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Communication

The uropygial gland microbiome of house sparrows with malaria infection

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Birds secrete preen oil from the uropygial (preen) gland which is used to maintain feather integrity and communicate through odour. The uropygial secretion is believed to influence host attractiveness to biting insects, thereby altering the risk of infection by vector-transmitted blood parasites. Previous studies have documented a presence of bacteria in the uropygial secretion; however, the microbial community in the gland is still largely unknown. In addition, we have no information yet as to whether there are any associations between these uropygial gland microbes and haemosporidian parasite infection. Here, we characterise the microbiome of the uropygial gland secretion in 23 wild-caught house sparrows *Passer domesticus* from Peru and investigate whether individuals with natural malaria *Plasmodium* infection have different preen oil microbiota than uninfected birds. We found no differences in microbial alpha diversity or richness, and malaria infection explained approximately 5% of the overall microbiome composition, however, this was not statistically significant. On the other hand, birds with malaria infection had significantly higher abundances of bacteria from the genera *Arthrobacter* and *Micrococcus* in their gland, whereas uninfected birds harboured higher abundances of *Rhodococcus*, *Phenylobacterium* and *Enhydrobacter*. These first results of the uropygial gland microbiome in malaria-infected birds illustrate the presence of a more specific relationship between certain members of the gland microbiota and *Plasmodium* parasites in birds, which opens up new questions on the role of the uropygial gland in avian health.

Keywords: Haemosporidian, microbiota, *Passer domesticus*, *Plasmodium*, preen gland, preen oil

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Introduction

The uropygial gland is a holocrine gland located in the synsacrum caudal region of birds. Also called the preen gland, it secretes an oily substance birds use during preening to maintain feather integrity (Jacob and Ziswiler 1982). This secretion is hydrophobic, enabling birds to stay dry and warm despite being potentially submerged in water (Montalti and Salibián 2000, Moreno-Rueda 2017). It also contains antimicrobial and antifungal properties (Soler et al. 2014b, Magallanes et al. 2016, Braun et al. 2018a) that could protect against feather-degrading microbes (Shawkey et al. 2003, Møller et al. 2009, Czirják et al. 2013, Jacob et al. 2018, Carneiro et al. 2020). Interestingly, the uropygial gland has been shown to house a diverse array of bacteria, some of which seem to produce these antimicrobial compounds (Soler et al. 2008, Martín-Vivaldi et al. 2010, Rodríguez-Ruano et al. 2015, Braun et al. 2016, Alt et al. 2020). The bacteria present in the gland may assist in structuring the skin and feather microbiota of birds (Javůrková et al. 2019), both of which can have important roles such as odour production (Maraci et al. 2018) and increased resilience to pathogens (Soler et al. 2014b, Jacob et al. 2018).

Avian body odour is believed to be primarily derived from the uropygial gland secretion (Hagelin and Jones 2007); therefore this substance could play an important role in host attractiveness to ectoparasites that find hosts primarily via chemoattractants (Fallis and Smith 1964, Moyer et al. 2003, Clayton et al. 2010, Møller et al. 2010, Pap et al. 2010). However, whether preen oil helps attract or defer biting insects is still inconclusive (Moreno-Rueda 2017). For example, Russell and Hunter (2005) found that cotton swabs with uropygial gland secretion attracted a greater number of *Culex* mosquitoes at 5 m elevation compared to blank control swabs. Conversely, a study by Tomás et al. (2020) found that the secretion and its bacteria may repel certain biting insects, and Díez-Fernández et al. (2020) showed that neither *Culex* or *Aedes* mosquitoes were attracted to uropygial gland secretions using a dual-choice olfactometer. In humans, the skin microbiota has been shown to be an important determinant of host attractiveness to *Anopheles* mosquitoes (Verhulst et al. 2009, 2011). Importantly, the degree of host attractiveness to biting insects will strongly affect the transmission potential for blood parasite infection (Cornet et al. 2013). In this sense, it has been recently shown that individuals with larger uropygial glands have lower prevalence of haemosporidian infection (Marzal et al. 2018, Magallanes et al. 2020), suggesting that uropygial gland secretions may be negatively associated with malaria infections.

