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Article

Innate and adaptive immune proteins in the preen gland secretions of male house sparrows

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Recent studies have demonstrated that preen oil acts to reduce or eliminate feather-associated bacteria. The mechanisms underlying this antibacterial activity, however, are incompletely understood. In addition to the activity of alcohols (i.e. 3,7-dimethyloctan-1-ol), recent research has suggested that antimicrobial peptides may underlie the antibacterial activity of preen oil. Here, we document the presence of innate and adaptive immune proteins, lysozyme and immunoglobulin Y (IgY), in the preen oil of house sparrows *Passer domesticus*. We suggest lysozyme functions as an antimicrobial agent, with potentially important impacts against Gram-positive feather degrading bacteria. Furthermore, both lysozyme and IgY likely act in local immune defence of the preen gland, and may also play a role in regulating the local microbiome, with potentially important consequences for chemical communication and signalling. Our findings suggest that the preen gland and its secretions should be considered an integral part of the body's first line of defence against invading infections.

Keywords: adaptive immunity, antimicrobial activity, constitutive immunity, local immunity, preen oil, uropygial gland

Introduction

Organisms are constantly exposed to a range of microorganisms in their environment, some of which may have detrimental impacts on their hosts (Steinert et al. 2000). To counteract the potentially negative effects of these microorganisms, animals have evolved a range of behavioural, mechanical and chemical defences to impede or defeat invading parasites and pathogens (Ganz 2002, Proksch et al. 2008, Gallo and Hooper 2012). The skin and mucosa provide a physical barrier to microbial invasion and, as such, act as the first line of defence against infectious agents. However, these barriers may also exhibit structures that incorporate specialised cells that secrete defence molecules (e.g. antimicrobial proteins) and play a role in local chemical defence. Such molecules can also contribute to systemic immunity (Diamond et al. 2009), and are thus a particularly important component of animal immune defences.

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The ecology and evolution of bird–bacteria interactions has received considerable attention over the last few decades, at least in part because wild birds may act as reservoirs for emerging zoonotic bacterial pathogens (Hubálek 2004, Benskin et al. 2009) and because of the potential for bacterial communities to influence host survival, reproduction and evolution (Benskin et al. 2009, Evans et al. 2017, Rowe et al. 2020). Most notably, recent studies have documented the presence of complex communities of bacteria inhabiting the feathers of birds (Shawkey et al. 2005, Czirják et al. 2010, Javůrková et al. 2019). Of particular interest are the keratinolytic bacteria (also referred to as feather-degrading bacteria), as these species have the capacity to digest β-keratin, a protein comprising > 90% of feather mass, via the secretion of keratinases (Gunderson 2008). Importantly, damage caused by these bacteria can alter feather integrity (Ruiz-Rodriguez et al. 2009, Verea et al. 2017, Azcárate-García et al. 2020) and impact plumage condition (Leclaire et al. 2014) and colour (Shawkey et al. 2007, Gunderson et al. 2009, Leclaire et al. 2014, but see Jacob et al. 2014). Thus, feather degrading bacteria appear to have the potential to generate significant fitness effects on their hosts via thermoregulatory stress, decreasing flight capacity or via a role in sexual selection.

