gland (Goutner *et al.* 2011).

Although accumulation of organochlorines has been associated with weakened health, we did not find any significant relationship between parasite infections and the relative proportion of DDE in European Blackbirds. Nonetheless, the effects of pollutants on the prevalence of infectious diseases are poorly known. The high exposure to organochlorines of pre-fledgling chicks of Caspian Terns *Hydroprogne caspia* and European Herring Gulls *Larus argentatus* has been associated with immunosuppression, in particular a decrease in T cells, which facilitates parasitic infections (Grasman *et al.* 1996). Because haemosporidians usually produce chronic infections, our results can be explained by birds being infected before DDE exposure or accumulation. Although parasite load and parasite infection status may reflect two different faces of parasite infections in birds (Westerdahl *et al.* 2011), we also did not find significant associations between parasitaemia and DDE in adult birds. Further studies with larger sample sizes and different age classes are necessary to fully understand this potential relationship in wild populations.

In conclusion, DDE could be easily detected using non-invasive and non-destructive methods in birds, thereby enabling the biomonitoring of contaminants as well as the identification of factors (e.g. habitat use and sex in our study) potentially affecting the exposure of birds to this pollutant.

Our results indicate that exposure to DDE is higher in the Corredor Verde del Guadiamar and that DDE accumulates preferentially in the uropygial secretions of females. Nonetheless, these results demonstrate the need to develop additional studies to evaluate the effect of pesticides on birds by using non-invasive methods to further increase our knowledge on this important research area.

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AUTHOR CONTRIBUTIONS

Alazne Díez-Fernández: Data curation (lead); formal analysis (lead); investigation (lead); methodology (equal); validation (equal); writing – original draft (lead); writing – review and editing (lead). **José Martín:** Data curation (lead); formal analysis (equal); supervision (equal); writing – review and editing (equal). **Josué Martínez-de la Puente:** Conceptualization (lead); formal analysis (equal); investigation (equal); methodology (equal); supervision (equal); validation (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 (supporting); 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).

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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ETHICS AND PERMISSIONS

This project was undertaken 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. Ringing licences 66 042 and 660 019. Bio-Ethics permit: 25–05–15-254. Regional Department of the Environment Permit 2013_21_22-2011_15 DGGMN 2014_2015.

Data Availability Statement

All data generated or analysed during this study are included in this published article. Data availability: the sequences from parasite lineages generated during the current study are available in MALAVI repository (http://130.235.244.92/Malavi/index.html) and GenBank (ncbi.nlm.nih.gov/genbank/). The lineages submitted are from *Plasmodium* SYAT05 (*Plasmodium vaughani*) and LINN1 (= pSPHUjJ 2), and three different lineages of *Leucocytozoon*: TUMER01, TUMER02 and NEVE01. The GenBank numbers are as follows: *Leucocytozoon*: ON730883 – Lineage NEVE01, ON730884 – Lineage TUMER02 and ON730885 – Lineage TUMER01; *Plasmodium*: ON730886 – Linage LINN1 and ON730887 – Lineage SYAT05.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Number of infected birds for each parasite genus and lineage found in European Blackbirds according with their locality.