Though the presence of bacteria and their antimicrobial products in the uropygial gland have been known for some time (Reneerkens et al. 2006, Soler et al. 2008), the uropygial bacterial community has only been evaluated in a few host species. The hoopoe *Upupa epops* uses microbes in the gland to cover its eggs and this bacterial coating protects the eggs from invading pathogens (Soler et al. 2014a, Martínez-García et al. 2016). In fact, hoopoes specifically alter the microbial structure in their uropygial glands during the hole-nesting period (Martín-Vivaldi et al. 2010, Rodríguez-Ruano et al. 2015, 2018, Díaz-Lora et al. 2019). In dark-eyed juncos *Junco hyemalis*, male and female pairs show similar microbial composition in the uropygial gland (Whittaker and Theis 2016, Whittaker et al. 2019), and disrupting this community alters the volatile compounds that are produced (Whittaker et al. 2019). A recent study evaluating great tits *Parus major* used co-culture experiments of uropygial bacteria with feather-degrading bacteria and pathogens to show that certain isolates might be able to suppress pathogenic growth (Bodawatta et al. 2020). Lastly, Pearce et al. (2017) examined the microbiota of the skin and feathers near the uropygial gland of Leach's storm petrels *Oceanodroma leucorhoa*, though not of the secretion itself. Besides in these species, little is known about the microbiome of the uropygial gland and how this unique microbial community varies with natural malaria infection.

Here we examined a potential association between the uropygial gland microbiota and haemosporidian parasites in birds. For this purpose we characterised the uropygial microbiome of wild-caught house sparrows *Passer domesticus*, and evaluated whether this microbial community differs in individuals that were naturally infected with *Plasmodium* parasites compared to uninfected birds.

Material and methods

Sample collection

All fieldwork was conducted in Peru (Manchay: 12°10′01.0″S, 76°51′22.4″W; Tarapoto 6°30′41.8″S, 76°22′07.4″W) 22nd and 24 of June 2017, respectively. We used mist-nets to capture 23 house sparrows (16 in Manchay and 7 in Tarapoto) (Supporting information). Secretion from the uropygial gland of all individuals was collected immediately after capture, following the method described by Martín-Vivaldi et al. (2009). Ethanol and hydrogen peroxide were used to clean the work area and new sterile gloves were used for each bird. Before collecting the preen oil, we cleaned the uropygial gland and surrounding skin with a cotton swab soaked in ethanol to minimise risk of contamination. After the alcohol had evaporated, the gland was softly pressed with a gloved finger to expel the secretion directly into a sterile cotton swab. The swab was placed in an empty sterile Eppendorf tube and kept at about 4°C in a portable ice box. Blood samples were collected with a microcapillary to obtain 50 µl of peripheral blood from the brachial vein and stored in 500 µl SET buffer in the ice box. We measured body weight of birds with a digital scale, and tarsus length and gland size (length, width, height) using a digital caliper with a precision of 0.01 mm (Galván and Sanz 2006). Relative body weight was calculated by dividing weight with tarsus length. All birds were released post-sampling. Swab samples were subsequently stored at −80°C and blood samples at −20°C within four hours after collection.

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Molecular detection of blood parasites

The blood samples were analysed using the molecular method described by Waldenström et al. (2004) to determine the presence of haemosporidian infection. We used the GeneJET Genomic DNA Purification Kit (Thermo Scientific, reference no. K0722) to extract DNA from each sample. Samples were diluted to 25 ng μl^{-1} and tested in a nested PCR. We included negative and positive PCR controls during the PCR for validation. Each sample that amplified in the nested PCR was subsequently sequenced using the haemosporidian detection methodology described by Hellgren et al. (2004). We identified the following *Plasmodium* lineages: SCHRUF1 (n = 2), PADOM9 (n = 1) and GRW4 (n = 6) (Supporting information). Detailed effects of specific lineages were not possible to evaluate due to limited sample size and different distribution among sites; instead birds were classified whether they were infected or uninfected with malaria *Plasmodium*.