Birds appear to have evolved a range of mechanisms to combat the negative effects of feather-degrading bacteria. For example, feather structure and biochemistry, dust and sunbathing, feather moult and preening behaviour have all been suggested to inhibit or limit the fitness impacts of bacterial-induced feather damage (Gunderson 2008). The preen (also called the uropygial or coccygeal) gland is a holocrine gland, exclusive to birds, that produces an oily secretion (hereafter, preen oil) that is spread over the plumage and other teguments during preening. Preen oil is predominately composed of lipids, such as monoester and diester waxes of fatty acids, as well as free alcohols, terpenes and fatty acids (Jacob and Ziswiler 1982, Salibian and Montalti 2009, Campagna et al. 2011, Jacob et al. 2018). A range of functions have been proposed to explain the preen gland and its secretions, though many of these are not mutually-exclusive and remain the topic of some debate (Moreno-Rueda 2017). Perhaps most notably, a considerable body of evidence supports the idea that preen oil provides antimicrobial protection against feather-associated bacteria and fungi, with several mechanisms of action having been identified or proposed. First, preen oil has been shown to have antibacterial (Bandyopadhyay and Bhattacharyya 1996, Shawkey et al. 2003, Czirják et al. 2013, Alt et al. 2020, but see Giraudeau et al. 2013) and antimycotic (Jacob et al. 1997, Bandyopadhyay and Bhattacharyya 1999) properties that are thought to reduce or eliminate feather-associated microbes. Additionally, in some cases antibacterial activity of preen oil is known to result from symbiotic bacteria in the preen gland that produce bacteriocins to effectively kill bacteria on feathers (Ruiz-Rodriguez et al. 2009, Martín-Vivaldi et al. 2010). Antimicrobial protection may also arise indirectly via hydrolyzation of ester waxes in preen oil by abiotic factors (e.g. UV radiation) or feather-associated esterase positive bacteria once the oil has been applied to plumage (Braun et al. 2018). Finally, preen oil may constitute a physical barrier to microbial attack (Reneerkens et al. 2008, Verea et al. 2017), thus preventing bacteria from directly interacting with feathers and causing damage.

Importantly, although antibacterial activity of preen oil has been demonstrated under both in vitro and in vivo conditions, the substances responsible for this activity remain generally unresolved. In gannets *Sula bassana*, the antimicrobial activity of preen oil appears based, at least in part, on the activity of an alcohol (i.e. 3,7-dimethyloctan-1-ol; Jacob et al. 1997). More recently, Braun et al. (2018), provided evidence that the antimicrobial activity of preen oil in turkeys *Meleagris gallopavo* does not originate from lipids, suggesting instead that it may be mediated by highly polar compounds such as antimicrobial peptides. The idea that antimicrobial peptides occur in preen oil, however, remains unexplored.

Here, we investigated the presence of proteins with potential antibacterial properties in the preen oil of house sparrows *Passer domesticus*. We chose to work with the house sparrow because antimicrobial activity of preen gland secretions has previously been reported in this species (Magallanes et al. 2016). Specifically, we hypothesised that in addition to waxes, preen gland secretions might contain immune-related proteins, such as those found in other mucosal membranes and associated secretions. We further hypothesised that lysozyme, an antibacterial enzyme that causes rapid cell lysis, especially in Gram-positive bacteria (Callewaert and Michiels 2010), would be a likely candidate as it has been reported in a broad range of body fluids (e.g. tears, saliva (Hankiewicz and Swierczek 1974), ejaculates (Otti et al. 2009, Rowe et al. 2013)). We therefore tested for the presence of lysozyme activity in preen oil samples from male sparrows. In addition, we tested for the presence of immunoglobulin Y (IgY), the avian equivalent to mammalian IgG, in preen oil of male sparrows. We chose to investigate an immunoglobulin because they have been documented in a range of animal mucosal tissues and secretions (Cerutti et al. 2011, Chen et al. 2020), and IgY in particular because it is the major circulating antibody in birds (Davison et al. 2008). Further, we investigated these two proteins because they play an important role in both innate and adaptive immunity (lysozyme and IgY, respectively). Given that these proteins represent different arms of the immune system, we tested for a relationship between lysozyme and IgY concentrations. Finally, given that previous studies have observed a relationship between preen gland size and bacteria loads (Leclaire et al. 2014, Fülöp et al. 2016, Giraudeau et al. 2016, but see Møller et al. 2009), we examined the relationships between both immune proteins and preen gland size, the secretory capacity of the gland, and body condition of males.

Methods

General methods and sample collection

Adult male house sparrows (n = 57) were trapped using mist nets over a 1-month period (15 May–16 June) during the

peak of the 2015 breeding season in Oslo between 7 a.m. and 2 p.m. Upon capture, birds were briefly held in small, individual cages prior to sampling. For each male, we measured body mass (± 0.1 g) and tarsus length (± 0.01 mm), as well as the length, width and depth of the preen gland (± 0.01 mm). Measurements of the preen gland were taken in triplicate, and as these were highly repeatable ($R > 0.85$, $p < 0.0001$; ANOVA-based repeatability following Nakagawa and Schielzeth 2010), we used the average of the three values for further analyses. Next, for each individual, we calculated the volume of the preen gland as, volume = length × width × dept (Fülöp et al. 2016). All measurements were performed by one person (DC), and each individual was banded with a unique numbered metal band to prevent resampling of the same individual.