Microbiome sequencing of uropygial gland secretion

The swabs with gland secretion were first soaked in 200 µl of TE buffer and incubated for one hour at 25°C. Samples were then sent to SAIUEx at the University of Extremadura, Spain, for purification, isolation and sequencing. GeneJET Genomic DNA Purification Kit (ref. K0721, Thermo Scientific) (95 µl) and proteinase K (10 µl) were added to the samples, which were subsequently incubated for one hour at 56°C. After incubation, swabs were removed and 30 µl of MagMAX CORE Nucleic Acid Purification kit (Applied Biosystems, Thermo Fisher Scientific) was added. The protocol for DNA isolation associated with this kit was followed. After DNA had been extracted, 16S amplicon libraries were prepared by following the instructions described by the Ion 16S Metagenomics Kit (Thermo Scientific), which includes two sets of primers that target seven hypervariable regions of the 16S rRNA gene (V2, V3, V4, V6–7, V8 and V9). Samples were added in groups of ten to a 530 chip using Ion 510, Ion 520 and Ion 530 Kit-Chef (Ion Torrent, Thermo Fisher). 16S amplicon reads were subsequently sequenced by SAIUEx using an Ion GeneStudio S5 System (model XL). The samples collected from Tarapoto were sequenced 20th of April 2018 and the samples from Manchay were split into two sequencing runs 27 April and 9 May 2018. This division of samples meant we were unfortunately unable to precisely distinguish locality effects from sequencing batch effects on the microbiomes. Both malaria-infected and uninfected samples were included in all three sequencing runs, as were six negative (blank) samples.

Data analyses

Sequences were quality-screened with FastQC (Andrews 2010) and subsequently prepared using QIIME 2 (v. 2020.2.0) (Bolyen et al. 2019). All 23 samples (n = 9 infected and n = 14 uninfected) yielded a high number of high-quality sequences (> 350 000 per sample) and were kept for analyses. DADA2 (v. 2020.2.0) (Callahan et al. 2016) was used to denoise and trim sequences evenly to 240 bp amplicon sequence variants (ASVs, also commonly referred to as OTUs: operational taxonomic units or sub-OTUs). Taxonomic classification was first tested against the SILVA (v. 132) full-length database (Quast et al. 2013), however, it resulted in 42.8% of ASVs unassigned. We therefore performed taxonomic classification instead using GreenGenes (v. 13.8) full-length database (McDonald et al. 2012), which successfully assigned 99.7% of ASVs. A phylogenetic tree was constructed inside QIIME 2 (v. 2020.2) using MAFFT (Katoh and Standley 2013) and FastTree2 (Price et al. 2010).

Statistical analyses were performed in R (v. 3.6.2) using phyloseq (v. 1.30.0) (McMurdie and Holmes 2013) and vegan (v. 2.5-6) (Oksanen et al. 2017). We first utilised Decontam (v. 1.6.0) (Davis et al. 2018) to eliminate potential contaminating ASVs found in six blank samples that were sequenced simultaneously as negative controls. A decontamination probability threshold of 0.5 was employed in order to be more stringent than the default setting of 0.1. In addition, we filtered ASVs that gave matches to mitochondria and chloroplast, as well as ASVs present in fewer than five individuals to further minimise potential contamination. Sequences were rarefied to 30 000 reads per sample and microbiome beta diversity was calculated with Bray–Curtis and weighted UniFrac distances. ASV richness was calculated as the total number of unique ASVs and alpha diversity was calculated with the Shannon index which takes into account both ASV richness and evenness. We used betadisper in vegan to calculate dispersion and adonis to perform a PERMANOVA both with and without sequencing run as a blocking factor. DESeq2 (v. 1.26.0) (Love et al. 2014) was used to test for differential ASV abundances between infected and uninfected birds while controlling for sequencing date. p-values were corrected for multiple testing with the Benjamini and Hochberg correction and ASVs were considered statistically significant at 0.05 false discovery rate.

Results

We detected a total of 299 ASVs (OTUs) in the uropygial gland microbiome. These sequences primarily belonged to the bacterial class Gammaproteobacteria, followed by Alphaproteobacteria, Actinobacteria, Betaproteobacteria and Bacilli (Fig. 1). Based on relative abundances, malaria-infected individuals had lower mean abundance of Gammaproteobacteria (35.3%) and higher mean abundance of Actinobacteria (29.4%) and Betaproteobacteria (16.4%), compared to the uninfected birds (Gammaproteobacteria = 58.9%; Actinobacteria = 14.2%; Betaproteobacteria = 12.2%) (Fig. 1). The most abundant ASVs in the uropygial gland were from a diverse set of bacterial genera belonging to the aforementioned classes (Supporting information). The majority of variation in the uropygial gland microbiome was explained by samples from different individuals (PERMANOVA using Bray–Curtis distances: $R^2 = 0.67$), followed by sequencing run ($R^2 = 0.11$), locality ($R^2 = 0.08$), malaria infection status ($R^2 = 0.05$), gland size ($R^2 = 0.04$), sex ($R^2 = 0.03$) and relative body weight ($R^2 = 0.03$) (Fig. 2, Supporting information); however, only sequencing run and

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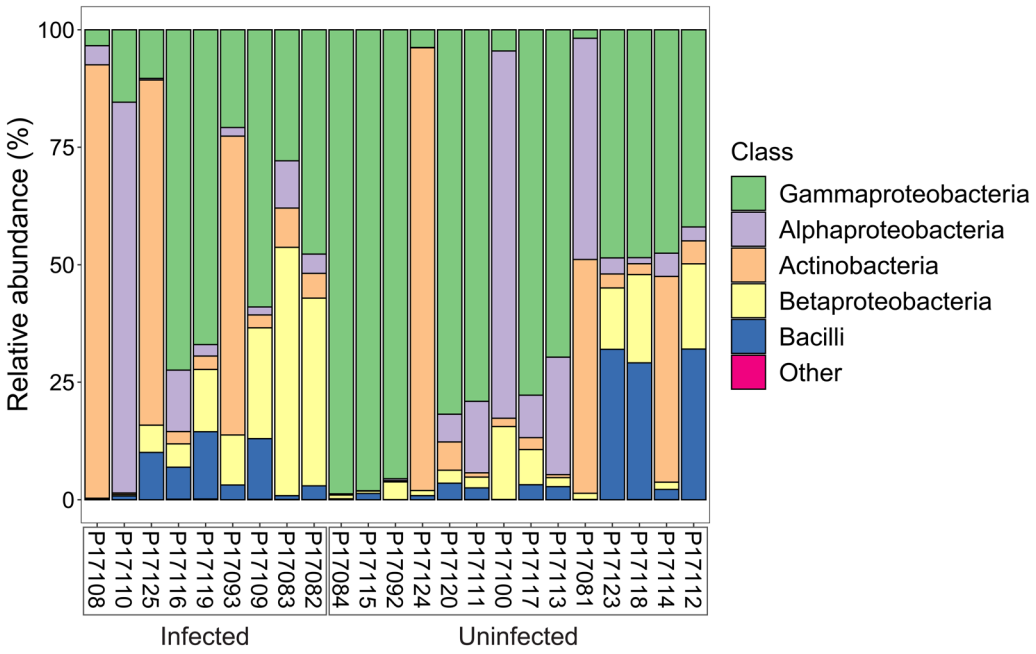


Figure 1. Relative abundances of bacterial classes in the uropygial gland microbiome of each host individual, sorted by malaria-infection status.

locality were statistically significant ($p=0.003$ and 0.042, respectively). Furthermore, we did not detect any heterogeneity of dispersion among the samples ($F=0.40$, $p=0.53$), and evaluating phylogenetic distances between microbiomes gave similar results (PERMANOVA using weighted UniFrac: individuals $R^2=0.64$, sequencing run $R^2=0.1$, locality $R^2=0.08$, gland size $R^2=0.08$, malaria infection status $R^2=0.05$, relative body weight $R^2=0.04$ and sex $R^2=0.01$) (Supporting information).