We first swabbed the preen gland and the surrounding skin with 70% ethanol in order to minimize the risk of bacterial contamination of the secretion, which might impact assay performance. We then extracted all the available preen oil by gently pressing the base of gland with a finger and collected the secretion into a capillary tube. Immediately after extraction, we measured the volume of the collected oil using a digital calliper, and used this measure as an estimate of the secretory capacity of the preen gland. The extracted preen oil was then transferred to a sterile Eppendorf tube and immediately placed on ice before being stored at −80°C until further processing.

Immune assays

Lysozyme concentration of preen oil was determined using the well-established lyso-plate assay, which takes advantage of the sensitivity of *Micrococcus lysodeikticus* to the action of lysozyme and is commonly used to measure lysozyme activity in a broad range of tissues and species (Osserman and Lawlor 1966, Millet et al. 2007, Rowe et al. 2013, Butler and Waite 2016). Specifically, we used the previously developed micromethod of the lysoplate assay (Rowe et al. 2013). For this assay, 30 ml of 1% Noble agar gel (A5431, Sigma) containing 50 mg/100 ml lyophilized *Micrococcus lysodeikticus* (M3770, Sigma) was pipetted into sterile 150 × 20 mm Petri dishes (82.1184.500, Sarstedt) and, after solidification, 1.5 µl of preen gland secretion was inoculated in test holes (1.8 mm diameter). Standard dilutions of crystalline chicken egg white lysozyme (L6876, Sigma) (0.5, 0.8, 1, 2, 4, 8, 10, 20 and 40 µg ml^{-1}) were used to prepare a standard curve in each plate. Plates were incubated at 37°C for 20 h; during this period, a clearing zone developed in the area of the gel surrounding the sample inoculation site as a result of bacterial lysis, with the diameter of these cleared zones being proportional to the log of the lysozyme concentration. Plates were photographed in a photobox (Imaging system; peqlab) and the diameter of the lytic area for each sample was measured three times using the digital image software ImageJ (ver. 1.48, http://imagej.nih.gov/ij/). These values were highly repeatable ($R > 0.99$, $p < 0.0001$; ANOVA-based repeatability following Nakagawa and Schielzeth 2010), and thus for each sample we calculated the mean diameter of each lytic area and converted these values into hen egg lysozyme equivalents (HEL equivalents, expressed in µg ml^{-1}) according to the standard curve. We have also previously shown this assay to be highly repeatable across plates (Rowe et al. 2013). Finally, we applied a heat treatment designed to inactive lysozyme (Masschalck et al. 2001) to a subset of the samples (i.e. those with sufficient sample volume remaining after the initial assays; $n = 10$). We also applied the same treatment to the chicken egg white lysozyme (L6876, Sigma) standard to confirm inactivation of lysozyme under our assay conditions. Heat treatment resulted in the complete elimination of enzymatic activities of all preen oil samples and eliminated or reduced enzymatic activity in the lysozyme controls (Supplementary information), supporting the identification of lysozyme proteins as being responsible for bacterial clearing in our assays.