Microbial richness (unique ASVs) and alpha diversity of the uropygial gland did not differ between birds infected with malaria and uninfected individuals (Wilcoxon rank sum test of Richness: $W=76$, $p=0.43$; Shannon diversity: $W=77$, $p=0.40$) (Fig. 2). We also did not see any correlation between alpha diversity and uropygial gland size (Spearman's rank correlation: $r=-0.26$, $S=2568$, $p=0.21$). Differences in microbiomes between infected and uninfected birds were, however, evident at a finer taxonomic scale. We found a total of 18 ASVs that showed significant differential abundances in birds with malaria infection, controlling for sequencing run (Fig. 2, Supporting information). In malaria-infected individuals, we found higher abundances of ASVs derived exclusively from the related Actinobacteria genera *Arthrobacter* and *Micrococcus*. Uninfected birds, on the other hand, harboured higher abundances of ASVs from the Proteobacteria genera *Enhydrobacter* and *Phenylobacterium* and the Actinobacteria genus *Rhodococcus* (Fig. 2).

Discussion

We found a significant but specific association between the uropygial gland microbiome and blood parasite infection in house sparrows. Individuals with malaria did not differ in microbial alpha diversity, richness and beta diversity, compared to uninfected individuals. Malaria infection explained approximately 5% of the overall beta diversity among individuals, while taking into account factors such as sex, weight, locality and sequencing run, however, this was not statistically significant. Instead, 18 ASVs showed significant differential abundances in malaria-infected birds, indicating a possible intricate interaction between malaria infection and specific bacterial genera.

An association between the uropygial microbiome and malaria infection could be due to one or several of the following hypothetical scenarios: 1) Specific members of the uropygial gland microbiome could potentially affect the prevalence of blood parasites in birds by structuring the feather or skin microbiome to alter host attractiveness to vectors via chemoattractants, thereby influencing the risk of getting infected. 2) Certain members of the uropygial microbiome might have the ability to affect host resistance to haemosporidian infection by triggering elevated immune responses in the host with indirect effects on blood parasites. Or vice versa, 3) malaria parasites could have indirect influence on specific taxa of the uropygial microbiome by activating immune gene expression levels in infected hosts. Thus, the specific associations we identified could be either seen as a result of an ongoing infection, or indirectly causative by influencing host attractiveness to vectors. Other factors that determine whether birds become infected with malaria can be for example host genetics (Sepil et al. 2013), age (van Oers et al. 2010), sex (Calero-Riestra and García 2016), vector abundance (Okanga et al. 2013), habitat characteristics (Ferraguti et al. 2018), and interactions between parasite lineage and host species (Garcia-Longoria et al. 2019).

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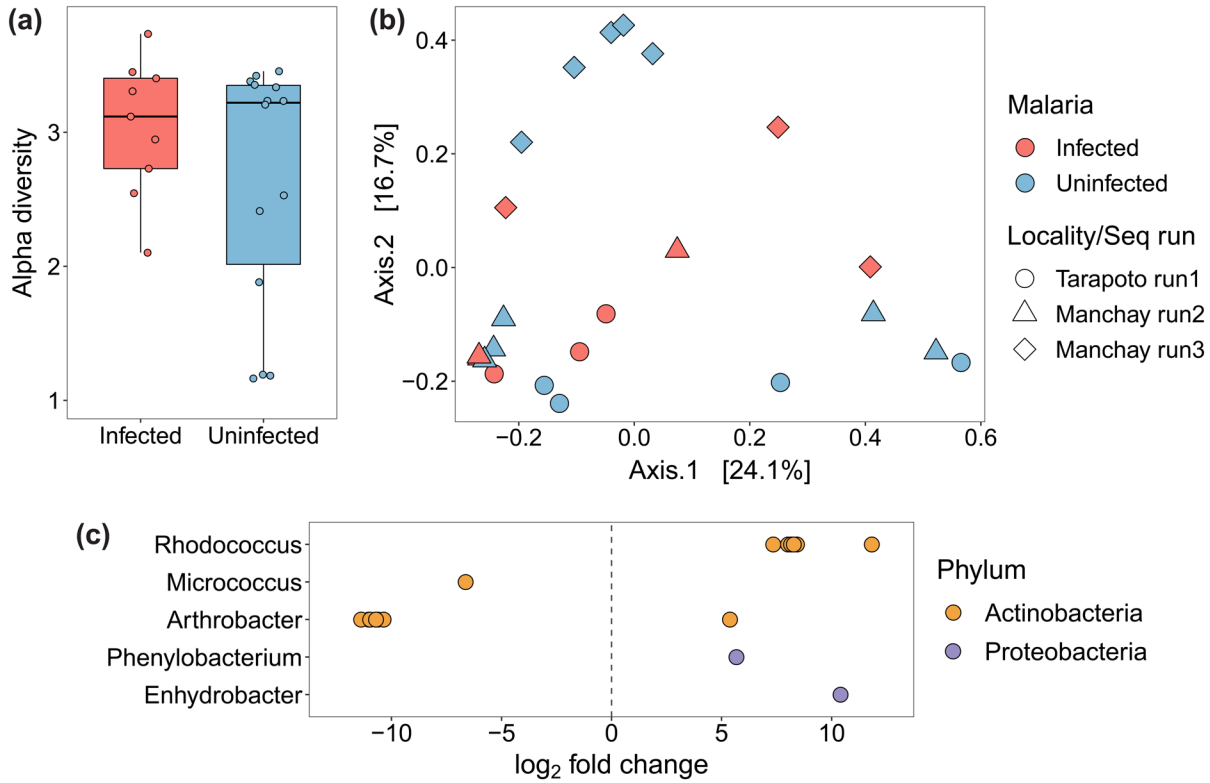


Figure 2. Uropygial microbiome analyses of birds infected with malaria and uninfected birds. (a) Alpha diversity in infected birds (red) does not differ from uninfected birds (blue). (b) PCoA of beta diversity (Bray–Curtis distances) among infected and uninfected individuals, with locality and sequencing run as symbols. (c) Eighteen ASVs (dots) were differentially abundant in birds with malaria infection (controlling for sequencing run), here coloured by phyla and labelled by genera. Negative $\log_2$ fold change indicates higher abundances in infected birds, and positive $\log_2$ fold change indicates higher abundances in uninfected birds.

Eight ASVs derived from *Arthrobacter* and *Micrococcus*, showed higher abundances in the uropygial gland of birds with malaria infection. Ten ASVs from *Rhodococcus*, *Phenylobacterium* and *Enhydrobacter* were instead overrepresented amongst the uninfected individuals. The closely related *Micrococcus* and *Arthrobacter* are sometimes part of the normal skin microbiome of vertebrates (Ross et al. 2019), and all the above-mentioned genera have previously been found in association with wild birds, either on the surface of eggshells (Potter et al. 2014), or as part of the normal feather and gut/cloacal microbiome (van Dongen et al. 2013, Musitelli et al. 2018). *Micrococcus* has been discovered in the uropygial glands of hoopoes and dark-eyed juncos (Soler et al. 2008, Whittaker et al. 2019), and other genera commonly found in our data have also been previously documented in the preen glands of birds. For example, *Kocuria* has been identified in the uropygial glands of great tits (Bodawatta et al. 2020), woodpeckers *Dendrocopos major* and barn owls *Tyto furcata* (Braun et al. 2018b, 2019), *Corynebacterium* in turkeys and dark-eyed juncos (Braun et al. 2016, Whittaker et al. 2019) and *Enterococcus* in hoopoes and great tits (Soler et al. 2008, Bodawatta et al. 2020). It is difficult to speculate as to why the aforementiond genera in particular had different abundances in infected individuals, though future studies of their functional roles inside bird hosts might generate clues.