The total amount of IgY in preen oil was assessed using a sensitive enzyme-linked immunoabsorbent assay (ELISA) with commercial anti-chicken antibodies (Martínez et al. 2003, Noreen et al. 2011). A previous study on the effectiveness of three secondary anti-avian IgY antibodies, showed that anti-chicken and anti-passerine antibody detected similar amounts of IgY in house sparrows (Fassbinder-Orth et al. 2016). After determining the appropriate dilution for the preen oil (1:500), 96-well high-binding ELISA plates (82.1581.200, Sarstedt) were coated with 100 µl of diluted preen gland secretion sample (diluted in carbonate–bicarbonate buffer, pH = 9.6) and incubated for 1 h at 37°C, and then overnight at 4°C. After incubation, the plates were emptied and then washed once with a 200 µl solution of Tween (0.05% Tween in PBS), before 100 µl of a solution of 1% gelatine in PBS–Tween was added. Plates were then incubated at 37°C for 1 h, washed with PBS–Tween and 100 µl of polyclonal rabbit anti-chicken IgY conjugated with peroxidase (A-9046, Sigma) at 1:250 (v/v) was added. Following 2 h incubation at 37°C, the plates were washed again with PBS–Tween three times. After washing, 100 µl of revealing solution [peroxide diluted 1:1000 in ABTS (2,20-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid))] was added, and the plates incubated for 1 h at 37°C. Finally, we measured absorbance at 405 nm using a photometric microplate reader (Biotek; µQuant Microplate Spectrophotometer) and used the resultant optical density (OD) measure as our estimate of total plasma IgY (Noreen et al. 2011). All samples were run in duplicate and duplicate values were significantly repeatable ($R > 0.98$, $p < 0.0001$; ANOVA-based repeatability following Nakagawa and Schielzeth 2010), we therefore used the average IgY concentration in all analyses.

Statistical analyses

All analyses were performed using R 3.6.1 (<www.r-project.org>). Both lysozyme concentration and preen gland volume were ln-transformed for all analyses. We estimated body condition of individuals using the scaled mass index (Peig and Green 2009) based on the relationship between body mass and tarsus length. Immune measures have previously been

shown to be influenced by both sampling (i.e. time of day the sample was collected) and handling (i.e. time elapsed between when bird was captured and sampling time) time (Zylberberg 2015). We therefore tested for an effect of both time variables on our data using separate linear models, with immune measure as the response variable and time as the predictor variable. We found no effect of either sampling time or handling time on lysozyme (sampling: $t=0.30$; $p=0.77$; handling: $t=-0.26$; $p=0.80$) or IgY (sampling: $t=0.27$; $p=0.79$; handling: $t=-1.61$; $p=0.12$) concentrations. Given that the amount of oil we could obtain from the preen gland might vary across the day, we also tested for an effect of sampling time on the secretory capacity of the gland, but again found no effects of sampling time ($t=0.21$, $p=0.84$). Finally, as the size of the preen gland may vary across the one-month sampling period, we tested for an effect of sampling date on preen gland size, but found no effect of date on preen gland volume ($t=1.25$, $p=0.22$).

We tested for a relationship between lysozyme and IgY concentrations using a Pearson correlation. Next, we examined the relationship between the size of the preen gland and the secretory capacity of the gland using a linear model with secretory capacity as the dependent variable and preen gland volume, body condition and their two-way interaction as predictor variables. Finally, to test whether immune activity (lysozyme or IgY) of preen oil was influenced by either gland size or the secretory capacity of the gland, we ran linear models with lysozyme and IgY as the response variable (separate models for each variable) and preen gland volume, secretory capacity and body condition, together with their two-way interactions, as predictor variables. We also included sampling time and sampling date as covariates in these models to account for potential effects of temporal variation on the relationships between protein concentration and preen gland size and preen oil volume.

For all linear models, non-significant interaction terms ($p > 0.05$) were removed from the model in a backwards stepwise procedure following Zuur et al. (2009). Model assumptions were verified through visual inspection of model plots (e.g. residuals versus fitted values, Zuur and Ieno 2016) and statistical tests of assumptions using the gvlma function in the R package *gvlma* (Peña and Slate 2006). Multicollinearity between variables was assessed for each model using the R package *car* (Fox and Weisberg 2019), but did not appear to bias our results; all variance inflation factors were < 1.5.

Results

We successfully collected preen oil from 56 males (oil volume: $\bar{x} \pm$ SD: 12.87 ± 5.03 µl; range = 4.53–28.22 µl). We detected immune activity (either lysozyme or IgY or both) in the preen oil of 53 males, while the remaining three males had insufficient volume of sample to perform the assays. From these samples, we detected lysozyme activity in 48 males; one male exhibited a lysozyme concentration below the assay detection limit (0.5 µg ml^{-1}), despite having a sufficiently large sample to perform the assay, and was excluded from all analyses. Across the remaining 47 males, lysozyme concentration varied more than 16-fold, ranging from 0.75 to 16.42 µg ml^{-1} ($\bar{x} \pm$ SD: 4.95 ± 4.03 µg ml^{-1}). For IgY, we successfully obtained data for 44 males, while IgY concentration of 12 males was below detection level. For these 44 males, IgY concentration varied considerably, ranging from 0.016 to 0.217 absorbance units (OD) (mean ± SD: 0.128 ± 0.06).