Due to the fact that samples in this study from Manchay and Tarapoto were prepared and sequenced separately (Supporting information), we were unable to precisely disentangle technical batch effects of sequencing run from effects of geographical locality. Our permanova analyses indicate that sequencing run had a significant influence on the uropygial gland microbial composition (Supporting information), despite stringent filtering measures and decontamination steps using blank samples. Samples containing relatively low microbial biomass, such as gland secretions and feathers, are particularly sensitive to technical variation and bacterial contamination that can arise during DNA extraction and library preparation (Eisenhofer et al. 2019). Therefore, future studies incorporating more samples and additional randomization approaches during library preparation and sequencing are needed to investigate the effects of malaria with greater precision.

Though our sample size is limited to 23 individuals, our study provides a first unique view into specific minor associations between avian haemosporidian infection and uropygial gland microbiota. This potential link is important to document because it will enable further explorations of the interactions between blood parasites and host-associated bacteria. Future studies evaluating time points prior to malaria infection are needed to establish whether the differential abundances of Actinobacteria and Proteobacteria resulted from altered

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immune levels in infected birds, or if these bacteria differed already prior to infection. A study by Grieves et al. (2018) was able to show that experimentally infecting song sparrows *Melospiza melodia* with malaria parasites *Plasmodium* induced changes to the wax ester profiles of their uropygial gland secretion. Whether the chemical composition of the gland was altered as a result of a shift in microbiome structure is, however, not known. Testing this hypothesis will yield important clues whether certain members of the uropygial gland microbiota are indirectly influenced by malaria via altered immune levels in infected hosts. It is possible that we were unable to identify a stronger effect on the uropygial microbiome due to the fact that wild birds with malaria usually harbour low chronic infection levels with little influence on fitness (Palinauskas et al. 2020). Another possibility is that different species or lineages of malaria parasites could have dissimilar associations with members of the uropygial gland microbiome. Our study incorporated three *Plasmodium* lineages (GRW4, SCHRUF1 and PADOM9), however, they showed entirely different prevalences at the two localities, making it impossible to disentangle lineage effects from locality effects.

In conclusion, our study did not identify any major associations between malaria parasites and the composition and diversity of the uropygial gland microbiota. However, we found a specific association in the form of five bacterial genera differentially abundant in infected birds. Further research is needed to investigate the precise mechanisms underlying this pattern. The outlined results and accompanying data will add to our collective knowledge on the relationship between host-associated microbiomes and blood infections, and open up new questions about the roles of uropygial microbiota in avian health.

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Conflict of interest – The authors declare no conflict of interest.

Author contributions

Elin Videvall: Data curation (lead); Formal analysis (lead); Software (lead); Validation (lead); Visualization (lead); Writing – original draft (lead); Writing – review and editing (lead). **Alfonso Marzal**: Conceptualization (lead); Funding acquisition (lead); Investigation (equal); Methodology (supporting); Project administration (lead); Resources (lead); Supervision (lead); Validation (supporting); Writing – review and editing (supporting). **Sergio Magallanes**: Conceptualization (equal); Investigation (equal); Methodology (equal); Writing – review and editing (supporting). **Rob Fleischer**: Funding acquisition (equal); Resources (supporting); Supervision (supporting); Writing – review and editing (supporting). **Kathya Espinoza**: Conceptualization (equal); Investigation (equal); Methodology (equal). **Luz García-Llongoria**: Conceptualization (equal); Investigation (equal); Validation (supporting); Writing – original draft (supporting); Writing – review and editing (supporting).

Data availability statement

Sequences have been uploaded to ENA at EMBL-EBI (accession no. PRJEB40061, <https://www.ebi.ac.uk/ena/browser/view/PRJEB40061>.

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