The two measures of immunity – lysozyme and IgY – were significantly positively correlated across individuals ($r=0.45$; $p=0.005$, Fig. 1). Next, we found that the secretory capacity of the gland was significantly positively correlated with gland volume ($t=2.65$; $p=0.01$), whereas there was no relationship between secretory capacity and body condition ($t=1.01$; $p=0.32$). Models examining the relationship between immune activity of preen oil and gland size, gland secretory capacity and body condition, showed that lysozyme concentration was significantly negatively associated with the secretory capacity of the preen gland ($t=-2.95$; $p=0.005$; Fig. 2A), but was not associated with gland volume ($t=0.83$; $p=0.41$), body condition ($t=-1.05$; $p=0.30$), sampling date ($t=-0.23$, $p=0.82$) or sampling time ($t=0.73$, $p=0.47$). Similarly, levels of IgY were significantly negatively correlated with the secretory capacity of the preen gland ($t=-3.50$; $p=0.001$; Fig. 2B), but were not associated with gland volume ($t=1.93$; $p=0.061$), body condition ($t=-0.47$; $p=0.64$), sampling date ($t=0.29$, $p=0.78$) or sampling time ($t=38$, $p=0.71$).

Discussion

We detected lysozyme and IgY in the preen oil of free-living house sparrow males. To the best of our knowledge, this is the first demonstration of immune defence proteins in avian preen oil. Previously, Jacob et al. (1997) reported the isolation of an alcohol (i.e. 3,7-dimethyloctan-l-ol) from preen oil exhibiting a particularly strong effect against dermatophytes, and, to a lesser degree, fungistatic effects against yeasts and moulds and antibacterial effects against Gram-positive bacteria. More recently, Braun et al. (2018) suggested that preen oil antimicrobial effects might originate from peptides. Our study builds on these earlier works by demonstrating that the antimicrobial peptide lysozyme contributes to the antimicrobial activity of preen oil in house sparrows. Furthermore, the presence of IgY in preen oil demonstrates that both innate and adaptive immune defence proteins are present in the preen gland secretions of house sparrows.

Lysozyme is one of the most common antimicrobial enzymes in animals (Osserman and Lawlor 1966), is particularly effective at controlling Gram-positive bacteria (Callewaert and Michiels 2010), and has been reported in a wide range of body tissues (e.g. blood, urine, saliva, tears, gastric juices, Hankiewicz and Swierczek 1974), including

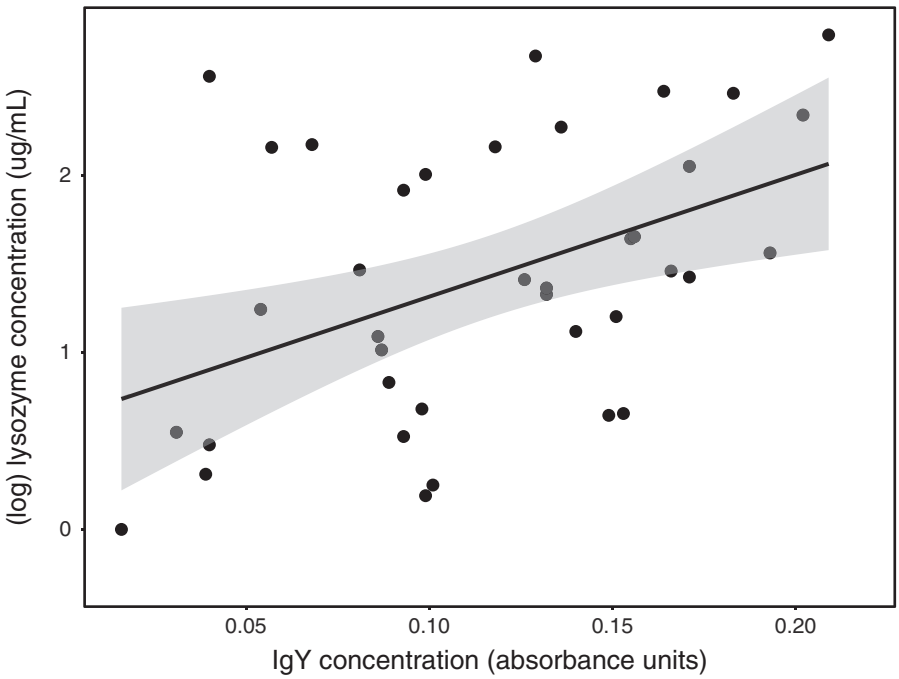

Figure 1. Relationship between the concentration of lysozyme and immunoglobulin Y in the preen oil of male house sparrows (n = 38, r = 0.45; p = 0.005). The line shows predicted values from a linear regression and shaded area the 95% confidence intervals.

the plasma, egg albumen and ejaculates of birds (Martin et al. 2010, Rowe et al. 2013, Butler and Waite 2016). Given that the enzyme is found broadly throughout the body, the presence of lysozyme in preen oil may simply reflect lysozyme in circulation, though comparison with lysozyme levels in circulation requires study. As many of the bacterial species currently identified as feather-degrading bacteria are Gram-positive taxa (Gunderson 2008), and given that lysozyme

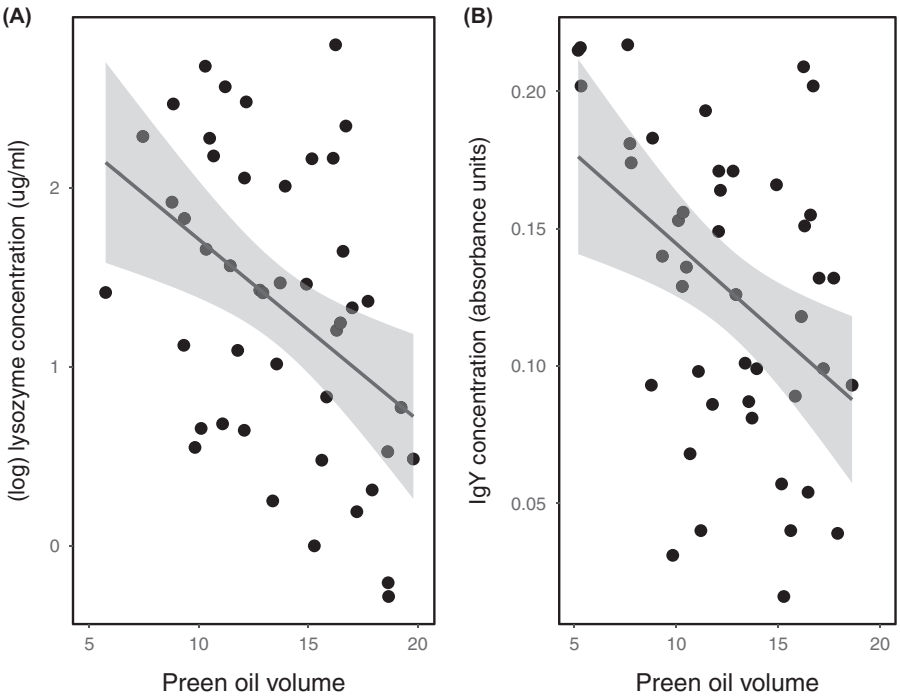

Figure 2. Relationship between preen oil volume and concentration of (A) lysozyme and (B) immunoglobulin Y in the preen oil of male house sparrows. For each plot, the line shows predicted values from a simple linear regression and shaded area the 95% confidence intervals.

appears to be stable across a broad range of temperatures and a broad pH range (Venkataramani et al. 2013), we suggest it is plausible that lysozyme from preen oil acts as an antibacterial defence mechanism against this specific group of feather-associated bacteria, though future tests of the stability and function of lysozyme on feathers are needed to establish this. Nonetheless, in such a capacity, lysozyme may regulate or reduce feather bacterial load or influence the community profile of plumage microbiota.

Within the preen gland, lysozyme and immunoglobulins may play a role in local immune defence as has been described for other mucosal surfaces (Tachibana et al. 1986, Li et al. 2011). The preen gland interfaces with the external environment, most notably through the action of preening, and thus represents a potential entry site for pathogenic and opportunistic microorganisms. The presence of lysozyme could thus defend the site against bacterial challenges and colonisation. Similarly, the secretion of immunoglobulins by mucosal B cells may function to prevent microbial intrusion into the gland.

Immunoglobulins can also play a fundamental role in the regulation of microbiota via the targeting of distinct commensal bacteria. For example, IgM secreted in the human intestine interacts with the intestinal microbiota and actively participates in maintaining its diversity (Magri et al. 2017). Thus, IgY in preen oil may shape or regulate the preen gland microbiome, which in turn may have consequences for the antibacterial activity of preen oil via effects on bacteria that produce bacteriocins. Additionally, given that volatile compounds in preen oil can be produced, at least in part, by symbiotic bacteria (Whittaker et al. 2019), immune protein regulation of the preen gland microbiome may influence odour-producing bacteria and thus chemical cues and signals in birds. Recent studies of the blue petrel *Halobaena caerulea* have also demonstrated links between individual odour profile, plumage microbiota and immunogenetic diversity (Leclaire et al. 2017, 2018). Thus, an intriguing possibility is that the presence of immune proteins in preen oil may play a role in mediating these relationships, though clearly this idea requires investigation.

We found that larger preen glands had a greater secretory capacity (i.e. greater preen oil volume), which is consistent with previous studies in house sparrows (Pap et al. 2010) and other passerines (Møller et al. 2009). Interestingly, however, the concentration of both lysozyme and IgY was negatively correlated with preen oil volume, suggesting a potential investment trade-off into different components of preen oil. Trade-offs between immune defences and other costly activities or tissues appear common in birds (Martin 2005, Moreno-Rueda 2014, Leclaire 2015). In this instance, immune proteins may trade-off against other preen secretions, which presumably require considerable energy in their anabolism. However, it is likely that both preen oil volume and immune protein concentrations are influenced by a range of factors that complicate the interpretation of these negative relationships. For example, preen oil volume is likely to reflect a balance between production and usage. Moreover, whether measures of immune indices reflect an individual's immunocompetence or its current status of pathogenic infection remains an open question (Adamo 2004, Biard et al. 2015), thus a clear interpretation of immune protein concentrations and their correlations with other traits is challenging. Variation in immunity among individuals may arise from variation in microbial pressures linked to different reproductive stages (e.g. nest build phase versus feeding nestlings), or variation in genetic background (Ardia et al. 2010), or may perhaps indicate fitness-related individual 'quality' differences (Tieleman et al. 2005). The idea that differences in quality may mediate variation in immune protein concentrations is underscored by our finding of a positive correlation between levels of lysozyme and IgY in preen oil of house sparrows, though at this time we refrain from drawing firm conclusions about factors that may drive inter individual variation in immune measures and their correlations with other traits.

In conclusion, our work shows presence of innate and adaptive immune proteins in preen oil, which we suggest may function as antimicrobial defences against Gram-positive bacteria, potentially including the feather-degrading bacteria, and local immune defence in the preen gland. Furthermore, our findings raise the intriguing idea that immune proteins in preen oil may play a role in regulating the local microbiome, with potential repercussions for chemical communication and sexual selection. Previous research shows that skin and mucosa represent an anatomical and chemical barrier to microbial invasion and, as such, act as the body's first line of defence against infectious agents. Our study supports the idea that the preen gland and its secretions should be considered an integral component of this barrier, functioning in the first line of defence against invading pathogens and opportunistic microorganisms.

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Author contributions – MR and GAC conceptualised the study; DC (lead) and MR (support) planned the study design and collected field data and samples; GAC analysed samples; DC and MR performed statistical analysis; all authors wrote the manuscript.

Conflicts of interest – The authors have declared that no competing interests exist.

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Data availability

Data will be made available at Dryad <https://doi.org/10.5061/dryad.kh189323s> (Carneiro et al. 2020).

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